Co-Transcriptional Splicing and Functional Role of PKCβ in Insulin-Sensitive L6 Skeletal Muscle Cells and 3T3-L1 Adipocytes

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Keywords: PGC1α, PPARγ, GLUT4, Akt, mTORC2

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<tbody>
<tr>
<td>15-d-PGJ2</td>
<td>15-deoxy prostaglandin J2</td>
</tr>
<tr>
<td>αMEM</td>
<td>Minimum essential medium alpha</td>
</tr>
<tr>
<td>AF-2</td>
<td>Activation function-2</td>
</tr>
<tr>
<td>AGC</td>
<td>cAMP-dependent, cGMP-dependent and protein kinase C</td>
</tr>
<tr>
<td>AICAR</td>
<td>Aminoimidazole carboxamide ribonucleotide</td>
</tr>
<tr>
<td>AKAP</td>
<td>A kinase anchoring protein</td>
</tr>
<tr>
<td>AMPK</td>
<td>5′AMP-activated protein kinase</td>
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<td>APPL1</td>
<td>Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1</td>
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<td>APS</td>
<td>Adaptor protein containing a PH and SH2 domain</td>
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<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>AS160</td>
<td>Akt substrate of 160kDa</td>
</tr>
<tr>
<td>ASM</td>
<td>Acid sphingomyelinase</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical PKC</td>
</tr>
<tr>
<td>C3G</td>
<td>CRK SH3-binding GEF</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaMK</td>
<td>Calcium/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>CAP</td>
<td>Cbl-associated protein</td>
</tr>
<tr>
<td>CAPP</td>
<td>Ceramide-activated protein phosphatase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>C/EBP</td>
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<td>Cdc42-interacting protein 4</td>
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<td>Clk1</td>
<td>CDC-like kinase 1</td>
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<td>cPKC</td>
<td>Classical PKC</td>
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<td>CREB</td>
<td>Cyclic-AMP-responsive-element-binding protein</td>
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<td>C-terminal domain</td>
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<tr>
<td>CTE</td>
<td>C-terminal extension</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
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<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
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<td>DBD</td>
<td>DNA-binding domain</td>
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<td>DEPTOR</td>
<td>dishevelled, egl-10, pleckstrin domains and interacts with mTOR</td>
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<td>DGK</td>
<td>Diacylglycerol kinase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DOC2B</td>
<td>Double C2-like domains, beta</td>
</tr>
<tr>
<td>DR1</td>
<td>Direct repeat 1</td>
</tr>
<tr>
<td>DV-GSC</td>
<td>Dispersed vesicular GLUT4 storage compartment</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related kinase</td>
</tr>
<tr>
<td>ESE</td>
<td>Exon splicing enhancer</td>
</tr>
<tr>
<td>ESS</td>
<td>Exon splicing silencer</td>
</tr>
</tbody>
</table>
Fe$^{2+}$  Ferrous iron
FFA  Free fatty acid
FOXO1  Forkhead box O1
FRAP  FKBP12-rapamycin-associated protein
GAP  GTPase activating protein
GEF  Guanyl nucleotide-exchange factor
GGA  Golgi-localized γ-ear-containing arf-binding protein
GLUT4  Glucose transporter 4
G6Pase  Glucose-6-phosphatase
GS  Glycogen synthase
GSK3  Glycogen synthase kinase 3
GSV  GLUT4 storage vesicle
HAT  Histone acetyltransferase
HETE  12- and 15-hydroxyeicosatetraenoic acid
hnRNP  Heterogeneous nuclear RNP
HODE  9- and 13-hydroxyoctadecadienoic acid
HSL  Hormone-sensitive lipase
IGF  Insulin-like growth factor
IL  Interleukin
IFN  Interferon
IP$_3$  Inositol triphosphate
IR  Insulin receptor
IRAP  Insulin responsive aminopeptidase
<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>IRM</td>
<td>Insulin-responsive motif</td>
</tr>
<tr>
<td>ISE</td>
<td>Intronic splicing enhancer</td>
</tr>
<tr>
<td>ISGT</td>
<td>Insulin stimulated glucose transport</td>
</tr>
<tr>
<td>ISR2</td>
<td>Insulin substrate receptor 2</td>
</tr>
<tr>
<td>ISS</td>
<td>Intronic splicing silencer</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand-binding domain</td>
</tr>
<tr>
<td>LDM</td>
<td>Low density microsome</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>LPAAT</td>
<td>Lysophosphatidic acid acyltransferases</td>
</tr>
<tr>
<td>MAGUK</td>
<td>Membrane-associated guanylate kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF2C</td>
<td>Myocyte-enhancer factor 2C</td>
</tr>
<tr>
<td>MEK</td>
<td>MAP kinase kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mammalian target of rapamycin complex 2</td>
</tr>
<tr>
<td>Myo1c</td>
<td>Myosin 1c</td>
</tr>
<tr>
<td>NCoR</td>
<td>Nuclear repressor co-repressor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>nPKC</td>
<td>Novel PKC</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO₂-LA</td>
<td>Nitrolinoleic acid</td>
</tr>
<tr>
<td>NO₂-OA</td>
<td>Nitrooleic acid</td>
</tr>
<tr>
<td>NDM</td>
<td>Nonsense-mediated decay</td>
</tr>
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<td>Abbreviation</td>
<td>Full Name</td>
</tr>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PAP</td>
<td>Phosphatidic acid phosphorylase</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidyl choline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<td>PDK1</td>
<td>3-phosphoinositide-dependent protein kinase 1</td>
</tr>
<tr>
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<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PGC1α</td>
<td>Peroxisome proliferator-activated receptor γ coactivator 1α</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PHLPP</td>
<td>PH domain leucine-rich repeat protein phosphatase</td>
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<td>Phosphoinositide-3-kinase</td>
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<td>PIAS1</td>
<td>Protein inhibitor of STAT1</td>
</tr>
<tr>
<td>PICK1</td>
<td>Protein interacting with C kinase 1</td>
</tr>
<tr>
<td>PIKfyve</td>
<td>Phosphoinositide kinase for five position containing a fyve finger</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol (4,5)-biphosphate</td>
</tr>
<tr>
<td>PI(3)P</td>
<td>Phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5)-triphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
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<td>Protein kinase D</td>
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<td>Protein kinase M</td>
</tr>
<tr>
<td>PLA</td>
<td>Phospholipase A</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PLD</td>
<td>Phospholipase D</td>
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</table>
PM  Plasma membrane
PMA  Phorbol 12-myristate 13-acetate
PP1  Type-1 phosphatase
PPARγ PPARγ Peroxisome proliferator-activated receptor γ
PPRE PPARγ response element
PR-GSC Perinuclear reticular GLUT4 storage compartment
PRAS40 Proline-rich Akt substrate 40 kDa
PROTOR Protein observed with Rictor
PTB  Phosphotyrosine-binding
PTPase Protein tyrosine phosphatases
PTEN Phosphatase and tensin homolog
PYR  Polypyrimidine tract
RabGDI Rab GDP dissociation inhibitor
RACK Receptor for activated C kinase 1
RAPTOR Regulatory-associated protein of mTOR
Rheb Ras homolog enriched in brain
RICCTOR Rapamycin insensitive companion of mTOR
RIP140 Nuclear receptor interacting protein 1
RNAPII RNA polymerase II
RRM  RNA recognition motif
RS  Arginine/serine
RUVBL2 RuvB-like protein 2
RXRα Retinoid X receptor α
<table>
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<tr>
<th>Acronym</th>
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<tr>
<td>S6K1</td>
<td>S6 kinase 1</td>
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<td>SH2-containing 5′-inositol phosphatase</td>
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<td>Stress-activated-protein-kinase-interacting protein 1</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SMS</td>
<td>Sphingomyelin synthase</td>
</tr>
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<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) Receptor</td>
</tr>
<tr>
<td>snRNP</td>
<td>Small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>SRC</td>
<td>Steroid receptor co-activator</td>
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<tr>
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<td>Substrates that interact with C kinase</td>
</tr>
<tr>
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<td>Diabetes mellitus type 2</td>
</tr>
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<td>Trans-Golgi network</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
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</tr>
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</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>VAMP2</td>
<td>Vessel-associated membrane protein 2</td>
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<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
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CO-TRANSCRIPTIONAL SPLICING AND FUNCTIONAL ROLE OF PKCβ IN INSULIN-SENSITIVE L6 SKELETAL MUSCLE CELLS AND 3T3-L1 ADIPOCYTES

Eden Kleiman

ABSTRACT

PKCβII is alternatively spliced during acute insulin stimulation in L6 skeletal muscle cells. This PKCβII isoform is critical in propagating GLUT4 translocation. PKCβ protein and promoter dysfunction correlate with human insulin resistance. TZD treatment ameliorates whole-body insulin-resistance. Its primary target is adipocyte PPARγ, which it activates upon binding. This causes both altered circulating serum FFA concentrations and adipokine secretion profile. How TZDs affect the intracellular signaling of skeletal muscle cells is unknown. RT-PCR and Western blot analysis showed that TZDs elevated PKCβII by a process that involves co-transcriptional splicing. PGC1α overexpression most closely resembled TZD treatment by increasing PKCβII protein levels and keeping PKCβI levels relatively constant. Use of a heterologous PKCβ promoter driven PKCβ minigene demonstrated that PPARγ could regulate the PKCβ promoter, but whether this is direct or indirect is unclear. SRp40 splicing factor has been shown to dock onto the PGC1α CTD and influence splicing. SRp40, through overexpression and silencing, appears to play a part in PKCβ promoter regulation.

PKCβ promoter regulation was also studied in 3T3-L1 cells. TZDs were experimentally shown to have no role in PKCβ promoter regulation despite PPARγ
activation. Chromatin immunoprecipitation assays revealed PU.1 as a putative PKCβ transcription factor that can cross-talk with the spliceosome, possibly through SRp40 which was also associated with the PKCβ promoter. 3T3-L1 adipocyte differentiation revealed a novel developmentally-regulated switch from PKCβI to PKCβII, using western blot and Real-Time PCR analysis. Pharmacological inhibition of PKCβII using CGP53353 and LY379196 blocked [3H]2-deoxyglucose uptake and revealed a functional role for PKCβII in adipocyte ISGT. CGP53353 specifically inhibited phosphorylation of PKCβII Serine 660 and not other critical upstream components of the insulin signaling pathway. Subcellular fractionation and PM sheet assay pointed to PKCβII-mediated regulation of GLUT4 translocation to the PM. Co-immunoprecipitation between PKCβII and GLUT4 allude to possible direct interaction. Western blot and immunofluorescence assays show PKCβII activity is linked with Akt Serine 473 phosphorylation, thus full Akt activity. Western blot and co-immunoprecipitation suggested that insulin caused active mTORC2 to directly activate PKCβII. Data support a model whereby PKCβII is downstream of mTORC2 yet upstream of Akt, thereby regulating GLUT4 translocation.
INTRODUCTION

Protein Kinase C: Brief History

Protein Kinase C (PKC) was discovered by Nishizuka and coworkers as a histone protein kinase isolated from the rat brain that could be activated by limited proteolysis [1], Ca\(^{2+}\) and (phospho)lipids [2] or phorbol esters and phospholipids [3]. Diacylglycerol (DAG), an early product of signal-induced inositol phospholipid breakdown, enhanced the affinity of PKC for calcium and thereby activating it [4]. Phorbol esters, which promoted tumors, were able to substitute for DAG in PKC activation [3]. This activation by phorbol esters was believed to be the reason PKC was a tumor promoter [5; 6], bringing it to the forefront on cancer research [7]. Early biochemical studies and purifications indicated PKC represented a group of several isoenzymes [8]. The major breakthrough came from cloning cDNAs of PKC isoforms, mostly from brain cDNA libraries. PKC\(\beta\) cDNA has been cloned from various sources including bovine, human, rat and mouse [9].

Signaling and Physiological Impact

PKC's role in signal transduction was first demonstrated in release of serotonin from platelets [10; 11]. A variety of agonist-induced cellular responses involve PKC including hormones, neurotransmitters and some growth factors [12]. One of the main pathways activated by PKC is the MEK-ERK pathway which promotes proliferation and differentiation [13]. However, there are many other signaling cascades of which PKC is
at the epicenter. Figure 1 illustrates the many proposed effects of protein kinase C activation [14].

In cancer signaling, PKC is also involved in cellular adhesion. This allows for cancer cell invasion via integrin binding, activation of metalloproteinases and expression of extracellular matrix proteins. Inhibition of PKC reduces invasiveness [14]. A PKCβ selective inhibitor, Enzastaurin, has recently been evaluated for its potential as an anticancer agent in lung cancer [15].

PKC is also involved in the PI3K-Akt pathway [14]. This places PKC in the middle of the insulin signaling cascade. PKC is involved in insulin-stimulated glucose uptake as a downstream effector of PI3K-PDK1 [16]. As proof of its central role in insulin signaling, PKC dysregulation is associated with many diabetic complications such as diabetic nephropathy, diabetic retinopathy and cardiovascular disease [17; 18].

**PKC Family and Structure**

The Protein Kinase C family are serine/threonine kinases that are members of the AGC (cAMP-dependent, cGMP-dependent, and protein kinase C) family of protein kinases which have structural features in common (includes PKA, PKB and PKC). AGC kinases contain highly conserved catalytic domains (motifs required for ATP/substrate-binding and catalysis) and a regulatory domain that keeps that enzyme in an inactive conformation. PKC regulatory domains occupy the amino terminal and contain an autoinhibitory pseudosubstrate domain (sequence where alanine substitutes for serine/threonine phosphoacceptor site) and two distinct plasma membrane targeting modules, C1 and C2. PKC isoforms are classified based on differences in their amino terminal regulatory domain (Figure 2) and cofactor requirement [14; 19; 20; 21].
The PKC family comprises 10 isozymes and their splice variants grouped into 3 classes based on co-factor requirement [21]. Conventional PKC (cPKC) isoforms include \( \alpha, \gamma, \beta I \) and alternatively spliced \( \beta II \). Starting at the N-terminal, cPKCs are allosterically regulated by the pseudosubstrate which as mentioned before resembles a substrate binding domain [20]. This was discovered by using peptides based on the sequence and showed that they were effective competitive inhibitors of PKC [22]. Activation of PKC (and downstream signaling) necessitates the release of the pseudosubstrate domain from the kinase core (Figure 3) [6; 23].

Antibodies targeting the pseudosubstrate domain resulted in co-factor independent PKC activation [24]. PKC is resistant to proteolysis when inactive (pseudosubstrate-kinase core binding) but highly sensitive to proteolysis in the pseudosubstrate domain (specific Arginine within) when active. The activation of PKC is dependent on pseudosubstrate unmasking [25]. cPKCs contain a C1 domain that binds DAG/PMA. The C1 domain consists of tandem (roughly 50) residue sequences, named C1A and C1B. C1A and C1B domains both have a zinc finger (Zn\(^{2+}\)) held by 6 cysteines and 2 histidines. This is used for DAG/phorbol ester binding [19]. The C1B domain contains a Tyrosine at position 22 making it a weak DAG responder [21]. The C2 domain binds calcium and subsequently binds anionic/acidic phospholipids [19]. Ca\(^{2+}\) is bound by a \( \beta \)-sandwich structure composed of eight \( \beta \)-strands creating two flat sheets connected with a short helix hinge. This \( \beta \) sandwich can bind 2-3 calcium ions. It is this positively charged ion that helps interact with negatively charged phospholipids such as phosphatidylserine [26]. The double positive symbol (Figure 2) in the cPKC C2 domain represents a basic patch (located in the distal area of the Ca\(^{2+}\)-dependent lipid binding
site) that specifically recognizes PIP$_2$ and distinguishes cPKC from other PKCs (among other things) in terms of activation [21]. Novel PKCs (nPKCs) include δ, ε, θ, η. δ/θ or ε/η could be further subdivided based on structural features. nPKCs have C1 and C2 domains, however, the order is switched relative to cPKCs (Figure 2). The nPKC C2 domain (C2-like) lacks the acidic residues necessary for calcium binding. This is the main pharmacological difference between cPKCs and nPKCs. As shown in Table 3, nPKCs are maximally activated by DAG/PMA and phosphatidylserine without the requirement for calcium [19]. nPKCs have stronger DAG responsiveness compared to cPKCs due to a tryptophan at position 22 (Figure 2) in the C1B domain [21]. Atypical PKCs (aPKCs) include ζ, ι/λ. They lack a calcium sensitive C2 domain and have an atypical C1 domain with only one cysteine-rich membrane-targeting structure that binds PIP$_3$ or ceramide (not DAG or PMA). Upstream of the C1 domain is a PB1 domain that mediates interactions with other PB1-containing scaffolding proteins such as PAR-6 and MEK5. The activity of aPKCs are regulated mainly by protein-protein interactions and PDK-1 mediated phosphorylation [19].

The PKC ATP-binding domain (C3) contains a lobe with β-sheets with a glycine-rich ATP-binding loop with the consensus GXGXXG. It has an invariant lysine which structures the enzyme for phosphoryl-transfer. The C4 domain is predominantly α-helical and contains the activation loop segment that positions magnesium and peptide substrates for catalysis. In between the C3 and C4 lobes of the kinase domain, there is a “gatekeeper” residue (methionine in PKCβII) that controls access to a cavity in the ATP binding pocket. The V5 domain contains highly conserved priming/regulatory
phosphorylation sites (discussed in further detail below) that play a crucial role in structuring the catalytic pocket [19].

PKC μ and ν are considered by some to constitute a fourth class of PKCs named Protein Kinase D [20]. Initially, human PKD (PKCμ) was discovered followed by the murine ortholog PKD [27; 28]. PKD is a serine/threonine kinase that consists of an N-terminal regulatory domain and a C-terminal catalytic domain. The N-terminal domain has 2 cysteine-rich, zinc finger-like motifs and a pleckstrin homology (PH) domain. Cys1 and Cys2 domains (Figure 2) bind DAG/PMA with high affinity [29]. The PH domain is involved in protein:protein interactions as well as PH domain-dependent autoinhibitory intramolecular interactions that maintain the enzyme in an inactive state with low basal activity in resting cells. An interesting connection exists between PKD and nPKCs. nPKCs activate PKD by phosphorylating them at highly conserved serine residues in the activation loop which relieves autoinhibition [30]. PKD2 and PKD3 have also recently been added to the list. PKD is distinct from PKC with respect to structural and enzymatic properties. For instance, PKD does not phosphorylate many bona-fide PKC substrates. PKD family members have been grouped into the calcium/calmodulin-dependent protein kinase (CaMK) based on catalytic domain structure and substrate specificity. However, unlike CaMK, PKDs are activated indirectly by calcium through DAG production [29].

Recently, another group of PKCs has been discovered. Protein kinase C-related kinase (PRK) consists of at least three members, PRK1-3. PRKs are insensitive to Ca^{2+}, DAG and phorbol esters but can be activated by limited proteolysis and phospholipids. Both PRK1 and PRK2 can bind activated RhoA GTPase proteins, which increases their
activity. This binding occurs in the HR1 domain. It is composed of three repeats of a 55
amino acid motif. In PRK1, the first HR1 repeat (HR1a) binds to activated GTP-RhoA
complex but not to the inactive GDP-bound form. HR1b is the second motif and binds
(less affinity) to either active or inactive RhoA. The HR1c repeat does not bind RhoA
and its function is unknown [31; 32].

PKCβ Gene

PKCβI and PKCβII are derived from alternative splicing of the same pre-mRNA
from the same gene (16p11.2 in humans). The region of splicing that determines the β
isoform is the V5 region. Rat and rabbit PKCβI and PKCβII cDNAs encode proteins
composed of 671 and 673 amino acids, respectively. The difference in the C-terminal V5
exon is a PKCβI product that is 50 amino acids and a PKCβII product that is 52 amino
acids (Figure 4) [33; 34]. This is also the case for human PKCβ [35].

Figure 5 depicts the possible products arising from PKCβ alternative splicing [33;
36]. Splice sites 1 & 2 are shown. However, it is predicted that additional splice sites
are possible. These additional splice sites may be involved in conferring additional
stability for the mature RNA (unpublished observation). The stop codon in the PKCβII
exon prevents the PKCβI exon from being translated (Figure 4 & 5). The intronic SRp40
binding site binds phosphorylated SRp40 (Figure 4). This splicing factor promotes
PKCβII exon inclusion [37; 38].

PKCβ V5 Domain

Since PKβI and PKCβII are identical up to the V5 region, then it is logical that
this domain distinguishes the two isoforms in terms of subcellular location (at rest and
activated) and function, even within the same cell type [19]. A classic example was
deciphered by the Cooper lab. In BC3H-1 myocytes, insulin treatment induced the alternative splicing of the PKCβII exon. Within 15 minutes of insulin treatment, basal PKCβI was replaced by the PKCβII isoform. It was hypothesized that PKCβII, as opposed to PKCβI, had a more significant role in glucose uptake. Subsequent overexpression of PKCβII in NIH-3T3 cells resulted in enhanced insulin-stimulated glucose uptake [39]. PKCβII’s unique role was further confirmed by experiments utilizing a C-terminal truncated PKCβII that acted as a dominant negative and pharmacologic inhibition via CGP53353 (a specific PKCβII inhibitor). Both mutant PKCβII and CGP53353 inhibited L6 skeletal muscle insulin-stimulated glucose uptake [40]. Another example of diametrically opposed PKCβ isoform function is in A10 vascular smooth muscle cells (rat). Here, PKCβI stimulates A10 growth while PKCβII inhibits A10 growth [41]. Distinct localizations are possible as well based on the V5 region. In cardiomyocytes, PKCβI localizes to the cytosol and perinuclear region and translocates to the nucleus following PMA treatment. PKCβII associates with fibrillar cytoskeletal structures at rest and translocates to the cell periphery and perinuclear region upon PMA treatment. Here, PKCβII co-localizes with RACK1 [42].

The V5 region shows specificity in its substrate targets. For instance, PKCβII activates PLD, whereas PKCβI does not [19].

**PKC Tissue Expression**

Select mammalian cells and tissues are listed below (Table 1) to give a general overview of PKC isoforms expression (either mRNA or protein detection) [11; 12; 43; 44; 45; 46; 47; 48; 49; 50; 51; 52]. Distribution may vary between species.
Table 1. PKC Isoform Tissue/Cell Expression

<table>
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<tr>
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<th>βI</th>
<th>βII</th>
<th>γ</th>
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PKC Size [12]

Table 2. PKC Isoform Size

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<td>λ</td>
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</table>

PKC Activation

Newly translated PKCs are unphosphorylated and associated with the cytoskeleton. Maturation (catalytic competence) must take place in order for PKC to translocate to the plasma membrane [53]. Before this maturation, interaction with the plasma membrane is very weak [19]. Using PKCβII as a model, physiological stimuli initiate one of three phosphorylations necessary for catalytic competence (Figure 6). First, threonine 500 in the activation loop is phosphorylated by PDK1. This is the rate-limiting step in the processing of PKC [20]. This phosphorylation aligns the active site with the downstream sites. Next, Threonine 641 is phosphorylated in the proline-rich turn motif via mTORC2 [54; 55]. Phosphorylation at this site locks the protein in a catalytically competent, thermally stable and phosphatase-resistant conformation. Also, this phosphorylation may serve as a docking pad for protein interactions [20]. Based on the sequence surrounding this motif, 14-3-3 protein is speculated to bind. Lastly, the hydrophobic motif is autophosphorylated at Serine 660 (S660). This hydrophobic site is the least conserved among PKCs. This phosphorylation is not essential for function, but without it they have less thermal stability and increased phosphatase sensitivity [20]. It should be noted that mammalian target of rapamycin complex 2 (mTORC2) is involved
in PKC’s intrinsic kinase activity by regulating phosphorylation of the turn motif which leads to autophosphorylation at the hydrophobic motif [21; 56].

Once the C-terminal serine and threonines are phosphorylated, PKCβII assumes its mature conformation. Subsequent dephosphorylation at Threonine 500 (T500) would not alter its mature status. However, dephosphorylation at the turn motif abolishes kinase activity. The three phosphorylation sites are conserved suggesting most PKCs undergo similar processing. The exceptions would be aPKCs. The aPKCs have a glutamic acid instead of serine and threonine in the hydrophobic motif. PKCδ, for example, T505 in the activation loop is not required because of the negative charge brought about by Glutamic acid at residue 500. Ultimately these phosphorylations are required for intracellular localization of PKCs [53].

In the traditional model of PKC activation, agonists promote phosphoinositide hydrolysis to produce DAG and IP$_3$ generation which mobilizes calcium. Calcium, a soluble ligand, binds to the C2 domain to increase PKC’s affinity for PhosphatidylSerine (PS). This interaction is relatively weak, however, once anchored to membranes the C1A domain interacts with DAG. The interaction between C1A and DAG would not be possible if it were not for PS binding to C2 which disrupts an electrostatic C1A/C2 interdomain binding. The C1A domain now penetrates the lipid bilayer to bind DAG. When both C1A and C2 domains are engaged with the membrane, the pseudosubstrate domain is expelled from the substrate-binding pocket which facilitates full PKC activation [19; 57]. DAG is thought to induce membrane alterations (inverted miscelles) improving the hydrophobic interaction with proteins that can integrate with the plasma membrane [58].
The traditional model of PKC activation was thought to be punctuated by cytoplasmic to plasma membrane translocation. However, this is not the case with every PKC isoform. PKCs can be targeted to many intracellular locations for activation [53]. Again, using PKCβII as an example, translocation to the plasma membrane is initiated by acute PLC-derived DAG accumulation. However, in cells that display a biphasic DAG response, catalytically active PKCβII is released from the plasma membrane to go to the pericentron region (subset of recycling endosomes containing small GTPase Rab11) [59; 60]. Pericentronic localization is due to PLD-derived DAG (Figure 7). PKCβII at this location can control the trafficking of continuously recycling membrane signaling proteins such as caveolin-1. PKCβII pericentron accumulation can be inhibited by PP1 (protein phosphatase 1) stimulation via ceramide. PP1 dephosphorylates PKCβII at the activation loop [19].

A detailed overview of cPKC activation is shown in Figure 8. * was inserted to remind the reader that mTORC2 does not constitutively phosphorylate PKCβII in all cases. It has been shown in mouse embryonic fibroblasts (MEFs) that this is the case [54; 55]. However, our data concerning 3T3-L1 adipocytes suggests that mTORC2 phosphorylation is mediated by insulin [52]. It has also been reported that in HEK293 cells, mTORC2 is activated via insulin stimulation [61]. This would suggest that whether cPKCs are constitutively or agonist-induced phosphorylated depends on the cell type.

As stated above, DAG can be produced by disparate mechanisms. De novo synthesis is one way DAG is induced (Figure 9). There are two main pathways for this biosynthesis. One is from glycerol-3-phosphate as a result of triacylglycerol mobilization. The other is use of dihydroxyacetone-3-phosphate, which is a glycolysis
intermediate. These two precursors undergo several modifications. These include two acylation steps that give rise to lysophosphatidic acid (LPA) and then phosphatidic acid (PA). PA can subsequently be transformed into DAG through phosphohydrolases (PAPs) [58]. DAG production in response to stimuli has many facets (Figure10). Acute DAG is produced via agonist-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate PIP2 by PLC. PLD-mediated hydrolysis of phosphatidylycholine (PC) produces another type of DAG [53]. The initial DAG (PLC-dependent) is polyunsaturated and consists of the following three types; 1-steroyl-2-arachidonoyl-sn-glycerol (SAG), 1-steroyl-2-docosahexaenoyl-sn-glycerol (SDG) and 1-steroyl-2-eicosapentaenoyl-glycerol (SEG) [62; 63; 64]. PKC isotypes are differentially activated by DAGs. SDG activates PKCα and PKCδ most powerfully. PKCβI is activated by SDG and SEG rather than SAG [53]. PLD-derived DAG is monounsaturated or saturated [64]. Polyunsaturated DAG is frequently transient with sustained DAG being predominantly monounsaturated (generally sc-2 acyl group) [65]. To generate DAG, PLD hydrolyzes phosphatidylcholine to PA and choline. PA is a bioreactive lipid whereas choline is not thought to participate in intracellular signaling [66]. PLD-derived PA is further processed by phosphatidic acid phosphohydrolases (PAPs) into DAG (1-palmitoyl 2-oleoyl-sn-glycerol). DAG can be converted back to PA via phosphorylation by diacylglycerol kinases (DGKs). In addition, PA can be deacylated by phospholipase A2 (PLA) to form monoacylated LysoPA. LysoPA can also be converted back to PA by lysophosphatidic acid acyltransferases (LPAAT) [67; 68]. PLA hydolization of phospholipids can also liberate free fatty acids [12]. Fatty acids activate PKCs in an isotype-specific manner [53; 69]. PKCβ (along with α, γ and ε) are activated by fatty
acids with carbon lengths between C13 and C18 \textit{in vitro}. Cis unsaturated fatty acids including oleic, linolenic, arachadonic and docosahexaenoic acids are all produced from phospholipids via PLA. These fatty acids allow PKC to exhibit almost full activity in the presence of Ca\(^{2+}\) concentrations less than 1\(\mu\)M. This effect is enhanced by the co-presence of DAG [12; 70; 71]. PC can also be converted to DAG by two other routes besides PLD-derived. One is a PC-PLC catalyzed pathway that releases choline phosphate to produce DAG. The other is the conversion of ceramide to sphingomyelin catalyzed by sphingomyelin synthase (SMS). SMS manufactures sphingomyelin from phosphatidylcholine by catalyzing replacement of a glycerol molecular by ceramide, resulting in the release of DAG [58; 65].

The following table (Table 3) summarizes the main activators of the various PKC isoforms [12; 20; 72; 73; 74; 75; 76; 77; 78] (activation may vary across species and cell/tissue type):

<table>
<thead>
<tr>
<th></th>
<th>(\alpha)</th>
<th>(\beta)I</th>
<th>(\beta)II</th>
<th>(\gamma)</th>
<th>(\delta)</th>
<th>(\epsilon)</th>
<th>(\eta)(L)</th>
<th>(\theta)</th>
<th>(\zeta)</th>
<th>(\lambda)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(Ca^{2+})</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DAG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FFA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LysoPC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Tyrosine phosphorylation can mediate PKC activation in the absence of PS. PKC\(\alpha\), \(\beta\)I, \(\delta\), \(\gamma\), and \(\zeta\) can be tyrosine phosphorylated induced by hydrogen peroxide [79]. In skeletal muscle, PKC\(\delta\) undergoes insulin-induced tyrosine phosphorylation (activation) directly via Src tyrosine kinase which leads to insulin receptor (IR) activation [80]. Almost all PKCs can be proteolytically cleaved at the V3 region by the calcium-activated protease calpain to produce a cofactor independent free catalytic subunit known...
as protein kinase M (PKM). Calpain exists as two distinct isoforms that have a low (μ) and high (m) requirement for Ca\(^{2+}\), millimolar and micromolar respectively [43; 81].

Even though proteolysis is normally responsible for PKC downregulation, cleavage at the hinge region renders PKC constitutively active [82]. All PKCs except PKCδ contain PEST sequences (hydrophilic polypeptide segments enriched in proline (P), glutamic acid (E), serine (S) and threonine (T)) that target them for degradation by the ubiquitin/proteosome pathway. Dephosphorylation of activated PKCs predisposes them to this degradation pathway [43; 83; 84].

Oxidation is another way to regulate PKC activity. PKCs isolated from tissues have been shown to be oxidized by a variety of agents including peroxide, N-chlorosuccinimide and periodate. This oxidation requires Fe\(^{2+}\) and the resultant PKC is constitutively active. For oxidized cPKCs, Ca\(^{2+}\) and phospholipids are not required for this type of activation [43; 85]. Oxidation can also negatively regulate PKC activity. N-chlorosuccinimide and hydrogen peroxide reduce phorbol ester binding to PKC, indicating oxidation of the C1A domain [86]. Oxidation represents a unique type of regulation whereby initial oxidation causes constitutive activity and further oxidation causes inactivation [43].

Yet another mode of activity regulation is nitrosylation. PKC contains thiol residues which may serve as substrates for S-nitrosylation. PKC disulphide bridge formation following nitrosylated thiols result in irreversible inactivation and loss of phorbol ester binding. S-nitrosylation also prevents calpain access to PKC [43; 81; 87; 88]. It was reported that 100μM peroxynitrite leads to tyrosine nitration of PKCβII and PKCα [89].
Steroid hormone binding has also been shown to activate certain PKC isoforms. The C2 domain of PKCα binds aldosterone whereas the C2 domain of both PKCα and PKCδ bind 17 β-estradiol. Both interactions result in PKC activation [90]. PKCs α, γ, and ε are activated by 1 α,25-dihydroxyvitamin D₃. In this instance, the C1 domain (most likely the zinc fingers) is responsible for binding, not the C2 domain. This interaction mediates a steroid hormone rapid “non-genomic” response [91].

**PKC Intracellular Distribution**

The traditional model of PKC subcellular localization comes from studies of PKCα. In this model, PKCα is cytosolic in the basal state. Upon activation, it translocates to the plasma membrane. However, this is an oversimplification since PKC isoforms are not restricted to these two compartments. As mentioned above, PKCβII translocates from the plasma membrane to the pericentron in a biphasic manner [19]. PMA induced ceramide (formed from the salvage pathway) prevents translocation of PKCβII to the pericentron (a.k.a. juxtanuclear compartment) [92]. PKCs can also translocate to specialized membrane compartments such as lipid rafts or caveolae. Lipid rafts and caveolae contain sphingolipids, cholesterol, saturated fatty acids and signaling proteins. The differences between the two are that lipid rafts do not contain caveolin and caveolae do not contain glycosyl-phosphatidylinositol-anchored proteins. Caveola form flashlike invaginations in the PM by oligomerization and association with lipid rafts. Caveola are detergent-resistant as opposed to lipid rafts. PKCδ and PKCζ exert their signaling effect through these domains. PKCδ is involved in ceramide production. Ceramide is formed in lipid rafts and is involved in raft fusion. PKCδ activates acid sphingomyelinase (ASM) which hydrolyzes sphingolmyelin to ceramide [93; 94; 95].
This ceramide accumulation at the plasma membrane leads to recruitment and activation of PKCζ [96]. Several cell lines have cPKCs and nPKCs (both activated and resting) recovered in the caveolae fraction [19; 97; 98].

The Golgi complex is another area where PKC can localize. For example, PKCδ and PKCθ (both ceramide activated) induce apoptosis from the Golgi [99; 100]. PKCε modulates secretion (via binding to RACK substrate) from the Golgi complex [19; 101]. Interleukin-3 (IL-3) treatment has been shown to induce PKCβI and PKCβII nuclear localization in hematopoietic cells [102]. PKCη localizes to the ER, nuclear membrane and the Golgi upon PMA treatment and serum starvation [103]. PKCδ activated by phorbol esters causes mitochondrial translocation from the cytoplasm, which causes cytochrome c release and subsequent apoptosis in human U-937 myeloid leukemia cells [104]. Lastly, PKCβII can localize to the cytoskeleton (F-actin), which in turn increases its autophosphorylation [105]. Clearly, PKC intracellular localization is dependent on many factors including isoform, cell type, agonist treatment, etc. Therefore, their direct effect (as well as indirect) would seem to extend to nearly every intracellular organelle.

**PKC Anchoring Proteins and Substrates**

The first anchoring protein discovered that could localize PKC near its intended substrate was RACK1 (Receptor for activated C kinase 1). RACK1 specifically binds activated PKCβII with a \( K_d \) of 1nM in vitro. This binding specificity is due to the V5 region (as well as the C2 domain) of PKCβII which differs from PKCβI (as mentioned above). However, RACK1 is not a substrate for PKCβII. This interaction allows for proper localization and targeting of the substrate [106; 107; 108; 109]. An example of altered subcellular localization would be TCDD (2,3,7,8-Tetrachlorodibenzo-p-dioxin).
activation of RACK1 in cerebellar granule cells. This activation causes RACK1-PKCβII association and subsequent PKCβII translocation from the cytosol to membrane fraction [110].

Another important example of PKCβII-RACK1 interaction that has implications for insulin signaling is found in CHO cells. Here, PMA or dopamine D2 receptor agonist induced PKCβII activation causes its association with RACK1 and subsequent translocation to the Golgi apparatus. This movement is inhibited by PLC inhibitor ET18OCH3 [111]. As mentioned above, PLC is needed to generate DAG and IP3 (inositol triphosphate) for PKC activation.

An example of a PKCβII substrate would be lamin B. During proliferative stimuli in human promyelocytic (HL60) leukemia cells, activated PKCβII (via nuclear membrane activation factor (NMAF)) translocates to the nucleus and phosphorylates lamin B which leads to mitotic nuclear envelope membrane breakdown [112]. Recently, PKCβII was shown to phosphorylate substrate MARCKS (myristoylated alanine-rich C-kinase substrate), perpetuating skeletal muscle cell ISGT (insulin-stimulated glucose transport) [113].

An example for PKCβII that would include both the anchor and substrate comes from cardiac myocytes. Here, activated PKCβII translocates from the cytosol to the plasma membrane as well as the perinucleus where it binds RACK1. This association brings PKCβII near the L-type calcium channels where it can phosphorylate and inhibit the channel [114; 115]. Regulation of L-type calcium channels is important for insulin release from pancreatic β islet cells [116].
Anchoring proteins regulate PKC distribution but do not necessarily have to bind activated PKCs. Unphosphorylated, phosphorylated but inactive and phosphorylated and active PKCs are all targets. RACKs may bind active phosphorylated PKCs, but CG-NAP anchoring protein binds and localizes newly synthesized (unphosphorylated) PKCε to the Golgi/centrosome [117]. AKAP (A Kinase Anchoring Protein) position phosphorylated inactive PKCs near the substrate [118]. STICKs (Substrates That Interact with C Kinase) bind phosphorylated inactive PKCs and then release them following their phosphorylation [119]. PICK1 (Protein Interacting with C Kinase 1) has been shown to bind activated PKCα positioning it for eventual phosphorylation of GluR2 (AMPA-type glutamate receptor subunit 2) which results in GluR2 release from synaptic anchors and in receptor transport from the synaptic membrane [120].

**PKCβ Promoter**

Much of the knowledge of PKCβ promoter comes from the work of Hannun lab. Here, up to 2200 bp upstream of the human transcriptional start site was analyzed. The region near the start site is very GC-rich (>80%) and lacks identifiable TATA or CAAT boxes. TATA and CAAT elements are found further upstream at -530 and -395, respectively. This is reverse of the normal order. The most important area for promoter activity was -111 to -40. Some of the factors identified in this study are included in the following table (Table 4):
Another important discovery is that phorbol esters transcriptionally up-regulate the PKCβ promoter (basal promoter element) in K562 erythroleukemia cells. Up-regulation of the PKCβ promoter may be a way for phorbol esters to relieve the negative regulation that they have on PKCβ protein down-regulation [121]. The PKCβ promoter and its regulators need more illumination. This is especially paramount given that a functional PKCβ promoter polymorphism in humans has been linked to insulin resistance with reduced expression of PKCβII [122].

**PKC Inhibitors**

PKCs are attractive targets for therapeutic intervention given their various cellular roles. However, the plethora of interacting proteins and many secondary messenger systems coupled with cellular and tissue-specific variability for each PKC isozyme renders specific drug targeting difficult. The following table (Table 5) from Twelves et al. represents the main PKC inhibitors in use today [14]:

### Table 4. PKCβ Promoter Transcription Factors

<table>
<thead>
<tr>
<th>Factor</th>
<th>Binding site</th>
<th>PKCβ promoter location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct BP</td>
<td>ATGCAAT</td>
<td>ATGCAAT (-76)</td>
</tr>
<tr>
<td>Sp1</td>
<td>GGGCGG</td>
<td>GGGCGG (-94,-63)</td>
</tr>
<tr>
<td>E12/47</td>
<td>GCAGGTGG</td>
<td>GCAGCTGG (-110, -26, +18)</td>
</tr>
<tr>
<td>AP2</td>
<td>CCCCACCCC</td>
<td>CCCCACCCC (-330)</td>
</tr>
<tr>
<td>CTF/NF-1</td>
<td>GCCAAT</td>
<td>CCAAT (-395)</td>
</tr>
<tr>
<td>AP1</td>
<td>TGAGTCA</td>
<td>TGAGTGAC (-442)</td>
</tr>
<tr>
<td>TFIID</td>
<td>TATAAA</td>
<td>TATAAA (-530)</td>
</tr>
</tbody>
</table>
### Table 5. PKC Inhibitors

<table>
<thead>
<tr>
<th>Drug</th>
<th>Class</th>
<th>Admin</th>
<th>Specificity</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>Phorbol ester</td>
<td>Intravenous</td>
<td>Non-specific</td>
<td>PKC activator; in a phase I trial in haematological malignancy</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>Indolocarbazole</td>
<td>Intravenous</td>
<td>Poor specificity, inhibits other Ser/Thr and Tyr kinases</td>
<td>Preclinical</td>
</tr>
<tr>
<td>PKC412 (midospaurin)</td>
<td>Indolocarbazole</td>
<td>Oral</td>
<td>PKCs α,β,γ,δ,ε,η; also inhibits Tyr pathways</td>
<td>Potentiates treatment with doxorubicin or vinblastine; in phase II trials</td>
</tr>
<tr>
<td>UCN01</td>
<td>Indolocarbazole</td>
<td>Intravenous</td>
<td>cPKCs&gt;nPKCs</td>
<td>Potentiates treatment with cisplatin, mitomycin C, camptothecin or 5FU; in phase II trials</td>
</tr>
<tr>
<td>Go6976</td>
<td>Indolocarbazole</td>
<td>Intravenous</td>
<td>cPKCs&gt;nPKCs</td>
<td>Potentiates treatment with cytosine arabinoside, paclitaxel, tamoxifen or vincristine</td>
</tr>
<tr>
<td>Byrostatin 1</td>
<td>Macrocyclic lactone</td>
<td>Intravenous</td>
<td>Activates cPKCs &amp; nPKCs Acts as antagonist in presence of agonist</td>
<td>Potentiates treatment with cytosine arabinoside, paclitaxel, tamoxifen or vincristine</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>Nonsteroidal anti-oestrogen</td>
<td>Oral</td>
<td>PKCs α,β,γ non selective</td>
<td>Used in treatment of diabetic retinopathy</td>
</tr>
<tr>
<td>Bisindoylmaleimide</td>
<td>Indolocarbazole</td>
<td>Oral</td>
<td>PKCβ</td>
<td>Potentiates treatment with gemcitabine, 5FU, cisplatin or radiotherapy</td>
</tr>
<tr>
<td>LY317615 (enzasautaurin)</td>
<td>Indolocarbazole</td>
<td>Oral</td>
<td>PKCβ</td>
<td>Phase I and Phase II trials</td>
</tr>
<tr>
<td>ISIS3521 (aprinocarsen)</td>
<td>Antisense oligo</td>
<td>Intravenous</td>
<td>PKCα</td>
<td>Phase I and Phase II trials</td>
</tr>
<tr>
<td>ISIS9606</td>
<td>Antisense oligo</td>
<td>Intravenous</td>
<td>PKCα</td>
<td>Not developed further in clinic</td>
</tr>
</tbody>
</table>

Other pertinent drugs include Ruboxistaurin (LY333531) which is a macrocyclic bisindolylmaleimide drug developed by Eli Lilly being being tested for use as therapy in
diabetic macular oedema and other diabetic angiopathies, including diabetic retinopathy, diabetic peripheral neuropathy and diabetic nephropathy [123]. It is a competitive reversible inhibitor of PKCβ [124]. LY379196 is an analog of LY33531 which will be used in studies detailed in the RESULTS section. The following table (Table 6) represents the IC₅₀ spectrum for LY379196 [125]:

**Table 6. LY379196 Inhibitor IC₅₀ Spectrum**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCα</td>
<td>0.6</td>
</tr>
<tr>
<td>PKCβI</td>
<td>0.05</td>
</tr>
<tr>
<td>PKCβII</td>
<td>0.03</td>
</tr>
<tr>
<td>PKCγ</td>
<td>0.6</td>
</tr>
<tr>
<td>PKCδ</td>
<td>0.7</td>
</tr>
<tr>
<td>PKCε</td>
<td>5</td>
</tr>
<tr>
<td>PKCζ</td>
<td>48</td>
</tr>
<tr>
<td>PKCμ</td>
<td>0.3</td>
</tr>
<tr>
<td>Cyclic AMP Kinase</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Ca²⁺-calmodulin Kinase</td>
<td>5</td>
</tr>
<tr>
<td>Casein Kinase</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Src Tyrosine Kinase</td>
<td>4.4</td>
</tr>
</tbody>
</table>

The other PKC inhibitor used is CGP53353 (4,5-Bis(4-fluoroanilino)phthalimide) synthesized by Novartis. It is a selective inhibitor of both Epidermal Growth Factor Receptor (EGFR) as well as PKCβII. The following table represents the IC₅₀ spectrum for CGP53353 [126]:
<table>
<thead>
<tr>
<th>Table 7. CGP53353 Inhibitor IC$_{50}$ Spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGP53353</td>
</tr>
<tr>
<td>EGF receptor intracellular domain</td>
</tr>
<tr>
<td>v-abi kinase</td>
</tr>
<tr>
<td>c-src kinase</td>
</tr>
<tr>
<td>c-lyn kinase</td>
</tr>
<tr>
<td>c-fgr kinase</td>
</tr>
<tr>
<td>Csk</td>
</tr>
<tr>
<td>TPK-II B kinase</td>
</tr>
<tr>
<td>Cyclic AMP-dependent protein kinase</td>
</tr>
<tr>
<td>Phosphorylase kinase</td>
</tr>
<tr>
<td>Protein kinase CK-1</td>
</tr>
<tr>
<td>Protein kinase CK-2</td>
</tr>
<tr>
<td>Cdc2/cyclin B</td>
</tr>
<tr>
<td>PKC$\alpha$</td>
</tr>
<tr>
<td>PKC$\beta$I</td>
</tr>
<tr>
<td>PKC$\beta$II</td>
</tr>
<tr>
<td>PKC$\gamma$</td>
</tr>
<tr>
<td>PKC$\delta$</td>
</tr>
<tr>
<td>PKC$\varepsilon$</td>
</tr>
<tr>
<td>PKC$\zeta$</td>
</tr>
<tr>
<td>PKC$\eta$</td>
</tr>
</tbody>
</table>

The Mochly-Rosen lab has been working on drugs that disrupt protein interaction domains. First generation drugs were short peptides that bound to specific PKC RACKs to disrupt anchoring and subsequent function of PKC isozymes. Second generation peptide design inhibits PKC binding to its substrate. This is expected to block the function related to phosphorylation of that substrate without affecting PKC translocation, binding to RACK or phosphorylation of another substrate. These peptides are 6-10 amino acids long and are derived from one of the interacting domains thereby blocking the association of two proteins. Even though the binding surface for PKC is large and flat, these peptides are highly selective and effective. They serve as potential drugs in treating human disease [26].
Glucose Transporter 4

Glucose is a fundamental source of energy for eukaryotic cells [127]. It is the precursor for the synthesis of glycoproteins, triglycerides and glycogen as well as providing an important source of energy by generating ATP through glycolysis. Glucose is polar so it does not readily cross the hydrophobic plasma membrane. Therefore, specialized carriers are needed to bring glucose into the cell [128]. The uptake of glucose into a cell involves a family of transport proteins called GLUTs (glucose transporter) which shuttle sugar across the cell surface [127]. GLUT4 is the major insulin-responsive glucose transporter. GLUT4 is highly expressed in striated muscle and adipose tissue. It is responsible for postprandial removal of glucose from the circulation [127; 129]. In the basal state, GLUT4 slowly but continuously cycles between the plasma membrane and other intracellular compartments. Only 5% of total GLUT4 is localized at the plasma membrane in the basal state. Insulin treatment (or exercise in the case of skeletal muscle) induces an acute response where within 2-3 minutes, GLUT4 exocytosis dramatically increases concomitant with a small decrease in endocytosis. At this point, 50% of the GLUT4 have been relocated to the plasma membrane. Removal of the insulin signal subsequently decreases the rate of exocytosis and the trafficking of GLUT4 returns to basal status [127; 129; 130; 131]. Exocytosis and endocytosis occur without the need for continuing protein synthesis [132]. Insulin-stimulated glucose transport (ISGT) due to irregularities in GLUT4 trafficking is severely disrupted in type 2 diabetes [130].

GLUT4, like other GLUTs (13 known), is a 12 transmembrane domain-containing protein [133]. Figure 11 shows GLUT insertion structure in the plasma membrane [127].
GLUT4 is widely dispersed to many organelles throughout the cell. These include the plasma membrane, sorting endosomes, recycling endosomes, the TGN (trans-Golgi network) and vesicles that mediate the movement of GLUT4 between these compartments [127].

Molecular regulation of GLUT4 via insulin involves several discrete steps (Figure 12) [134]. The first step would be biogenesis of GLUT4 storage vesicles (GSVs). This is where the majority of GLUT4 is found and where the majority of GLUT4 translocates following insulin treatment (skeletal muscle and 3T3-L1 adipocytes) [135]. The second step would be translocation. Translocation is thought to involve cytoskeletal elements such as actin and microtubules [134]. In 3T3-L1 adipocytes, cortical actin remodeling/polymerization and well as actin comet tailing (representative of actin-based motility) on GLUT4-containing vesicles was critical for insulin-stimulated translocation [136; 137]. Also in 3T3-L1 adipocytes, the kinesin motor (moving toward plus end [plasma membrane] of microtubule) was critical for GLUT4 translocation [138]. The third step is tethering. This is a low-affinity interaction between GSVs and the plasma membrane arbitrated by a tethering complex [134]. Tethering involves what is referred to as the “exocyst complex.” This complex is responsible for the initial interaction of GLUT4 vesicles with the plasma membrane in 3T3-L1 adipocytes. Exo70, Sec6 and Sec8 are some of the recently identified components of the exocyst complex [139]. The fourth step is docking. This is the assembly of the trans SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) Receptor) complex. It is the final committed step before fusion between GSVs and the plasma membrane [134]. In 3T3-L1 adipocytes, VAMP2 (vesicle-associated membrane protein 2) and
VAMP3/cellubrevin represent v-SNARE proteins (protein complexes in the vesicle compartment) and syntaxin 4 represents t-SNARE proteins (receptor complex at the plasma membrane) [140]. The fifth step is fusion. This is where the lipid bi-layers of the GSV and the PM amalgamate [134]. Frohman et al. has shown that activated PLD1 (phospholipase D1) plays a rate-limiting step in insulin-stimulated fusion of GSVs with the PM in 3T3-L1 adipocytes [141]. Corroborating this finding is the observation that PA (product of PLD1 mediated hydrolysis) serves as an anchor for mammalian components of the SNARE complex [142]. PA has also been purported to act as a fusogenic lipid by lowering the activation energy for membrane bending during generation and expansion of fusion pores [143]. PA is also capable of activating phosphatidylinositol 4-phosphate 5-kinase thereby increasing the levels of phosphatidylinositol 4,5-bisphosphate, critical for exocytosis [141]. In L6 skeletal muscle cells, insulin-stimulated PKCβII causes retention of PLD1 at the PM and subsequent fusion of GLUT4 [113]. GLUT4 can translocate and fuse with both caveolae and non-caveolar lipid rafts [144]. The sixth step is endocytosis [134]. GLUT4 has been shown to be internalized via clathrin-mediated endocytosis (CME). This has been demonstrated in 3T3-L1 adipocytes and L6 myotubes [132]. Interestingly, caveolae associated GLUT4 internalized faster than non-caveolar lipid raft after removal of insulin [144].

3T3-L1 adipocytes have been used as the gold standard for GLUT4 trafficking. In these cells, GLUT4 is mostly located in the perinuclear region and the cytoplasm to a lesser extent [134; 145]. Insulin induces a 10-fold increase in glucose uptake. Rat adipocytes have an even greater response (20-fold increase in glucose uptake) whereas
human adipocytes only respond with a 2-3 fold increase in insulin-stimulated glucose uptake. In L6 myotubes (the only skeletal muscle cell line that expresses GLUT4), GLUT4 is expressed mostly in perinuclear regions as well as being dispersed along discrete bodies in the cytosol. Insulin induces a 2-fold increase in glucose uptake in this cell line [145].

In 3T3-L1, as mentioned above, most GLUT4 reside in GSVs near the TGN. Even before GLUT4 is expressed in differentiated adipocytes, there is a reservoir compartment (what will eventually be the GSV). A protein called insulin-responsive aminopeptidase (IRAP) is localized to this storage compartment and has very similar trafficking to that of GLUT4. Once GLUT4 is expressed, it enters this storage compartment [130]. Regardless of whether insulin stimulation has occurred or not, GLUT4 and IRAP are not retained at the plasma membrane. They are rapidly internalized with half times of 3-10 minutes [130; 146]. GSV formation requires Golgi-localized γ-ear-containing Arf-binding protein (GGA) in order to sort GLUT4 from the TGN [147]. Sortilin (also part of the TGN and endosomal membrane) is also necessary for GSV formation as well as for GLUT4 protein stability [148]. Sortilin most likely serves as the cargo adaptor linking GLUT4 to GGA coated transport vesicles. Sortillin does this by binding to GGA’s VHS cargo-binding domain with its VHS consensus binding motif (DxxLL) [129]. In differentiating adipocytes, sortilin is expressed prior to GLUT4 [148].

The GLUT4 N-terminal FQQI domain and C-terminal LL domain are both required for proper trafficking of GLUT4. The FQQI domain is important in transferring GLUT4 away from the endosome system [149]. The LL domain is important in shuttling
GLUT4 between the TGN and GSVs [150]. Both domains are required for proper endocytosis [130]. Trafficking steps of GLUT4 can be summarized by Figure 13 [130]. It is important to note that the pericellular reticular GLUT4 storage compartment and dispersed vesicular GLUT4 storage compartment are further sub-classifications of GSVs. It is also noteworthy to point out that v-SNARE VAMP2 (in addition to IRAP) colocalizes with GLUT4 in PR-GSC. The ratio between the two proteins is kept fairly constant in the DV-GSC. This would suggest a role for VAMP2 in direct fusion with the PM [151]. aPKCs have been suggested to serine phosphorylate VAMP2, possibly localizing GSVs at sites of actin remodeling and subsequent PM insertion [152].

Figure 13 is not a hundred percent accepted model. There are two main models concerning GLUT4 storage and cycling in 3T3-L1 adipocytes [153]. Model 1 (which more closely resembles Figure 13) asserts that GLUT4 from every compartment eventually reaches the PM in the basal state, GLUT4 intracellular storage is dynamic and that GSV is distinct from the transferrin-positive TGN [154]. Model 1 also asserts that insulin promotes two routes for GLUT4 mobilization towards the PM. The direct route comes from the GSV. The indirect route goes from the GSV to the ERC to the PM [153]. The two routes possible to get to the PM are supported by the presence of VAMP2 in insulin-dependent GLUT4 recycling [140; 155]. Model 2 proposes that only a fraction of GLUT4 reaches the PM in the basal state, that insulin increases GLUT4 amount available for translocation, and that part of the transferrin receptor negative GLUT4 compartment interfaces with the TGN [156]. Increasing insulin causes more GLUT4 to accumulate at the non-cycling pool, which is then translocated to the PM through a single exit route (GSVs to PM). The non-cycling pool contains few “latent GLUT4 molecules” which are
not mobilized in response to insulin. It is hypothesized that these latent GLUT4 molecules are synthesized early in cellular life and exhibit non-insulin responsiveness [157]. The main differences between the two models are the extent to which GLUT4 recycles in the basal state, the presence of a static/latent pool of GLUT4, the TGN being a storage site for GLUT4 and the number of routes insulin-stimulated GLUT4 can take to get to the PM [153].

**Akt**

Protein Kinase B (Akt) belongs to a family of ubiquitously expressed serine/threonine kinases that was discovered by cloning using a probe specific for PKA [158]. Akt shares 65% homology with PKA and 77% homology with PKC. Three mammalian isoforms exist, α (1), β(2), γ(3) (Figure13), which are products of different genes [159]. The three isoforms share 90% homology between each other [160]. All three isoforms contain an N-terminal PH domain, which is a roughly 100 amino acid domain that can bind phosphatidylinositol lipids (e.g. PIP2) as well as PKC and other proteins during signaling [161]. Downstream of the PH domain is the catalytic domain and finally the C-terminal domain [159]. Rat Akt3 contains a truncated C-terminal domain whereas human Akt3 contains a full-length C-terminal domain [160; 162].

Akt was identified as an oncogene early on. First, Akt1 amplification was detected in gastric adenocarcinoma [163]. It was then shown that Akt2 was amplified in two ovarian carcinoma cell lines [164]. The breakthrough in Akt research came using the PI3K inhibitor, wortmanin. This led to the discovery that Akt was a downstream target of PI3K activated by PDGF and EGF [165; 166]. The lipid-binding PH domain of Akt was essential for this PI3K-mediated activation in response to these ligands [166].
Binding of the PH domain to lipids is not the only way Akt is activated. Phosphatase inhibitor experiments showed that Akt activity was controlled by reversible phosphorylations on both serine and threonine residues [167]. The next step was showing that insulin stimulation resulted in Akt phosphorylation at residues T308 and S473 [168]. At this point, complete Akt activation necessitates lipid binding via the PH domain as well as phosphorylation by at least one upstream kinase. Previous observations indicate that Akt kinase activity requires PI3K and that Akt is potently activated by products of PI3K, PIP2 and PIP3. This led to the discovery of PDK1 (3-phosphoinositide-dependent protein kinase 1) [169]. This ended up being the kinase which is responsible for phosphorylation of Akt at T308. However, the kinase responsible for phosphorylating Akt on S473 has remained controversial and only recently been proposed (mTORC2). Mutation of either T308 or S473 in Akt1 does not prevent the other residue from being phosphorylated in response to insulin, suggesting the phosphorylations can occur independently [170]. Akt phosphorylated at only the PDK1 site has 10% of the activity compared to when it is phosphorylated at both T308 and S473 [171]. However, the threshold of activity required for substrate phosphorylation may differ so that Akt phosphorylated at only T308 may be sufficiently active to phosphorylate certain targets [172]. Figure 15 illustrates the mechanism of Akt activation [57; 169; 173; 174; 175]. A controversial additional pathway for Akt activation has been proposed for skeletal muscle cells. In this two-step model for Akt activation, insulin triggers the assembly of β-arrestin-2, c-Src and Akt. This allows c-Src to phosphorylate Akt on Tyrosine 315 (Y315) and Y326 which is required for downstream phosphorylations of Akt at S473 and T308. This may not apply to fat cells
because in muscle and liver, but not fat, β-arrestin-2 expression is reduced ~50% in insulin-resistant animal models [176].

As with any kinase, there is a mechanism to turn it on as well as one to turn it off. Recently discovered PHLPP proteins put the brakes on Akt and PKC activation. PHLPP is a Ser/Thr-specific phosphatase (PH domain leucine-rich repeat protein phosphatase). Once Akt is phosphorylated, its activity no longer relies on secondary messengers. In contrast, PKC is only active when bound to DAG. Dephosphorylation of PKC would promote degradation [177]. PHLPP1 and PHLPP2 specifically dephosphorylate the hydrophobic motif (S473) of Akt, resulting in decreased activity. PHLPP needs the PDZ-binding motif in order to accomplish this dephosphorylation [177]. PHLPP1 and PHLPP2 dephosphorylate the hydrophobic motif of cPKCs and nPKCs but not aPKCs because they contain a Glutamic acid at this position [171]. As alluded to earlier, cells deficient in the mTORC2 complex have decreased PKC phosphorylation at the hydrophobic motif which suggests that this complex contributes to phosphorylation of PKC at this site [178]. This possibly puts PHLPP in opposition of mTORC2 [177].

There is abundant evidence that Akt plays a role in insulin-dependent glucose disposal by directing GLUT4 vesicles to the PM. Initially it was reported that insulin rapidly and persistently activates Akt in traditional target tissues such as muscle and fat [179]. The connection between Akt and insulin's metabolic actions was firmly established when constitutively active Akt mimicked insulin in eliciting high levels of glucose transport and GLUT4 translocation in adipocytes in the absence of insulin [180]. More specifically, insulin causes Akt association with GLUT4 vesicles in rat adipocytes which results in phosphorylation of associated proteins [181; 182]. Suspected proteins
phosphorylated by Akt include sortilin and SCAMPs (secretory carrier-associated membrane proteins) which are involved in membrane trafficking and fusion of GLUT4 [182]. SNARES are also thought to be an important Akt substrate [183]. Inhibiting Akt activity by antibodies, substrate peptides and dominant-negative constructs partially blocks insulin-stimulated GLUT4 translocation in fat and muscle [184; 185].

Insulin is responsible for a host of outcomes, besides glucose homeostasis, that are mediated at least in part via Akt. For instance, insulin mediates vasodilation of blood vessels promoting peripheral tissue glucose disposal [186]. Akt is able to exert its effect here by phosphorylating/activating endothelial nitric oxide (NO) synthase, thus increasing NO production [187].

Insulin mediates protein synthesis in muscle and fat cells by enhancing initiation and elongation steps in protein translation. Evidence suggests that Akt modulates the activity of translational components [179]. Constitutively active Akt increases protein synthesis in L6 muscle cells and 3T3-L1 adipocytes [188; 189]. Insulin-stimulated protein synthesis is blocked by dominant inhibitory Akt mutant in 3T3-L1 adipocytes [190].

Lipogenesis (converting incoming sugar to fatty acids) is an insulin regulated event. Akt is thought to play a crucial role by inhibiting GSK-3 (glycogen synthase kinase 3). GSK-3 normally phosphorylates ATP citrate lyase (decreasing its activity), an enzyme that catalyzes the conversion of citrate and CoA to acetyl-CoA and oxaloacetate in the cytosol. This step is a major source for the biosynthesis of fatty acids, cholesterol and acetylcholine [191]. In addition, Akt also increases the transcription of fatty acid synthase, an enzyme that catalyzes several steps converting malonyl-CoA and acetyl-
CoA to long-chain fatty acids [192]. Constitutively active Akt has been shown to induce high levels of lipogenesis in quiescent 3T3-L1 adipocytes [193]. At the same time Akt is turning on genes for lipogenesis, it has been reported to help block lipolysis in adipocytes by inhibiting hormone-sensitive lipase (HSL). HSL is activated by PKA phosphorylation, and PKA activity is activated by cAMP. Use of a dominant-negative mutant as well as constitutively active kinase showed Akt reduces cAMP concentrations by stimulating cyclic nucleotide phosphodiesterase PDE-3B via phosphorylation [193; 194].

Insulin promotes glycogen synthesis from the glucose that is driven into the cell. It does this by activating glycogen synthase (GS) which adds glucosyl groups to growing polysaccharide chains, which is the final step in glycogen synthesis. Akt is able to exert its effect on this pathway. GS is negatively regulated by C-terminal serine phosphorylations by GSK3. Akt is able to inhibit GSK3, thus relieving the inhibitory phosphorylation and activating GS [179]. In adipocytes, a GSK3β mutant that is insensitive to Akt results in insulin-stimulated GS suppression [195]. Similarly, constitutively active Akt in L6 skeletal muscle dramatically blocks GSK3 activity and can activate GS without insulin. In the same cells type, dominant-negative Akt resulted in over 50% inhibition of insulin-stimulated GS activation [189; 196].

Blood sugar levels are maintained by insulin through promotion of glucose uptake as well as suppressing hepatic glucose output. The liver is the main organ to respond to insulin in terms of reducing its glucose production. Akt is thought play a role here by suppressing glycogenolysis but promoting glycogen synthesis (likely similar to skeletal muscle and fat). Akt may exert influence on PEPCK (phosphoglucone
CARBOXYKINASE and G6Pase (glucose-6-phosphatase). PEPCK catalyzes the early committed step in gluconeogenesis and G6Pase regulates the terminal step for both gluconeogenesis and glycogenolysis before glucose is released from the liver. There is still controversy as to whether Akt really inhibits these genes [179]. Very recently, Akt activation was shown to stimulate APPL1 (Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1), which potentiates insulin-stimulated inhibition of hepatic glucose output [197]. In 3T3-L1 adipocytes, the interaction of Akt with APPL1 is required for insulin-stimulated GLUT4 translocation, even though insulin causes their dissociation [198].

A role for Akt in pancreatic cells has also been proposed. This is because pancreatic regeneration is associated with an increase in IRS2 and activated Akt in proliferating duct cells [199]. Overexpression of active Akt1 in β cells of transgenic mice leads to a significant expansion of β-cell mass (cell number and cell size) [200]. Insulin signaling via PI3K and Akt was critical in preventing ER stress and thus β cell dysfunction [201].

As if Akt did not do enough, it has also recently been implicated (Akt1) in adipocyte differentiation [202]. Figure 16 summarizes the pivotal role Akt plays across various tissues [179].

Physiological relevance of Akt in insulin resistance, and thus T2DM, extends beyond cell culture. Akt activity has been found to be reduced in adipocytes [203] and muscle [204] obtained from T2DM patients. However, there have also been antagonizing reports that Akt activity is normal from diabetic muscle tissue where PI3K activity is reduced [205]. These contradictory findings have also been reported in rodent
models of obesity and insulin resistance [179]. Mice with adipocyte insulin resistance had been found to have reduced IRS and PI3K activity but normal Akt activity [206]. In Zucker rats that were insulin resistant obese, activation of PI3K was much more impaired than either Akt1 or Akt2. In adipocytes, Akt2 activation actually increased slightly [207]. Etiology of T2DM is not well understood and this may have something to do with the heterogeneity of results. However, targeted disruption of Akt2 (expressed highly in muscle and fat) in mice presents a phenotype resembling human impaired glucose tolerance [208]. Akt2 knockout mice display severe impairment in whole-body glucose disposal [179]. Interestingly, Akt1 knockout mice do not display insulin resistance [209]. This suggests distinct physiological roles for different Akt isoforms.

Akt can regulate alternative splicing by acting as an SR protein kinase and directly phosphorylating the RS domain [210]. This has a major impact on the insulin signaling cascade as Akt can phosphorylate SRp40 [211] thus regulating PKCβ alternative splicing. Akt can also regulate other SR kinases such as Clk/Sty (CDC-like kinase 1) (LAMMER family of protein kinases) giving it an even broader role in insulin-mediated alternative splicing [212].

**Insulin Signaling and Involvement of PKC, GLUT4 and Akt**

To give a broad general overview, insulin signaling involves two distinct branches. One defined by the Rho-family GTPase TC10 (also referred to as CAP/Cbl pathway) and the other by phosphatidylinositol 3-kinase (PI3K) [213]. Briefly, the insulin receptor (IR) is a member of the family of transmembrane receptors with intrinsic tyrosine kinase activity [214]. Some or all of the insulin receptor is constitutively bound to lipid rafts [215]. The mature IR is composed of two extra-cellular α- and two
transmembrane β-subunits disulfide linked into an α₂β₂-heterotetrameric structure [216]. Alternative splicing gives rise to two IR isoforms, IR-A and IR-B [216]. IR-A is expressed in the developing fetus as well as adult pancreatic β-cells, whereas IR-B is expressed in adult muscle, adipose and liver. IR-A is regulated by IGF-II (insulin-like growth factor-II) whereas IR-B is regulated by insulin. Insulin binds to the extracellular α-subunits which induces a conformational change that allosterically regulates the intracellular β-subunit tyrosine kinase domain. Ensuing is a series of intermolecular trans-autophosphorylations generating multiple phospho-tyrosine sites [133; 216]. Tyrosine phosphorylation at the juxtamembrane Y960 is required for binding IRS1-4. This is important for propagating the insulin signaling cascade. Phosphorylation at Y1146, Y1150, Y1151 in the kinase activation domain relieves pseudosubstrate inhibition further enhancing the tyrosine kinase activity [216]. Other scaffolding proteins recruited to the IR are Gab1, Shc, SIRPS, Cbl and APS [133]. IRS2 knockout cells show a major defect in insulin-stimulated glucose transport [217]. IRS is able to bind IR via its C-terminal PH domain and phosphotyrosine-binding domain (PTB) [218]. This facilitates binding to PI3K, which has a pivotal role in metabolic and mitogenic actions of insulin and IGF1 [219]. Because of PI3K’s crucial role in propagating the insulin signaling pathway, it deserves further digression.

PI3Ks catalyze the transfer of the γ-phosphate of ATP to the D3 position of phosphoinositides. PI3Ks can be grouped into three classes based on substrate specificity [220]. Only class I PI3Ks have been shown to activate Akt in cells. In cells, class I PI3Ks prefer PtdIns(4,5)P₂ as a substrate. The resulting PtdIns(3,4,5)P₃ can then go on to give rise to PtdIns(3,4)P₂ via 5’ inositol phosphatases. Class I PI3Ks are heterodimers
made up of a roughly 110 kDa catalytic subunit (p110) and an adaptor/regulatory subunit. Class I PI3Ks that bind tyrosine kinases and heterotrimeric G-protein coupled receptors are referred to as Class IA and Class IB PI3Ks respectively. Class IA PI3Ks are diverse with three catalytic p110 isoforms (p110α, p110β and p110δ, each encoded by different genes) and seven adaptor proteins (generated by alternative splicing of three genes: p85α, p85β and p55γ) [170]. The p85 adapter subunit, starting from the N-terminus, contains a Src homology 3 (SH3) domain, a breakpoint-cluster-region homology (BH) domain flanked by two proline-rich regions and two C-terminal SH2 domains spaced by an inter-SH2 (iSH2) region. The iSH2 mediates tight binding between p85 to the catalytic subunit [221; 222; 223]. The catalytic subunit possesses intrinsic serine kinase activity. The regulatory subunit p85α S608 can be phosphorylated by the p110α catalytic subunit which reduces lipid kinase activity [224]. P110δ can not phosphorylate regulatory subunits but can undergo autophosphorylation [224]. As far as insulin stimulation is concerned, class IA PI3K activation is accomplished by translocation to the plasma membrane and binding with its two SH2 domains (one N-terminal, the other C-terminal) to tyrosine-phosphorylated pYMXM and pYXXM motifs on IRS [220; 225].

As mentioned, PI3K produces PIP₃, which binds to the PH domain of a variety of signaling molecules altering their activity and/or subcellular localization [226]. Phosphatidylinositol-3-phosphates regulate three main classes of signaling effectors: the AGC family of serine/threonine protein kinases (which include PKCs), guanine nucleotide-exhange proteins of the Rho family of GTPases and the TEC family of tyrosine kinases. PI3K might also activate the mTOR/FRAP pathway and be involved in PLD1 regulation, which leads to hydrolysis of phosphatidylcholine and increases in PA.
and DAG [225; 227]. The best characterized AGC target of PI3K is phosphoinositide-dependent kinase 1 (PDK1), one of the serine kinases that phosphorylates and activates Akt. PDK1 has an N-terminal kinase domain and a C-terminal PH domain which has a higher affinity (in vitro) for PtdIns(3,4,5)P3 and PtdIns(3,4)P2 than other PIs such as PtdIns(4,5)P2. Its affinity for PIs is higher than that of Akt [170]. PDK1 was discovered for its ability to phosphorylate Akt Thr 308 in vitro [169]. In unstimulated cells, PDK1 is mainly cytosolic with relatively little PM localization [228]. With agonist stimulation (e.g. insulin) PDK1 phosphorylates Akt1 on T308 and the equivalent residues in Akt2,3. This phosphorylation of Akt is enhanced over 1000-fold in the presence of lipid vesicles containing miniscule amounts of PtdIns(3,4,5)P3 or PtdIns(3,4)P2 but not PtdIns(4,5)P2 or other PIs [169]. Akt binds with its PH domain to PtdIns(3,4,5)P3 or PtdIns(3,4)P2 which alters its conformation so that T308 becomes accessible to PDK1 [170].

For a long time, there was no consensus as to the identity of the kinase “PDK2” or “hydrophobic motif kinase” responsible for S473 phosphorylation of Akt. Several candidates had been proposed including PDK1, integrin-linked kinase (ILK) and Akt itself and DNA-PKcs. However, the identity of the kinase complex responsible has now been firmly established as mTORC2 [174; 229]. Due to the pertinence of mTOR in this study, it necessitates further digression.

mTOR is a member of the phosphoinositide 3-kinase-related kinase (PIKK) family of proteins [230]. mTOR is a target of rapamycin, a macrolide antibiotic and immunosuppressant of the phosphoinositide kinase family. The mTOR kinase exists in two complexes, mTORC1 and mTORC2. mTORC1 consists of mTOR, mLST8, RAPTOR (regulatory-associated protein of mTOR) and PRAS40 (proline-rich Akt
RAPTOR positively regulates mTOR activity and acts as a scaffold recruiting mTORC1 substrates. PRAS40 negatively regulates mTORC1 activity depending on its phosphorylation state. Activated mTOR can phosphorylate PRAS40 relieving mTORC1 of substrate competition. The best known targets of mTORC1 include ribosomal protein S6 kinases (S6K1 and S6K2 in mammals) and eukaryotic initiation factor 4E (eIF-4E)-binding protein 1 (4E-BP1). S6K1 is activated by phosphorylation and regulates ribosomal protein translation and ribosome biogenesis. S6K1 also acts as a feedback inhibitor of insulin induced PI3K-Akt pathway. It does this by directly phosphorylating and inhibiting IRS1 on S270. Phosphorylation of 4E-BP1 by mTOR decreases 4E-BP1 affinity for eIF-4E leading to translation of cap-dependent mRNAs.

The mTORC2 protein complex consists of mTOR, RICTOR (rapamycin insensitive companion of mTOR), mLST8/GβL (mammalian LST8/G-protein β-subunit like protein), SIN1 (stress-activated-protein-kinase-interacting protein 1) and Protor (protein observed with Rictor) (collectively called mTORC2). Rapamycin acutely inhibits mTORC1 but is needed for much longer incubations to have an effect on mTORC2. mTORC2 can phosphorylate Akt S473 in 3T3-L1 adipocytes. This along with phosphorylation of T308 results in fully activated Akt. mTORC2 also phosphorylates Akt at the turn motif T450. mTORC2 regulates organization of the actin cytoskeleton through phosphorylation of PKCα. SIN1 maintains mTORC2 complex integrity thereby regulating phosphorylation of Akt S473. It is not known how the interaction between mTORC2 and Akt is facilitated. Alternative splicing of mSin1 gives rise to at least five isoforms, three of which
assemble into mTORC2 to generate three distinct mTORC2s. Two of these mTORC2s are regulated by insulin [239]. mTORC2 is also regulated by two tumor suppressors called tuberous sclerosis complex protein 1 and 2 (TSC1 and TSC2). Within the TSC1-TSC2 complex, TSC1 stabilizes TSC2, while TSC2 acts as a GTPase activating protein (GAP) for the small GTPase Rheb (Ras homolog enriched in brain). GTP bound Rheb activates mTORC1 which is involved in promoting cell growth and proliferation through its main substrate ribosomal S6 kinases (S6K1 and S6K2). The mTORC1 complex is inhibited if the TSC1-TSC2 complex is active by stimulating the conversion of Rheb-GTP to Rheb-GDP. The TSC1/TSC2 complex activates mTORC2 and thus Akt in a manner that is independent of Rheb, mTORC1 and mTORC1-mediated feedback effects on PI3K. Endogenous TSC1 and TSC2 co-immunoprecipitate with exogenously expressed mTOR [234; 240]. However, the TSC1-TSC2 complex can only associate with mTORC2, not mTORC1 [240]. In response to growth factors, Akt phosphorylates TSC2 directly. Phosphorylation of TSC2 impairs the ability of the TSC1-TSC2 complex to act as a GAP towards Rheb, which allows Rheb-GTP to accumulate and potently activate mTORC1. This will eventually lead to negative regulation of Akt because activated mTORC1 will target S6K1/2 which will block insulin signaling as mentioned above [234]. DEPTOR (dishevelled, egl-10, pleckstrin (DEP) domains and interacts with mTOR) is an mTOR interacting protein whose expression is negatively regulated by mTORC1 and mTORC2. DEPTOR normally functions to inhibit mTORC1 and mTORC2 pathways. However, when overexpressed, it relieves the negative feedback inhibition of Akt, thus causing Akt activation [241].
Many additional factors converge on mTORC1 (including mTORC2) thereby regulating protein synthesis as well as insulin signaling. Akt phosphorylates TSC2 at S939, S981 and T1462. The serine phosphorylations allow binding of the cystolic anchor 14-3-3. This disrupts binding between TSC1 and TSC2. Akt can phosphorylate PRAS40 at T246 resulting in dissociation of PRAS40 from mTORC1. PRAS40 is also a substrate of both mTORC1 and mTORC2. S183 and S221 are phosphorylation residue targets of mTORC1 and mTORC2, respectively. TNFα signaling to Akt, induces IKKα (inhibitor of nuclear factor-κB kinase α), which is able to activate Raptor and thus mTORC1. IKKβ, also downstream of TNFα signaling pathway, is able to inhibit TSC1 by phosphorylation at S487 and S511 [230]. mTOR is able to sense cellular energy levels by monitoring cellular the ATP:AMP ratio via AMP-activated protein kinase (AMPK) [242]. Cellular stress activates AMPK, especially when AMP levels are high. AMPK (phosphorylated and activated by LKB1) phosphorylates TSC2 at S1345, increasing its GAP activity towards RheB-GTP, inhibiting mTORC1. AMPK can also phosphorylate Raptor at S792, leading to 14-3-3 binding and inhibition of mTORC1 [230]. The Wnt pathway, involved in cell growth control, is linked to mTORC1 activation. Wnt signaling inhibits GSK3β. GSK3β phosphorylates and inhibits TSC2 on S1341 and S1337 (after priming phosphorylation of TSC2 on S1345 by AMPK). Akt and RSK (ribosomal S6 kinase) can also phosphorylate and inhibit GSK3β. Hypoxic conditions work to inactive mTORC1 through hypoxia-inducible factor 1α (HIF1α) and REDD1. REDD1 competes with TSC2 for 14-3-3 binding. Low nutrient conditions inhibit mTORC1. Rag proteins (Ras small GTPases comprised of four members) are capable of activating mTORC1 in an amino acid sensitive manner. RAG proteins form heterodimers of RAGA or RAGB
with RAGC or RAGD. During conditions where there are sufficient amino acids GTP-bound RAG complex activates mTORC1, likely by changing the subcellular localization of mTORC1 and bringing it into close proximity with RheB. The Ras-ERK pathway activates mTORC1 by phosphorylating TSC2 at S664 and S1798 by ERK and RSK (ribosomal S6 kinase), respectively. RSK can also activate Raptor via phosphorylations at S719, S721 and S722. Some of the many pathways converging on mTORC1 are displayed in Figure 17 [230; 242].

The conversion of PA from PC by PLD1 is integral for both mTORC1 and mTORC2 activation. Structural studies have shown that PA binds to the FRB (FKBP rapamycin binding) domain of mTOR. PA is in competition for this site with rapamycin which is associated with FKBP12 (FK506 binding protein 12). PLD1 through PA facilitates formation of mTOR complexes. mTORC2 binds PA more strongly than mTORC1 which explains why higher concentrations of rapamycin are needed to inhibit mTORC2 versus mTORC1 [243].

Growth factors stimulate mTORC2 activity with some mTORC2 subunits undergoing phosphorylation. The kinases responsible for these phosphorylations are unknown. There is preliminary evidence that Ras may be responsible for some of these phosphorylations [238]. mTORC1 and mTORC2 activity can be distinguished based on the phosphorylation status of mTOR. Activated mTOR is phosphorylated between its catalytic domain and FATC (Frap, ATM, TRRAP, C-terminal) domain near the C-terminus at T2446, S2448 and S2481 [61]. T2446 is phosphorylated in response to nutrient availability [244]. mTORC1 is predominantly phosphorylated on S2448 while
mTORC2 is predominantly phosphorylated at S2481. S2481 is a rapamycin-insensitive autophosphorylation site [61; 245].

Activated Akt has many target substrates involved in glucose homeostasis. SNARE associated protein synip has been identified as an Akt substrate. Phosphorylation of synip via Akt might allow for insulin-dependent dissociation of synip from syntaxin 4 which would allow SNARE pairing between syntaxin 4 and VAMP2 thereby facilitating GSV fusion with the PM in adipocytes [246; 247]. A very promising Akt substrate was identified using a phosphor Ser/Thr Akt substrate (PAS) antibody. This protein was AS160 (Akt substrate of 160kDa), originally named TBD1D4 [246]. AS160 is abundant in muscle and adipose tissues [246; 248]. There are six putative Akt substrate motifs on mouse AS160. In 3T3-L1 adipocytes, insulin stimulated (likely through Akt) phosphorylation in five of the six putative Akt substrate motifs (S318, S341, S570, S588, T642, T751) [249]. In muscle cells, insulin, AMPK agonists or contractile activity have been shown to cause AS160 and GLUT4 translocation [250]. AS160 contains two N-terminal PTB domains and a C-terminal Rab-GAP (Rab GTPase-activating protein) domain. This led to the hypothesis that Akt-mediated AS160 phosphorylation (and inactivation) regulates GLUT4 translocation via regulation of GAP activity towards Rab proteins [251]. A second Rab-GAP substrate of Akt is TBC1D1 (tre-2/USP6, BUB2, cdc16 domain family member 1), which regulates insulin-stimulated GLUT4 trafficking [252]. RUVBL2 (RuvB-like protein 2) has been identified as an AS160 substrate critical for insulin-stimulated phosphorylation of AS160 in 3T3-L1 adipocytes [253]. The current thinking is that Rab-GAP activity promotes hydrolysis of GTP to GDP by Rab proteins on GSVs. In the inactive form, GDP-bound Rabs are
unable to cause GLUT4 translocation to the PM. However, with insulin treatment, AS160 is phosphorylated which leads to inactivity of Rab-GAP. This would enable GSV-associated Rabs to load up on GTP and elicit GLUT4 translocation. So far, the Rab14 isoform has emerged as the most likely Rab responsible for GLUT4 translocation in 3T3-L1 adipocytes [252]. Rab 10 has also been implicated in adipose cells [254]. In L6 muscle cells, the AS160-substrate Rab8A isoform seems to be involved in GLUT4 translocation [255]. Rab8A and Rab10 are both able to bind myosin Va and Vb, molecular motors responsible for localization of Rabs and GLUT4 translocation [254]. Munc18c is a putative target of Akt-controlled Rab activity. Munc18c is a member of the Sec1p/Munc18 (SM) family of proteins. Munc18c is thought to keep syntaxin4 (t-SNARE) in a closed conformation. Rab activity causes dissociation of Munc18c from syntaxin4 allowing syntaxin4 to interact with VAMP2 (v-SNARE) and SNAP23 (23 kDa synaptosomal-associated protein) (t-SNARE) [129; 183]. Recent in vitro experiments also point to a Munc18c role in fusion by anchoring VAMP to syntaxin [129].

In adipocytes AS160 phosphorylation at Thr642 promotes interaction with 14-3-3 protein. This interaction is critical in insulin-stimulated (Akt-dependent) suppression of AS160's inhibitory role in GLUT4 translocation in adipocytes [252; 256]. 14-3-3 binding to AS160 in L6 cells is not enhanced as a result of insulin-stimulation. However, AMPK-mediated phosphorylation of AS160 Ser237 did enhance binding [257].

Using the same PAS antibody mentioned above, phosphoinositide 5-kinase PIKfyve (phosphoinositide kinase for five position containing a Fyve finger) was identified as a potential Akt substrate. It is phosphorylated at S318 (inactivated) in
response to insulin. This effect is blocked by wortmannin. Inactivated PIKfyve is associated with enhanced insulin-stimulated GLUT4 translocation [258].

In addition to Akt and its downstream effectors, PKCλ/ζ is downstream targets of PI3K signaling that have been suggested to play a role in insulin-induced GLUT4 translocation [259; 260; 261]. However, there is some controversy as to PKCλ/ζ's role in insulin-stimulated GLUT4 translocation [262]. After phosphorylation of PKCλ/ζ by PDK1, it is recruited to lipid rafts in a TC10-dependent manner via Par3 and Par6. Par6 and PKCλ/ζ both contain PB1 (Phox and Bem1) domains required for heterodimer complex formation. Par3 has three PDZ domains that specifically bind to both Par6 and PKCλ/ζ [263].

Before moving on to the other half of the insulin pathway, it should be noted that PI3K activation can be attenuated by PIP3 dephosphorylation via 3′ phosphatases such as PTEN (phosphatase and tensin homolog) [264] or 5′ phosphatases such as SHIP2 (SH2-containing 5′-inositol phosphatase 2) [265]. PTEN action on PIP3 yields phosphatidylinositol 4,5-bisphosphate whereas SHIP2 action on PIP3 yields phosphatidylinositol 3,4-bisphosphate [152]. Both PTEN and SHIP2 can negatively regulate insulin signaling [266; 267]. However, in 3T3-L1 adipocytes, PTEN, and not SHIP2, is able to inhibit PI3K-dependent insulin signaling [268]. Insulin action can also be attenuated by protein tyrosine phosphatases (PTPases) which catalyze dephosphorylation of the receptor and its substrates (e.g. IRS). The most prevalent is the cytoplasmic PTP1B. PTP1B−/− mice are resistant to diet-induced obesity [225]. In L6 skeletal muscle cells, Ghosh et al. have shown that ceramide-activated protein phosphatase (CAPP) is able to dephosphorylate Akt and SRp40 resulting in decreased
PKCβII alternative splicing. The PP1-like CAPP, in this case, is stimulated by TNFα, which in turn stimulates de novo and hydrolysis pathways of ceramide generation. This ceramide allosterically activates the CAPP responsible for suppressing PKCβII expression [269].

The other pathway critical for insulin-stimulated glucose uptake is the CAP/Cbl pathway. Upon insulin stimulation, Cbl is phosphorylated with the help of APS (adapter protein containing a PH and SH2 domain). APS has a PH domain required for membrane localization and an SH2 domain that interacts with the phosphorylated receptor. APS needs to be phosphorylated in order to recruit Cbl. It interacts with an atypical SH2 domain of Cbl. APS and Cbl are recruited to the receptor as dimers. Once at the insulin receptor, Cbl is tyrosine phosphorylated in the C-terminus. This tyrosine phosphorylation also requires another adaptor protein, CAP (Cbl-associated protein). CAP interacts with Cbl through a C-terminal SH3 domain [136; 270]. Once Cbl has been phosphorylated, Cbl-CAP dissociate from the receptor and translocate to lipid raft/caveolae microdomains in the PM. This is mediated by interaction of the CAP SoHo (sorbin homology) domain with flotillin [271]. Lipid raft-bound Cbl recruits another adaptor protein named CrkII. An SH2 domain of CrkII interacts with phospho-Cbl. CrkII forms a constitutive complex with C3G (CRK SH3-binding guanyl nucleotide-exchange factor (GEF)). C3G (acting as an insulin-stimulated GEF) catalyzes the exchange of GTP for GDP for the G protein TC10 [272]. TC10 has a C-terminal CAAX sequence specifying farnesylation and palmitoylation responsible for targeting to lipid rafts [273]. TC10 localization to lipid rafts (caveolin positive) is required for insulin-induced activation [273; 274]. Activated TC10 provides a second signal to GLUT4 in
parallel with activation of the PI3K pathway [272]. This may involve stabilization of cortical actin, critical for GLUT4 translocation [225]. Rac is the G protein equivalent to TC10 in skeletal muscle cells that is responsible for actin remodeling [275]. Several effectors of TC10 have been proposed. One is CIP4 (Cdc42-interacting protein 4) which contains 1 FCH domain, 2 coiled-coil domains and 1 SH3 domain. The FCH domain (part of larger F-BAR domain) interacts with microtubules, the F-BAR domain regulates membrane curvature and the second coiled-coil domain interacts with GTP-bound TC10. In addition, CIP4 interacting proteins include Gapex-5, a RasGAP and VPS9 domain-containing protein that functions as a guanine nucleotide exchange factor (GEF) for Rab31. Rab31 is a Rab5 subfamily GTPase involved in TGN-to-endosome trafficking. Insulin recruits the CIP4/Gapex-5 complex to the PM decreasing Rab31 activity in 3T3-L1 adipocytes [276]. Rab31 may possibly function in retention of GSVs, therefore, inhibition of Rab31 would allow for release of this GLUT4 population [129]. Gapex-5 is also part of the TC10/Gapex-5/Rab5 axis that mediates insulin-stimulated production of phosphatidylinositol 3-phosphate [PI(3)P]. PI(3)P is an important regulator of GLUT4 vesicle trafficking, regulating translocation of GSVs to the PM. To accomplish this TC10 (via Gapex-5) must activate Rab5, a multifunctional GTPase that recruits a network of effectors regulating internalization of proteins from the cell surface, homotypic fusion of early endosomes, formation of clathrin-coated vesicles and motility of early endosomes on microtubules. Phosphoinositide synthesis and turnover are also regulated by Rab5 via recruitment of two distinct PI3-kinases, VSP34 and PI3K-p110β as well as PI phosphatases including type II PI5-phosphatase and type 1α PI(3,4)P2 4-phosphatase. Because spatial and temporal distribution of phosphoinositides is critical
for ISGT, Rab5 is a critical regulator of insulin action. In order to accomplish Rab5 activation, several upstream processes must occur. Gapex-5 is bound to Rab31 (active) in the unstimulated state which promotes intracellular retention of GLUT4. Insulin activates TC10 which recruits CIP4/Gapex-5 to the PM. Dissociation of Gapex-5 from Rab31 (now inactive) allows for GLUT4 translocation to begin. At the PM, Gapex-5 (likely acting as a GEF) activates Rab5 by causing dissociation of Rab5 from RabGDI (Rab GDP dissociation inhibitor). Rab5, which is now GTP loaded (active), can assist in ISGT [277; 278].

TC10 also interacts with Exo70, a component of the exocyst complex. This is important for tethering and docking of secretory vesicles. Additional components of the exocyst complex are Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo84 and the C-terminal of a protein called Snapin which binds to the coiled-coil domain of Exo70. This complex is necessary for glucose uptake in 3T3-L1 adipocytes [152; 279]. Other factors important for propagating TC10-regulated tethering and docking include SAP97, a MAGUK (membrane-associated guanylate kinase) family member. Sec8 associates with the PDZ domain of SAP97 and recruits it to lipid rafts. SAP97 is expressed in lipid rafts (localization not influenced by insulin) and anchors the exocyst complex to lipid rafts [215]. The exocyst complex is non-fusogenic. Fusion involves the SNARE complex which is capable of inducing the fusion reaction. This includes VAMP2 (v-SNARE on GSVs) and Syntaxin4 (t-SNARE) and SNAP23 (t-SNARE). Syntaxin4 interacting partners are Munc18c, Synip and Tomosyn, all of which appear to inhibit syntaxin from binding VAMP or SNAP. Insulin-dependent Akt2 phosphorylation of Synip on S99 leads to dissociation of Synip from syntaxin4, allowing for assembly of SNARE-
complex. Activated PKCζ complexes with 80K-H and Munc18c to promote VAMP2 binding to syntaxin4 that enhances GLUT4 fusion. This suggests both a negative and positive regulatory effect of Munc18c. PLD1 product, phosphatidic acid (PA) accelerates the rate of fusion when incorporated with the syntaxin/SNAP23 acceptor membrane [129]. DOC2B (double C2-like domains, beta), a SNARE related protein, is a very recent discovery. It is a positive SNARE regulator of insulin-stimulated GLUT4 fusion. It has two C2 domains and translocates to the PM during insulin treatment in a Ca^{2+} dependent manner [280]

TC10 is also thought to influence actin cytoskeleton dynamics. 3T3-L1 adipocytes contain cortical actin that lines the surface of the PM. This cortical actin contains punctate filamentous F-actin that emanate from the organized caveolae-rosettes where TC10 is segregated. This F-actin structure is referred to as Cav-actin. Interference with TC10 abolished cav-actin formation. TC10 is thought to regulate both Cav-actin and cortical actin structures [281]. The organization of F-actin is entirely different in myotubes, which are present as stress fibers running longitudinally [152]. It is also thought that TC10 influences massive actin polymerization in the peri-nuclear regions via downstream effector N-WASP protein (neural Wiscott-Aldrich syndrome protein). TC10, along with caveolins and flotillin, is detected in the TGN endosomes localized in the peri-nuclear regions. This region (as mentioned above) is implicated as a major storage site for GSV in 3T3-L1 adipocytes [282; 283]. TC10 inhibition using c. difficile toxin B completely shuts down both cortical actin and peri-nuclear actin rearrangements and greatly reduces insulin-stimulated GLUT4 translocation [284]. N-WASP protein contains a VCA (Verprolin, cofilin, acidic) domain. When N-WASP is bound to TC10,
the VCA domain is exposed and activates the Arp2/3 complex resulting in de novo actin polymerization in response to extracellular stimuli [285]. This actin polymerization (actin comet-tails) is the driving force for vesicle movement and is involved in membrane trafficking events [286]. Activated TC10 differentially regulates two distinct population of F-actin in 3T3-L1 adipocytes. It depolymerizes cortical F-actin beneath the PM and greatly increases F-actin polymerization in the perinuclear region [282].

Insulin stimulation causes GSVs to migrate toward cortical actin along microtubules. The kinesin motor thought to be responsible for this is KIF5B (kinesin family member 5B). KIF5B is responsible for transporting GSVs along microtubules in a PI3K independent manner in 3T3-L1 adipocytes [287]. Work from several groups has helped to bridge the myosin motor, actin cytoskeleton and the exocyst complex as it relates to 3T3-L1 GLUT4 translocation to the PM. After GSVs have migrated along microtubules, translocation requires both the actin cytoskeleton and the rapid movement of GLUT4 along linear tracks which is likely mediated by molecular motors. A key component of the molecular motor near actin filaments is the unconventional myosin Myo1c (Myosin 1c) protein which is present in GSVs. Myo1c functions independent of PI3K and helps control the movement of GSVs to the PM [288]. Myo1c travels along the actin cable in order to reach the PM. This will likely take GSVs to F-actin where TC10 (and the exocyst complex) is located [136]. GTPase RalA mediates the coordination between Myo1c and the exocyst complex. RalA that is GTP-loaded (via insulin) interacts with the exocyst complex. RalA interaction with Myo1c is GTP-independent. Calmodulin binds GSVs and modulates the association between RalA and Myo1c through IQ motifs in a manner that depends on calcium-bound calmodulin. Myo1c may
recognize RalA as a cargo receptor on GSVs [289]. CaMKII (calcium/calmodulin-dependent kinase II)-mediated phosphorylation of Myo1c is necessary for ISGT to proceed. Myo1c phosphorylation is associated with elevated 14-3-3 binding and reduced calmodulin binding [290]. Binding of 14-3-3 to Myo1c does not inhibit its ATPase activity (which it obtained via phosphorylation). Insulin causes a calcium influx just below the plasma membrane which activates CaMKII. Interestingly, Rictor associates with Myo1c during insulin signaling and may provide a link for the regulated interaction of Myo1c and actin [291]. The interaction between Rictor and Myo1c is insulin independent. Their association is necessary for phosphorylation of actin filament regulatory protein paxillin and promotes cortical actin remodeling [292]. In addition to Myo1c, myosin Va and myosin Vb are also actin-based motors implicated in GLUT4 trafficking. Va is phosphorylated by Akt2, enhancing its association with actin [152]. Vb can bind Rab8A which is critical for localization of Rab8A and GLUT4 translocation [254]. How GSVs associate with the cytoskeleton (cortical actin mesh) is not clearly known. A prime candidate is α-actinin 4 (ACTN4). This protein is found only within remodeled actin but not along filaments in unstimulated cells. Insulin promotes colocalization of actin filaments, ACTN4 and GLUT4 as well as physical association between GLUT4 and ACTN4. It is thought that insulin-stimulated release of TUG (tether, containing a UBX domain, for GLUT4) from GLUT4 enables its interaction with ACTN4 [152]. TUG is responsible for sequestration of GLUT4 in GSVs in unstimulated 3T3-L1 adipocytes [293; 294].

The preceding insulin signaling cascades as it relates to glucose uptake are illustrated in Figure 18 [129; 133; 175; 225; 252; 276; 289; 290; 293; 295].
**Peroxisome Proliferator-Activated Receptor γ**

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of transcription factors that exist as three isoforms. PPARα is expressed mainly in brown adipose tissue, liver, kidney, heart and skeletal muscle and plays a major role in lipid catabolism [296]. PPARδ, expressed in many tissues, is involved in lipid metabolism and energy utilization [297]. PPARγ exists as two isoforms, PPARγ1 and PPARγ2 resulting from alternative promoter usage and mRNA splicing. PPARγ1 is more ubiquitously expressed, whereas PPARγ2 is to be found mainly in adipocytes. PPARγ2 is critical for adipogenesis and regulates genes involved in lipid storage and glucose metabolism. To list a few; acyl-CoA oxidase, aP2, PEPCK, malic enzyme, leptin, resistin, lipoprotein lipase and adiponectin [296; 298]. PPARγ1 has also shown to have some adipogenic action but not as much as PPARγ2 [299]. PPARγ3 has also been observed. Its expression seems to be confined to macrophages [300].

The structure of PPARγ is shown in Figure 19 [297; 301]. At the N-terminal end of PPARγ is the AF-1 region which is a ligand-independent activation function (AF-1) domain. The AF-1 domain can functionally synergize with the AF-2 domain [302]. The AF-1 region contains serine and threonine residues that can be phosphorylated. For example, MAPK (mitogen-activated protein kinase) family member ERK (extracellular signal-regulated protein kinase) can phosphorylate PPARγ at serine112 and cause inhibition of transcriptional activation [303].

After the AF-1 domain is the DNA-binding domain (DBD). Glucocorticoid receptor (GR) DBD is the current model representing the nuclear receptor superfamily.
Briefly, DBD folds into a globular domain made up of two nonequivalent zinc-finger structures. Each zinc atom is coordinated by four cysteines. The zinc-finger structure is necessary for DNA-binding activity [304]. Important are two α-helices. The N-terminal helix (P-box) directly interacts with the major groove of each DNA half-site (base specific contact). The C-terminal helix aids in stabilization. Residues that make up the dimer interface are located in the C-terminal zinc finger (D-box) (Figure 19) [301]. DNA binding is coupled to structural changes necessary for heterodimer formation. Heterodimerization depends on helical unfolding which occurs in the C-terminal extension (CTE) of the DBD. In the case of thyroid receptor (TR), two helices within the CTE are crucial for DNA binding and heterodimer assembly. A helix in the T-box completes the heterodimeric interface with the second zinc-finger helix of retinoid X receptor (RXR). A helix in the A-box makes extensive contacts with the DNA [301].

Next is the ligand binding domain (LBD). It is composed of many 12 α-helical globular domains. These domains form three anti-parallel helical sheets that combine to make an α-helical sandwich. The ligand binding pocket is located in the interior of this structure. Binding strength and specificity are based on hydrophobic interactions, hydrogen bonding and the steric size and shape of the binding pocket. For hormone receptors, the smaller the size the more discerning the binding pocket is towards ligands [301]. PPARγ has a relatively disordered pocket to accommodate different ligands [305]. At the end of the LBD is the ligand-dependent activation function (AF-2) that can bind coactivators. The coactivator interface of this domain is a hydrophobic groove formed by several helices of the LBD including helix 12 (called AF-2 helix). Coactivators can bind this groove through a LXXLL motif [301]. Agonists regulate LBD-coactivator
interactions by modulating both LBD and helix 12 conformations. Non-agonist bound PPARγ would inactivate the LBD causing it to exist in many conformations, few of which are the active [306]. Corepressor binding to PPARγ can also inhibit coactivator recruitment. Corepressors also contain LXXLL binding motifs enabling them to bind to the groove formed by the LBD and helix 12. However, unlike coactivators, the corepressor binding motif forms a long three-turn helix sterically blocking helix 12 from obtaining an active conformation [307]. Further stabilization of the LBD-corepressor complex occurs via antagonist ligand binding. This prevents helix 12 from gaining an active conformation as well as creates a larger binding surface for the corepressor LXXLL motif [301].

PPARγ heterodimerizes with RXR alpha (RXRα) to regulate transcription of PPARγ-responsive genes. PPARγ/RXR heterodimers bind to direct repeats (DR) of the consensus sequence (AGGTCA) separated by a single nucleotide sequence (DR1) called the PPARγ Response Element (PPRE). PPARγ binds to the 5’ half site while RXRα binds to the 3’ half site (polarity) [296; 308]. The PPRE contains an additional AAACCT motif upstream of the DR1 [309]. Generally, PPREs reside in upstream enhancer regions as opposed to the proximal promoter [310]. The heterodimer binds to the PPRE in a head-to-tail orientation, allowing them to accommodate small changes in the number of nucleotides spacing the two hexanucleotides [301]. In fact, binding of the heterodimer pair has been observed in DR0 and DR2 PPRE’s [311]. The heterodimer pair is able to bind to DNA without ligand activation. However, gene activation is inhibited due to the binding of corepressors. Upon ligand binding, corepressors are released and coactivators are recruited leading to transcriptional activation [309].
The PPARγ-RXRα complex structure was recently reported. In PPARγ, the CTE forms significant DNA interactions and is followed by two helical segments that reach the LBD. The RXRα CTE forms one of the dimer contacts with the PPARγ DBD. Otherwise, RXRα has no secondary structure which may be the reason it can promiscuously bind other nuclear receptors. Both DBDs have α-helices that directly bind to the half sites. Hydrogen-bonding occurs between the DBD and the major groove of the half-sites. The PPARγ LBD and DBD are closely positioned whereas the RXRα LBD and DBD are far apart with the space between them being occupied by PPARγ LBD. PPARγ LBD is the target of most drugs since it is the centerpiece around which all other domains are positioned.

Polarity (as mentioned above) is mainly determined by the PPARγ CTE’s affinity for the 5’ flanking sequence. Another determinant of polarity is that both receptors have tight binding between their DBDs. Both DBDs have α-helices that can bind with the half sites. Hydrogen-bonding occurs between the DBD and major groove of the half sites. PPARγ makes more base and phosphate backbone contacts than RXRα. The PPARγ hinge region also has extensive DNA interactions, binding to the upstream AAACT sequence. Besides the DBD-DBD interface, an additional dimer interface is created by the two LBDs. A third interface occurs between PPARγ LBD and the DBD CTE region of RXRα.

Heterodimerized PPARγ has a “Y” shaped pocket in its LBD that binds ligands. As alluded to before, the active conformation for the PPARγ-RXRα have their helix 12 properly positioned by ligands thereby facilitating coactivator docking. Coactivator binding is far from other protein-protein interaction sites. The PPRE is also far from
coactivator binding. This suggests that in order to recruit promoter-specific coactivators, PPREs may have to cooperate with other promoter elements [309]. Adding more complexity is the fact that an intact PPRE is not required for heterodimer binding and subsequent activation for some PPREs [296]. A phenomenon called the phantom effect has been reported whereby an RXR ligand can cause dissociation of corepressors and recruitment of coactivators. Here, the binding of a ligand to one receptor can cause a similar activating conformational change in the other receptor. The RXR-RAR (retinoic acid receptor) heterodimer has reported to undergo this type of activation [312]. However, it has not been observed yet for the PPARγ heterodimer.

Polyunsaturated fats and their metabolites have been identified as PPARγ ligands although none have established physiological relevance. The best characterized is 15-deoxy prostaglandin J2 (15-d-PGJ2). It binds PPARγ with a Kd (dissociation constant) in the low micromolar range and can activate PPARγ target genes at concentrations near the Kd. The problems with this ligand are that it has never been shown to exist in vivo and it may not be specific to PPARγ [313; 314]. Other natural ligands of PPARγ include component of oxidized low-density lipoprotein 9- and 13-hydroxyoctadecadienoic acid (HODE) and 12- and 15-hydroxyeicosatetraenoic acid (HETE) [313]. Recently, unsaturated nitrated fatty acids (nitroalkenes) have been shown to activate PPARγ. Specifically, nitrolinoleic acid (NO2-LA) and nitrooleic acid (NO2-OA) are potent PPARγ activators. They can activate PPARγ-dependent transcription in the nanomolar range [315]. The regioisomer 12-NO2-LA was identified as the most potent with an IC50 (0.41-0.6 μM) comparable to synthetic PPARγ agonist Rosiglitazone (IC50 = 0.25 μM)
Lysophosphatidic acid has also been suggested to be an endogenous PPARγ ligand [317].

PPARγ shares many of the same coactivators with other members of the nuclear receptor family. These include CBP/p300, the SRC family, TRAP220, PGC1α etc. [318]. Some coactivators, such as PGC1α, can bind in a ligand-independent manner [319]. Despite this, PGC1α’s activating role for PPARγ displays gene selectivity. Some of the corepressors capable of suppressing PPARγ activity include SMART, NCoR and RIP140 (nuclear receptor interacting protein1) [318]. The general mechanism for PPARγ associated gene regulation is shown in Figure 20 [320]. Ligand bound PPARγ heterodimer activates target promoters whereas without ligand activation the heterodimer is bound by corepressors that repress target promoters [320]. Another mode of PPARγ-mediated gene suppression is called ligand-dependent transrepression. This involves repression of pro-inflammatory genes such as nuclear factor-κB (NF-κB). Here, activated PPARγ monomer blocks the clearance of the corepressor complex. The mechanism is not precisely defined but is thought to involve ligand-dependent association with PIAS1 (protein inhibitor of STAT1). PIAS1 binding would induce SUMOylation of the PPARγ LBD which would enable it to maintain NCoR (nuclear repressor corepressor) on the promoter of inflammatory genes [297].

One of the most critical functions for PPARγ is being the “master” regulator of adipogenesis. The adipocyte is at the nexus of energy balance and whole-body lipid homoeostasis. PPARγ is induced during the differentiation from fibroblasts to adipocytes (both white and brown fat) [298; 318]. Adipose cells can not form without PPARγ. PPARγ advances adipogenesis by inducing a transcriptional cascade that includes such
members as CAAT/enhancer binding proteins (C/EBP) [318]. Adipogenesis initially starts by induction of C/EBPβ and C/EBPδ. These proteins bind to the PPARγ promoter and induce its expression [321]. PPARγ, upon ligand activation, induces expression of target genes involved in lipogenesis and adipogenesis. It also activates C/EBPα which can positively regulate PPARγ expression [318]. C/EBPα cannot induce adipogenesis without PPARγ [299]. Many genes are involved in lipogenesis and insulin sensitivity which have PPREs. Of interest is GLUT4, which is responsible for attaining full insulin responsiveness. Deletion of PPARγ in mature adipocytes leads to lipodystrophy and insulin resistance [322]. PPARγ2 deletion in obese and insulin-resistant ob/ob mice leads to decreased fat mass, severe insulin resistance and dyslipidemia [323].

**Thiazolidinediones**

Thiazolidinediones (TZDs) are a class of drugs that are used to treat T2DM by reversing insulin resistance in target tissues and reduce hyperinsulinemia [324]. Today, two members of this drug class, rosiglitazone and pioglitazone, are currently approved by the FDA to treat T2DM [318]. Lehmann et al. was the first to discover that TZDs were direct ligands for PPARγ [325]. TZDs are believed to increase insulin sensitivity mainly through binding PPARγ. There are three main reasons for this. First, clinical potencies of different TZDs correlate with potency of PPARγ activation [326; 327]. Second, non-TZD agonists for PPARγ also improve insulin sensitivity [328]. Third, mutations in PPARγ in humans are associated with insulin resistance [322; 329; 330].

Current evidence suggests that adipose tissue is the consequential tissue target responsible for TZD beneficial effects (Figure 21). The “lipid steal” hypothesis has been used to explain the mechanism of TZD. The rationale is as follows. Type 2 diabetes
correlates with increased plasma free fatty acids (FFA) and their inappropriate deposition on liver and skeletal muscle [318; 331; 332]. FFA and triglyceride (TG) accumulation in skeletal muscle translates to insulin resistance and compromised systemic glucose homeostasis [333]. Nonalcoholic fatty liver disease (NALFD) has a high correlation with the metabolic syndrome (which includes insulin resistance) [334]. White fat cell PPARγ activation is believed to boost its capacity to store dietary FFAs. As a consequence, the FFAs are turned into fat deposits and partitioned away from tissues thereby enhancing peripheral tissue insulin sensitivity. Consistent with this model, TZDs lower circulating FFA levels [335]. It has also been proposed that TZDs exert their anti-diabetic effect through modulation of cytokine expression.

The “lipid steal” contends that the primary site of TZD action is adipose tissue. This is evidenced by a study showing that the presence of white adipose tissue (WAT) is necessary for TZDs effects to materialize. However, in this study, TZDs was still able to reduce circulating lipid levels in mice suggesting other sites may play a role in lipid removal via fatty acid oxidation etc. [336]. Also, adipose specific PPARγ knockdown mice do not respond to TZD treatment. In this study, TZDs were not able to lower plasma FFAs without adipose tissue that expressed PPARγ [322]. Compensatory mechanisms such as oxidation may kick in when WAT is not around.

Another question that arises from the “lipid steal” hypothesis is whether there is adipose hyperplasia or hypertrophy. In cultured cells, PPARγ agonists induce adipogenesis. Patients taking TZDs tend to gain weight, some of which is attributable to an increase in fat mass [337]. This might seem at odds with helping improve insulin sensitivity. However, TZDs simulate fat cell differentiation of smaller, more insulin-
sensitive adipocytes that promote adipocyte glucose uptake [338; 339]. In addition, the
added adipocyte load is predominantly in the subcutaneous fat with a slight decrease in
visceral fat [338; 340]. Individuals with upper-body (central) obesity (excess
subcutaneous abdominal and especially visceral fat) tend to be have metabolic and
cardiovascular complications [341]. This can partly be explained due to the fact that
visceral fat is more lipolytically active. This means excess FFAs are released from
triglyceride stores into the circulation. This is compounded by the fact that visceral fat
drains directly into the hepatic portal vein, thereby delivering FFA to the liver causing
hepatic insulin resistance [341]. The excess FFA will also reach skeletal muscle [338].

There is another mechanism by which activated PPARγ can induce whole-body
insulin sensitivity which does not contradict “lipid steal.” This involves alteration of the
adipokines secreted from adipocytes. One of the most critical is adiponectin.
Adiponectin is produced exclusively from adipocytes and is a direct target for regulation
by PPARγ [342]. TZDs induce adiponectin mRNA and plasma protein levels in rodents
and humans [343]. Further, TZDs induce a preferential switch to the high molecular
weight adiponectin (as opposed to the low molecular weight type), which is the active
form responsible for reducing serum glucose levels [344]. In mice, adiponectin
treatment leads to suppression of hepatic glucose output and improved glucose disposal
[345]. Mice lacking adiponectin display impaired TZD responses [346]. TZD activation
of PPARγ can also repress expression of adipokines inversely related to insulin
sensitivity. These include tumor necrosis factor (TNF) α and resistin which are thought
to cause insulin resistance [347].
Skeletal muscle is the largest importer of glucose. TZD ability to improve overall insulin sensitivity must affect skeletal muscle glucose uptake. However, PPARγ has very limited expression levels in skeletal muscle. This means that the TZD effect is likely indirect. The “lipid steal” hypothesis would explain this through less circulating FFA and increased insulin sensitizing adipokines. Liver also express low levels of PPARγ and its activation in liver is associated with steatosis. However, overall TZD benefits most likely outweigh this side effect [318].

Another area of PPARγ activation affected by TZDs is macrophages. Infiltration of adipocytes by macrophages is thought to lead to increased inflammatory factors that cause insulin resistance such as IL-1β, TNFα and IL-6. PPARγ is induced during the differentiation of monocytes into macrophages and is highly expressed in activated macrophages [348]. Macrophages can undergo activation to inflammatory (M1) macrophages which are characterized by production of IFN-γ (Interferon) and IL-12. They can also undergo alternative activation to anti-inflammatory M2 macrophages characterized by the production of arginase I and IL-10 [348]. Macrophage-specific PPARγ expression was shown to inhibit inflammation and increase insulin sensitivity (muscle and liver) as well as reduce atherosclerotic lesion size [349; 350]. Figure 21 illustrates the global as well as molecular insulin sensitizing benefits of TZD action on the major TZD target tissues.

**Peroxisome Proliferator-Activated Receptor γ Coactivator 1 α**

Peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) was originally identified as the coactivator of PPARγ [319]. PGC1α has since been shown to increase the transcriptional activity of PPARγ and variety of nuclear receptor families.
including oestrogen receptor, retinoid X receptor, mineralocorticoid receptor (MCR), glucocorticoid receptor (GR), liver X receptor (LXR), pregnane X receptor (PXR), the constitutive androstane receptor (CAR), vitamin D and thyroid hormone receptor families [351; 352]. PGC1α can also bind unliganded nuclear receptors such as PPARγ, orphan hepatocyte nuclear factor (HNF) 4α, farnesoid X receptor (FXR), and oestrogen-related receptor (ERR) α [352; 353]. PGC1α also targets non-nuclear receptors involved in the insulin signaling pathway including FOXO1 (forkhead box O1), whose activation is required for gluconeogenesis [354]. Most receptor binding takes place through PGC1α’s three LXXLL (L1-L3) motifs (Figure 22). The L3 motif marks the beginning of the negative regulatory (NR) regions which aids in anchoring to PPARγ [352]. Recently, Li et al. showed that rosiglitazone-stimulated PPARγ has a preference for the PGC1α ID1 motif (L1 aa 144-148). This combined with the fact that PGC1α expression increases with Rosiglitazone treatment (via coactivation of PPARγ on the PGC1α promoter), suggests that the interaction between PPARγ and PGC1α is critical in mediating TZD benefits [355; 356]. After the PGC1α NR region is the central hinge region (amino acids 400-500), which contains the tetrapeptide DHDY. This is the host cell factor docking site [352]. Of interest, PGC1α has the MADS box transcription enhancer factor (MEF) 2C binding site which is required for coactivation of GLUT4 [357].

Besides being a powerful transcriptional coactivator, PGC1α can also bind histone acetyltransferase (HAT)-containing proteins at their N-terminal such as CBP, p300 and SRC-1 [358]. HATs acetylate histones and remodel chromatin allowing access for factors of transcription. Concomitant with recruitment of transcription activators, PGC1α is able displace repressor proteins such as histone deacetylase (HDAC) and small
heterodimer partner (SHP) [359; 360]. The C-terminal region of PGC1α binds the mediator complex (TRAP/DRIP complex) which is responsible for coupling pre-mRNA splicing and transcription due to its Ser/Arg-rich domain as well as its RNA binding domain [361; 362]. PGC1α's C-terminal region also has two RS domains (to bind SR proteins) and an RRM domain. Binding to SR proteins influences splice site selection during splicing. The RRM is implicated in the control of translational elongation as well as splicing. Both the RS and RRM synergize with the nuclear localization sequence (NLS) in translocating PGC1α to the nucleus [363]. PGC1α is diffusely distributed in the nucleoplasm including nuclear speckles, where hypophosphorylated inactive SR proteins concentrate [362]. Just like the RS of other proteins (e.g. SRp proteins), those of PGC1α harbor several Akt consensus site (RXRXXS/T) [352]. SRp40, for example, (which can bind to PGC1α) can be phosphorylated in this manner and participates in alternative splicing of PKCβII in an insulin-dependent manner [211; 362]. The ability of PGC1α to bind proteins involved in transcription and splicing suggests that it is a key mediator of co-transcriptional gene processing [362]. PGC1α affects alternative splicing only when it is recruited to complexes that interact with gene promoters [362].

Coactivation of mitochondrial gene transcription factors allows PGC1α (as well as PGC1β) to stimulate mitochondrial biogenesis. Some of the most critical are NRF-1, NRF-2, PPARα, PPARδ, ERRα and TR [364]. NRF-1 and NRF-2 are not only targets as transcription factors but their genes are subject to regulation by PGC1α. These genes are able to simulate the expression of mitochondrial transcription factor A (Tfam), a mitochondrial matrix protein necessary for replication and transcription of mitochondrial
DNA [365]. Control of both nuclear and mitochondrial genes leads to increased activity in fatty-acid β-oxidation, Krebs cycle, and oxidative phosphorylation (OXPHOS) [364].

White fat cells are needed for energy storage while brown fat cells are used for energy dissipation. PPARγ is needed for formation of brown fat and white fat. TZD stimulation has even been shown to promote the differentiation of brown fat [366]. PGC1α was discovered to turn on adaptive thermogenesis in brown fat. These processes include fuel intake, mitochondrial fatty-acid oxidation and heat production through uncoupling protein-1 (UCP1). PRDM16 is a coregulator necessary and sufficient for brown fat cell differentiation. PRDM16 dramatically increases PGC1α expression, UCP1 and other brown fat genes. PGC1α and PGC1β exhibit complementary function during brown fat differentiation through genes such as UCP1 as well as mitochondrial biogenesis. However, after development of brown fat, PGC1α and PGC1β do not always share complementarity in terms of function. For instance, PGC1α regulates cold inducible UCP1 in mature brown fat, PGC1β does not. Recently, LRP130 has been shown to be critical for the complementary actions of the PGC1’s during brown fat development [367].

Exercise training promotes switching of muscle type fiber. It increases the number of type I and type IIA muscle fibers which are red in appearance and contain a large number of mitochondria, more myoglobin and vascularization than type II B and type IIX muscle fibers. The former muscle type are resistant to fatigue and contract slower with a low peak force [368]. Endurance training or strength training both result in the release of calcium from the sarcoplasmic reticulum. This activates several important transcription factors such as cyclic-AMP-responsive-element-binding protein (CREB),
myocyte-enhancer factor 2C (MEF2C) and MEF2D, and members of the nuclear factor of activated T cells (NFAT) family. These factors lead to an increase in PGC1α which is probably involved in modulating metabolic fluxes in skeletal muscle in response to a decrease in ATP and altered fuel demands [369; 370]. PGC1α's interaction with AMP-activated protein kinase (AMPK) may mediate these metabolic fluxes. Activated AMPK phosphorylates PGC1α and induces de novo synthesis of additional PGC1α [370; 371]. Transgenic mice encoding PGC1α in skeletal muscle at levels of those in type I muscle fibers, show a definite switch towards both type I and type IIA muscle fiber [372]. These fibers are more resistant to fatigue than wild-type mice and are characterized by an increase in mitochondrial density and function, increased oxidative metabolism, increased expression of myofibrillar proteins characteristic of type I and type IIA muscle fibers [372]. PGC1α also increases glucose uptake which leads to an increase in glycogen stores post-exercise for future expenditure [373]. Substrate usage for energy shifts to fatty-acid oxidation [374]. Mice with skeletal muscle ablation of PGC1α have more type IIB and type IIX (glycolytic) muscle fibers and a lower capacity for endurance exercise compared with wild-type mice [375]. There is wide variation in the PGC1α expression in people's human muscle. This may have implications in terms of susceptibility to metabolic diseases such as insulin-resistance [376].

Serine/Arginine-rich Proteins

Serine/arginine-rich (SR) proteins are splicing factors characterized by their arginine-serine (RS) dipeptide rich domain [377]. SF2/ASF was the first SR protein identified [378]. The term ‘SR protein’ was coined following identification of RS domain-containing proteins using the antibody mAb 104. These SR proteins were bound
to active sites of RNA polymerase II transcription [379]. Those SR proteins were SRp20, SRp40, SRp55 and SRp75, named after their molecular mass on an SDS/PAGE gel [380]. SR proteins have a modular structure with one or two copies of an N-terminal RRM (RNA recognition motif) that provides RNA-binding specificity (SRp40 has two RRMs). The C-terminal RS domain facilitates protein-protein interactions that bring the SR proteins to the spliceosome [381; 382]. Once localized to pre-mRNA, SR proteins can make contact via the BP (branch point) and the 5′ ss (splice site) [363; 383]. Also in the RS domain repertoire is the ability to act as an nuclear localization sequence (NLS) which shuttles SR proteins into the nucleus via the SR protein nuclear import receptor, transportin-SR [384; 385].

Classical SR proteins include SF2/ASF, SC35, SRp20, SRp75, SRp40, SRp55, 9G8. The criteria used to define classical SR proteins are structural similarity, dual function in constitutive and alternative splicing, presence of a phosphoepitope recognized by mAb104 and purification using magnesium chloride [377]. Additional SR proteins have been classified as well as SR-related proteins that may have additional roles besides splicing such as chromatin remodeling, transcription and cell cycle progression [386].

SR proteins are concentrated in nuclear speckles and are recruited to RNA polymerase II (RNAPII) during transcription [387]. Interactions between SR-related proteins and the CTD (C-terminal domain) of RNAPII have been reported [388]. Also, SR proteins are among the hundreds of proteins present in the RNAPII complex [389]. SC35 has recently been reported to promote RNAP II elongation in certain genes. This demonstrates the potential of SR proteins to couple transcription and splicing, even bidirectionally [390]. The importance of the transcriptional machinery in splicing has
produced a kinetic co-transcriptional coupling model whereby the rate of transcriptional elongation determines splice site selection, and this rate is partially determined by the recruitment of splicing factors to the CTD of RNAP II [391].

SR proteins influence splicing by binding to ESE’s and ISEs (exonic and intronic splicing enhancers) or ESSs and ISSs (exonic and intronic splicing silencers). Binding of SR protein to ESEs prevents exon skipping [392]. Cooper et al. has shown SRp40 binds to an ISE downstream of the PKCβII exon and promotes insulin-stimulated PKCβII exon inclusion [38]. Two models have been proposed to explain how SR proteins regulate exon inclusion. The ‘recruitment model’ proposes that ESE-bound SR proteins recruit and stabilize binding of U1 snRNP (small nuclear ribonucleoprotein) at the 5’ ss as well as U2AF65 at the 3’ss [393]. This is referred to as exon definition [394]. In the second model, ‘inhibitor model’, ESE-bound SR proteins antagonize activity of hnRNP-ESE recognition. The hnRNP (heterogeneous nuclear RNP) family of proteins binds RNA and is known to negatively regulate alternative splicing [377]. In addition to these two models, it has been shown that SR proteins can form a network of protein-protein interactions spanning the intron boundaries early in spliceosomal assembly. They can also bind ISEs at the branch point to promote pre-spliceosomal assembly [363; 383]. SR-related proteins can recruit U4/U6.U5 tri-snRNP to the pre-spliceosome via the RS domain [395].

SR proteins can also function in mRNA processing, mRNA nuclear export, NMD (nonsense-mediated decay) and translation [377]. Some of these processes are partly aided by nuclear SR proteins localized in nuclear speckles [396]. A subset of SR proteins (SF2/ASF, SRp20 and 9G8) are able to shuttle between the nucleus and the cytoplasm
that can further progress these processes [396]. Increased expression of SRp40, along with others, is implicated in nuclear aspects of NMD [397].

SR protein phosphorylation dynamics have an important part in pre-mRNA splicing. The RS domain of SR proteins is heavily phosphorylated on serine residues [377]. This regulates both subcellular localization and activity. Phosphorylation of the RS domain in SF2/ASF stimulates protein-protein binding with other RS domain-containing splicing factors such as U1-70K [398]. Dephosphorylation of SR and SR-related proteins is required for the splicing reaction to progress [399]. Insulin-induced PKCβII alternative splicing occurs as a result of SRp40 phosphorylation by Akt [211]. The phosphorylation status of the RS domain is important in post-splicing activities of SR proteins. A hypophosphorylated RS domain is needed for the interaction of nucleocytoplasmic shuttling of SR proteins with TAP/NFX1 nuclear export receptor [400]. SR protein kinases re-phosphorylate the RS domains enabling reentry into the nucleus [401]. Both shuttling and non-shuttling SR proteins associate with the pre-mRNA when hyperphosphorylated. Non-shuttling SR proteins are released after initial spliceosomal assembly. Dephosphorylation of the RS domain determines the sorting within the nucleus. Shuttling SR proteins are likely dephosphorylated during the transition from prespliceosomal complexes (E and A) to mature spliceosomal complexes (B and C). The shuttling SR protein may remain bound to aid in RNA export. Shuttling SR proteins must be dephosphorylated to be moved to the cytoplasm. Shuttling and non-shuttling SR proteins are recycled via different pathways [402].
PU.1/Spi1

PU.1/Spi1 is a hematopoietic-specific ETS (E26 transformation-specific) family member transcription factor involved in the development of all hematopoietic lineages [403]. ETS proteins are characterized by a conserved 85-residue domain that binds DNA. This domain recognizes purine-rich sequences containing 5′-(A/T)GGA(A/T)-3′ consensus. ETS proteins can tolerate variation in the flanking bases [404]. PU.1 is able to partner with spliceosomal proteins [405]. One such factor is TLS (translocated in liposarcoma) which binds PU.1 in vivo. TLS is an RNA-binding protein that influences the choice of alternative splice sites by favoring the selection of the proximal 5′-splice site of E1A pre-mRNA. In addition, the C-terminal region of TLS can bind SR proteins which will also influence splicing [406]. The DBD of Spi-1 is able to interact with poly(A) + RNAs and homoribonucleotide poly (G) polymers. However, it does not have RNA recognition specificity [405; 407].

Co-transcriptional Splicing

Pre-mRNA processing (capping, splicing and cleavage/polyadenylation) can be tightly coupled to transcription through RNA polymerase II allowing for much greater efficiency, both spatially and energetically [408]. However, co-transcriptional splicing is not obligatory. Introns can not serve as splicing substrates until both the 3′ and the 5′ ends are synthesized. Therefore, the time it takes for RNA pol II to synthesize each intron, defines a minimal time and distance along the gene where splicing factors can be recruited and spliceosomes formed. The time that it takes for RNA pol II to reach the end of the TU (transcription unit) defines the maximal time that splicing could occur co-transcriptionally [409]. Introns in the 5′ part of the transcript will likely be co-
transcriptionally spliced. However, modulation of RNAP II elongation rates by transcriptional activators influences alternative splicing [408].

Co-transcriptional splicing occurs due to RNA pol II being able to recruit both transcription and splicing factors by acting as a landing pad. In particular, the CTD of RNA pol II plays a central role in the coupling process. Dynamic changes in CTD phosphorylation play a role in RNA processing. Transcriptional activation of RNA pol II results in recruitment of splicing factors to sites of transcription [408]. This does not occur if the CTD is mutated [387].

Promoter identity is critical in determining alternative splicing. Factors recruited to the promoter affect the type of SR proteins recruited [408]. PGC1α, as mentioned before, is a pertinent example because it can bind SRp40 [362]. Promoters, through the factors they recruit, also affect RNA pol II elongation rates. This is important because a slower pol II elongation rate or internal pauses favors inclusion of alternative exons where there is a weak 3′ ss upstream of a strong 3′ ss [408]. It has been suggested that the rate of RNA synthesis affects its secondary structure which in turn affects splicing. This model suggests that pre-mRNA is free to fold within a limited timeframe after transcription [410].
Figure 1. Proposed effects of protein kinase C activation. The PKC family influences many pathways. Some isozymes can be activated by several different pathways, such as calcium or DAG production. Others are activated by one pathway such as ceramide. Many isozymes have overlapping substrate specificities in vitro and thus may interact to control signaling pathways that mediate cell-cycle control, proliferation, apoptosis, cellular adhesion and metastasis.
Figure 2. Domain structure of PKC isoforms. PKCs have a conserved kinase domain (blue) and ATP binding domain (turquoise) and more variable regulatory domains. All PKC regulatory domains (except PKCD,v) have a pseudosubstrate motif (yellow) upstream of the C1 domain (light blue). C1 domains bind DAG/PMA in cPKCs & nPKCs. aPKC C1 cannot bind DAG/PMA. C2 domain (dark green) binds Ca$^{2+}$ and phospholipids in cPKCs. nPKC C2-like domain does not bind Ca$^{2+}$. The Hinge region (cPKC, nPKC, aPKC) separates the regulatory and catalytic domain and can be cleaved (scissors). aPKC PB1 (orange) domain is for protein:protein interaction. PKCD PH domain (red) is involved in protein:protein interactions. ++ (cPKC C2) represents a basic patch that recognizes PIP2. W (tryptophan nPKC C1B) residue confers high DAG/PMA affinity. Tyrosine (Y in cPKC C1B) residue can be phosphorylated in place of phosphatidylserine binding.
Figure 3. PKC pseudosubstrate autoinhibition. Newly synthesized PKC is unfolded with the pseudosubstrate unbound to the substrate binding domain. However, upon phosphorylation, PKC adopts a closed conformation (left). Signals causing PKC engagement with its C1 and C2 domains results in pseudosubstrate release, allowing downstream signaling (right).
Figure 4. PKCβ secondary structure. Rat PKCβ gene structure (top). Close up of the C-terminal region (middle). The approximate sizes of introns and exons are given. Polyadenylation sites are designated AATAA for PKCβII and ATTAAA for PKCβI. Stop codon (TAA) 159bp into PKCβII exon. Possible splice site 2 is shown. Close up of PKCβII exon and 3′ UTR (lower). CATG/GTGACAT and CATG/GTGGCAT define the exon/intron junction of splice site 1 & 2, respectively. Polypyrimidine tract (pyr) between ss1 and ss2 is a 30bp pyrimidine-rich tract important for spliceosome assembly. Black dots represent short (10-14bp) purine-rich splicing enhancers. Stem loop structure reflects 44bp AUUUA stem loop structure. SRp40 depicts binding site for phosphorylated SRp40. Exon Splicing Enhancer (ESE) represents a 30bp purine-rich exon splicing enhancer.
Figure 5. PKCβ alternative splicing and alternative polyadenylation. 4 possible splice variants (I-IV) from rat brain are shown. Splice variant I reflects mature PKCβI mRNA. Splice variant II reflects mature PKCβII mRNA. Splice variant III reflects mature PKCβII mRNA with additional splice site. Splice variant IV reflects PKCβII mRNA from alternative polyadenylation selection.
Figure 6. PKC isoform phosphorylation sites. Alignment of activation loop, turn motif and hydrophobic motif phosphorylation sites in PKC isozymes. PKCβII structure is shown on top along with residues of phosphorylation. Below is an alignment of PKC isoform phosphorylation sites.
Figure 7. Biphasic cPKC translocation in short- and long-term exposure to agonists. (A) Activation of either G protein-coupled receptor or tyrosine kinase receptor (e.g., insulin receptor) leads to activation of PLC and subsequent hydrolysis of PIP₂ to membrane-bound DAG and soluble IP₃. IP₃ leads to an elevation of Ca²⁺ which then recruits PKC to the plasma membrane where it binds DAG and PS in the presence of Ca²⁺ and becomes activated. Activated PKC can then phosphorylate target substrates. PKCs return to the cytosol within 120s, which correlates with the metabolism and loss of DAG and the autophosphorylation of PKC C-terminus. (B) In chronic PKC stimulation, stimulation of receptors leads to activation of PLD. PLD cleaves PC to generate PA and free choline. PA can be converted to DAG via PAP. This type of DAG (mimicked by long-term PMA treatment) is stable in the plasma membrane for over 1 hour and induces prolonged PKC translocation. PKC can translocate (among other areas) to a novel juxtanuclear compartment, termed the “pericentron.”
Figure 8. Spatial, temporal and conformational regulation of cPKC. Newly synthesized cPKC associates with PM in an open conformation in which the pseudosubstrate (orange rectangle) is dislodged from the substrate-binding cavity on the kinase domain (green circle). The upstream kinase, PDK1 (light blue circle with rectangular PH domain) is docked on the C-terminal tail and initiates phosphorylation at the activation loop (Thr 500 in PKCβII). This is followed by mTORC2 mediated phosphorylation at the turn motif (Thr 641 in PKCβII) and then autophosphorylation of the hydrophobic motif (Ser 660 in PKCβII). Fully phosphorylated “mature” PKC is released into the cytosol in a closed conformation in which the pseudosubstrate occupies the substrate-binding cavity, thereby inhibiting the kinase (bottom left). External signals causing hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP2) cause PKC translocation to the PM. Binding of Ca2+ to the C2 domain (red) recruits PKC to the PM via a low-affinity interaction where the C1 (brown) domain binds DAG. The C1 domain (post calcium binding) also interacts with PIP2 and phosphatidylserine. Engagement of both C1 and C2 domains on the PM results in pseudosubstrate release allowing downstream signaling to take place. This membrane translocation is reversible determined by second messenger levels. The membrane-bound PKC conformation is highly sensitive to phosphatases. Prolonged exposure in this conformation results in dephosphorylation of PKC by PH domain Leucine-rich repeat protein phosphatase (PHLPP) (red) as well as PP2A which can potentially lead to degradation. Binding of Hsp70 (dark yellow) to the dephosphorylated turn motif on the PKC C-terminus causes stabilization and the ability to be re-phosphorylated, thus re-entering the pool of signaling-competent PKC. The phosphorylation step is constitutive* and the translocation and dephosphorylation are agonist-induced. PKC that is not rescued by Hsp70 is ubiquitinated by E3 ligases (e.g. RINCK) and degraded.
Figure 9. The main pathways of DAG generation and catalysis. DAG is inside the blue box (dashed). The principle enzymes involved are green. The enzymes dependent of extracellular signaling are circled. Groups that are changed during the reaction are shown in red (OH is hydroxyl; R, R' and R'' are fatty acids; P is a phospho group). The three-carbon backbone is in black. AT, acyltransferase; CDP, cytidine diphosphate; CEPT`, choline/ethanolamine phosphotransferase 1; CPT1, choline phosphotransferase; DGAT, diacylglycerol acyltransferase; TAG, triacylglycerol; MAG, monoacylglycerol; LPA, lyso-phosphatidic acid; PLA, phospholipase A; PAP, phosphatidic acid phosphohydrolase; LPP, lipid phosphate phosphatase; LPAAT, lyso-phosphatidic acid acyltransferase; DGK, diacylglycerol kinase; PLD, phospholipase D; PLC, phospholipase C; DGK, diacylglycerol kinase; SMS, sphingomyelin synthase; SM, sphingomyelin.
Figure 10. Agonist-induced membrane phospholipid degradation for acute and sustained PKC activation. Phospholipase C is responsible for acute DAG production by converting phosphatidyl 4,5 bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3). Phosphatidylcholine (PC) is the starting material for sustained agonist-induced DAG production. Phospholipase D converts PC to DAG. Phospholipase A_2 hydrolysis of PC generates free fatty acids (FFAs) or lysophosphatidylcholine (LysoPC). These products help potentiate PKC activation in the presence of DAG.
Figure 11. GLUT family PM insertion structure. There are 13 GLUT family members. All span the plasma membrane 12 times with both amino- and carboxyl-termini cytosolic. GLUT4 belongs to Class I GLUTs (1-4) which are glucose transporters. Class II GLUTs (5, 7, 9, and 11) are fructose transporters. Class III GLUTs (6, 8, 10, 12 and HMIT1) are structurally atypical and poorly defined. This diagram shows a homology plot between GLUT1 and GLUT4. Residues shown in red are unique to GLUT4.
Figure 12. GLUT4 trafficking itinerary. GLUT4 trafficking can be divided into six discreet steps. 1) Budding (biogenesis of GLUT storage vesicles (GSVs)), 2) Movement, 3) Tethering, 4) Docking, 5) Fusion and 6) endocytosis.
Figure 13. Proposed GLUT4 compartments and trafficking. GLUT4 (purple) and other rapidly recycling proteins such as transferrin receptors (red) are removed from the plasma membrane in clathrin-coated vesicles and enter the endosome system (1). Depending on cell-type, this recycling endosomal system can be both beneath the plasma membrane and perinuclear. GLUT4 can either enter the endosomal recycling compartment (2) or be sorted away from transferrin receptors and shuttled to the perinuclear reticular GLUT4 storage compartment [PR-GSC] (3). The PR-GSC is similar to the Trans-Golgi network (TGN) (5) but has a more restricted population of stored proteins. GLUT4 is in constant flux between the PR-GSC and the dispersed vesicular GLUT4 storage compartment [DV-GSC] (4). The DV-GSC is available for quick fusion with the PM. Newly synthesized GLUT4 from the TGN mostly goes to the PR-GSV. The PR-GSV can become saturated and is available within the endosome system for rapid translocation in response to insulin. Mutated GLUT4 (alterations in targeting domains) are targeted to the late endosomes and lysosomes (6). Black arrows indicate trafficking steps that lead to GLUT4 storage. This includes the biosynthetic route (TGN) as well as from the PM via the endosome system. Red arrows indicate probable route from storage to the PM and the route stimulated by insulin. Blue arrows indicate possible route of GLUT4 from storage to the endosomes and to the PM. Green arrows indicate routes mutant GLUT4 might take if it can not be retained in the PR-GSC or endosome compartment.
**Figure 14.** Domain structure of mammalian PKB/Akt isoforms. The N-terminal region contains the PH domain. The lysine (K) indicates the residue involved in catalysis. Regulatory phosphorylations in the catalytic and C-terminal domain are indicated.
Figure 15. Mechanism of Akt activation. Newly synthesized Akt (light blue circle) is phosphorylated on the turn motif (Thr 450 in Akt1) in an mTORC2 (dark orange) dependent manner. Signals generating phosphatidylinositol-3,4,5-triphosphate (PIP₃) engage Akt PH domain at the PM. Now the Akt activation loop (Thr 308) is exposed leading to PDK1 mediated phosphorylation and subsequent phosphorylation of hydrophobic motif (Ser 473) via mTORC2. Fully phosphorylated Akt is locked in an active conformation and translocates throughout the cell influencing downstream signaling (bottom right). Signaling is terminated by dephosphorylation of the lipid second messengers (via PTEN) and direct dephosphorylation of Akt. PHLPP (red) directly dephosphorylates the hydrophobic motif. Protein phosphatase 2 (PP2A) (red) has been shown to dephosphorylate Thr 308. Whether phosphorylation of Thr 308 or Ser 473 comes first is still being debated. It has recently been proposed that Ser 473 precedes phosphorylation of Thr 308.
Figure 16. Functional role of Akt in various tissues. The role of Akt in liver, pancreas, muscle and adipocyte is shown. Images courtesy of iStockphoto.com.
Figure 17. Mechanism of mTORC activation. mTORC1 phosphotransferase activity is stimulated by GTP-bound RHEB. RHEB is negatively regulated by the TSC heterodimer complex. TSC2 converts RHEB to its GDP-bound form (inactive). Amino acids cause the Rag protein complex to be in its GTP (active) form and subsequently activate mTORC1. The PI3K-Akt, Ras-ERK and IKK pathways converge and activate mTORC1 by inhibiting the TSC complex or PRAS40. RSK kinase and IKKα can activate mTORC1. The AMPK and GSK3β pathways negatively regulate mTORC1 by activating TSC2. AMPK can also directly inhibit Raptor. Both mTORC1 and mTORC2 can turn off PRAS40 inhibition of mTORC1. Activated by growth factors, mTORC2 is the kinase complex responsible for phosphorylating Akt on Serine 473 and thus full Akt activation. However, by activating mTORC1, mTORC2 causes a negative feedback loop that will blunt the PI3K-Akt signaling pathway. This is because mTORC1 activates S6K1 which will phosphorylate and inhibit IRS1. Downstream targets of mTORC1 are components of the translational machinery. mTORC1 is potently inhibited by rapamycin. Green arrows indicate activation. Red arrows indicate inhibition.
Figure 18. Model for insulin signaling. PI3K and TC10 signaling pathways converge on GLUT4 storage vesicles to cause their translocation, docking, tethering and fusion with the PM. Green arrows indicate activation. Red arrows indicate inactivation. Black oval arrows indicate change in activity level.
Figure 19. **Generalized structure of PPARγ1 and PPARγ2.** Starting at the N-terminus, the AF-1 domain is a ligand-independent transcriptional activation function domain. Then the DNA-binding domain consisting of two highly conserved zinc-finger motifs. In the middle is a hinge region allowing flexibility for PPARγ to dimerize and bind DNA. Next is the ligand-binding domain (LBD) which has a second dimerization interface. Finally, the ligand-dependent activation function domain (AF-2). Numbers on top indicate amino acid number. Below is the detailed structure of the DBD.
Figure 20. Ligand-dependent transactivation and active repression. PPARγ heterodimerizes with RXRα. (A) In the presence of ligands (natural or synthetic), PPARγ binds to coactivator complexes which result in the activation of target genes. (B) In the absence of ligands, PPARγ associates with corepressor complexes on target genes. These corepressors that also include histone deacetylases (HDACs) which lead to active repression of the promoter.
Figure 21. Improved insulin sensitivity via TZD mediated adipocyte PPARγ activation. TZD activation of PPARγ leads to lipid storage in adipose tissue through target gene activation. Adipokine secretion profile (center blue box) is also altered leading to increased peripheral tissue insulin sensitivity. Overall effects of reduced serum FFAs and beneficial adipokines include reduced hepatic glucose output and increased skeletal muscle glucose uptake. PPARγ also siphons macrophages towards an anti-inflammatory M2 phenotype and reduces their infiltration into fat. Reprinted with permission, from the *Annual Review of Biochemistry*, Volume 77 © 2008 by Annual Reviews [www.annualreviews.org](http://www.annualreviews.org)
Figure 22. Human PGC1α protein structure. The N-terminal region contains the activation domain (AD). Two of the LXXLL motifs are present in the AD. The third LXXLL motif lies in the negative regulatory region. After L3 there are three p38 MAPK phosphorylation sites located within the negative regulatory (NR) region. Host cell factor (HCB) is in a region that binds MEF2C protein. A novel DEAD box is located in human PGC1α (not mouse or rat). Directly downstream of the DEAD box are two putative casein kinase (CK) phosphorylation sites (CK1 and CK2). Rat and mouse contain only CK2. RS protein interaction domains followed by a nuclear localization (NL) signal and the RNA recognition motif (RRM). The C-terminal region has been shown to bind the TRAP220 mediator complex, splicing factors (U1-70K) and some transcription factors.
EXPERIMENTAL PROCEDURES

Cell Culture

Mouse 3T3-L1 preadipocytes obtained from American Type Tissue Culture repository, ATCC (Manassas, VA) were maintained and passaged as preconfluent cultures in DMEM high glucose 4.5g/L (Invitrogen, Carlsbad, CA) with 10% newborn calf serum (Sigma-Aldrich, St. Louis, MO) at 37°C and 10% CO₂. Once confluent, cells were differentiated (day 0) in DMEM high glucose with 10% fetal bovine serum (Atlas Biological, Fort Collins, CO), 10μg/mL bovine insulin (Sigma), 1mM dexamethasone (Sigma), and 0.5mM isobutyl-1-methylxanthine (Sigma). On day 2, media was replaced with DMEM high glucose, 10% FBS, and bovine insulin. Day 4 and afterwards, cells were cultured in DMEM high glucose plus 10% FBS. Media was changed every two days. Prior to insulin treatment for glucose uptake or other assay, 4 hour serum starvation was accomplished by using DMEM high glucose without FBS.

L6 rat skeletal myoblasts (obtained from Dr. Amira Klip, The Hospital for Sick Children, Toronto, Canada) were grown in αMEM (Invitrogen) with 10% FBS to confluence at 37°C and 5% CO₂. Myoblasts were fused into myotubes by changing media to αMEM with 2% FBS for 2-4 days post confluence. Serum starvation was accomplished by using αMEM for 6 hours.
Rat aortic vascular smooth muscle cells (A10, ATCC CRL 1476) were grown in DMEM low glucose with 10% FBS at 37°C and 5% CO₂. Once confluency was reached, cell synchronization was achieved by serum deprivation (with 0.5% FBS) for 48 hours.

HeLa cells (ATCC CCL-2) were grown in MEM (Invitrogen) with 10% FBS until confluent at 37°C and 5% CO₂. Serum starvation was achieved by incubation with MEM (no serum) for 6 hours.

**Overexpression/Minigene Transient Transfection**

Transient transfection was accomplished using TransIT-LT1 transfection reagent (Mirus Bio Corporation, Madison, WI) according to manufacturer’s protocol. Briefly, L6 cells were cultured in 6-well plates until 60-75% confluent. 250μL serum-free αMEM was mixed with 2.5μL TransIT-LT1 transfection reagent per 1μg DNA. DNA was added to this mixture and incubated for 30 minutes at room temperature. TransIT-LT1 Reagent – DNA complex was added to cells (with complete growth medium) and incubated for 48-72 hours. Plasmids used were as follows:

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<thead>
<tr>
<th>Gene of Interest</th>
<th>Donor</th>
<th>ID #</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1/myc-His A vector control</td>
<td>Invitrogen</td>
<td>V800-20</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Addgene Inc., (Cambridge MA)</td>
<td>8895</td>
</tr>
<tr>
<td>PPARγ E499Q</td>
<td>Addgene</td>
<td>8896</td>
</tr>
<tr>
<td>PGC1α</td>
<td>Addgene</td>
<td>1026</td>
</tr>
<tr>
<td>PGC1α delta CTD</td>
<td>Addgene</td>
<td>1030</td>
</tr>
<tr>
<td>SRp40 cDNA</td>
<td>Dr. Rebecca Taub</td>
<td>PMID: 9199345</td>
</tr>
<tr>
<td>SRp40 (myc)</td>
<td>Hercules Apostolatos</td>
<td>PMID: 15684423</td>
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<tr>
<td>HRS (myc)</td>
<td>Hercules Apostolatos</td>
<td>N/A</td>
</tr>
<tr>
<td>pCMV GFP</td>
<td>Addgene</td>
<td>11153</td>
</tr>
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</table>
siRNA Knockdown in L6 Skeletal Muscle Cells

L6 skeletal muscle cells were grown in 6-well plates. Cells were ready for transfection at 60-75% confluency. In one tube (per well), 5μL siPORT NeoFX Transfection Reagent (Applied Biosystems/Ambion, Austin, TX) was mixed with 95μL αMEM (no serum). This was incubated 10 minutes room temperature. In another tube, RNA and αMEM (no serum) were combined up to 100μL. Two tubes were mixed and incubated 10 minutes. siPORT NeoFX – RNA solution was added to cells. αMEM (no serum) was added to a final volume of 2.5mL. After 6-8 hours, media was replaced with serum containing αMEM.

<table>
<thead>
<tr>
<th>Gene silenced</th>
<th>Species</th>
<th>5′ – 3′ Silencer siRNA (Ambion) Sense Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ (ID#197812)</td>
<td>Rat</td>
<td>GCAUUUGUAUGACUCAUACtt</td>
</tr>
<tr>
<td>PGC1α (ID#114748)</td>
<td>Human</td>
<td>GCCAACACUCAGCUAGUUtt</td>
</tr>
<tr>
<td>SRp40 (ID#198849)</td>
<td>Rat</td>
<td>GCUCUUUAUAACUAACGtt</td>
</tr>
<tr>
<td>Silencer Negative Control #1 siRNA (#4611)</td>
<td>Rat, Mouse, Human</td>
<td>Proprietary</td>
</tr>
</tbody>
</table>

siRNA Knockdown in 3T3-L1 Adipocytes

Transfection of differentiated 3T3-L1 adipocytes was attempted using DeliverX Plus siRNA Reagent Solution (Panomics Inc., Fremont, CA). Day 8 3T3-L1 adipocytes were trypsinized and thinned on a 6-well plate. Transfection was performed the next day. The following is for transfection of 1 well of a 6-well plate. The siRNA working stocks were prepared in one tube by adding siRNA and Buffer-1 up to 50μL. The DeliverX Plus siRNA Transfection Reagent was sonicated (P/N DX0400, Panomics) for 5 minutes before usage to achieve a homogeneous solution. In another tube, 8μL DeliverX Plus
siRNA Transfection Reagent and 42μL Buffer-2 were combined. This second tube was vortexed and sonicated for 5 minutes as before. The two tubes were combined, vortexed and incubated at 37°C for 20 minutes. 100μL Buffer-1 and 100μL Buffer-2 were added to the siRNA-DeliverX Plus Transfection Reagent mixture. Just prior to usage, cells were washed several times with DMEM high glucose (no serum) to remove traces of serum. 300uL mixture was added to each well along with an additional 300μL DMEM high glucose (no serum) and incubated for 4 hours. After, 1mL of DMEM high glucose with 10% FBS was added. Medium was changed the next day. Knockdown was assessed at time points described. This transfection protocol was also attempted on cells not trypsinized (100% confluent) without success.

<table>
<thead>
<tr>
<th>Gene Silenced</th>
<th>Species</th>
<th>5’ – 3’ Silencer Select siRNA (Ambion) Sense Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCβII</td>
<td>Mouse</td>
<td>CAAUCAGAAUUCGAAGGAUtt (Custom)</td>
</tr>
<tr>
<td>PKCβII</td>
<td>Mouse</td>
<td>AGAGCUAAGUAGAUCCGUAtt (Custom)</td>
</tr>
<tr>
<td>Silencer Select</td>
<td>Rat,</td>
<td>Proprietary</td>
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<tr>
<td>Negative Control #1</td>
<td>Mouse,</td>
<td></td>
</tr>
<tr>
<td>siRNA (#4390843)</td>
<td>Human</td>
<td></td>
</tr>
</tbody>
</table>

**Oil Red O Staining**

3T3-L1 adipocytes were cultured on chamber slides and fixed with 10% formalin in PBS, washed with PBS, and stained for 1 hour at room temperature with 0.15% Oil Red O (Sigma) (60:40 mix of isopropanol and water). Slides were evaluated for the accumulation of lipid droplets.

**Cloning the Minigene**

Construction of the PKCβ heterologous minigene was initiated by Hercules Apostolatos (H.A.). PCRs were performed on rat cell DNA extracts to obtain the βII and
βI exonic sequence and their flanking intronic sequence (as depicted in Figure 36). The primers (sequences listed with rest of primers) used and the products (visualized in Figure 36) obtained are summarized in the following table (performed by H.A.):

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Restriction Enzymes (RE) Inserted</th>
<th>5’ intronic length (not including RE)</th>
<th>Exon length</th>
<th>3’ intronic length (not including RE)</th>
<th>Total size</th>
</tr>
</thead>
<tbody>
<tr>
<td>BII sense &amp; ss1 antisense</td>
<td>5’ XhoI, 3’ BamHI</td>
<td>103bp</td>
<td>216bp</td>
<td>80bp</td>
<td>399bp</td>
</tr>
<tr>
<td>BII sense &amp; ss2 antisense</td>
<td>5’ XhoI, 3’ BamHI</td>
<td>103bp</td>
<td>216bp</td>
<td>120bp</td>
<td>439bp</td>
</tr>
<tr>
<td>BII sense &amp; ss3 antisense</td>
<td>5’ XhoI, 3’ BamHI</td>
<td>103bp</td>
<td>216bp</td>
<td>346bp</td>
<td>665bp</td>
</tr>
<tr>
<td>BII sense &amp; ss4 antisense</td>
<td>5’ XhoI, 3’ BamHI</td>
<td>103bp</td>
<td>216bp</td>
<td>684bp</td>
<td>1003bp</td>
</tr>
<tr>
<td>BI sense &amp; BI antisense</td>
<td>5’ BamHI, 3’ XbaI</td>
<td>422bp</td>
<td>150bp</td>
<td>7bp</td>
<td>579bp</td>
</tr>
</tbody>
</table>

BII and BI fragments were digested with BamHI and ligated to produce a BII-BI fragment with relevant flanking intronic sequence (H.A.). These fragments were in turn digested with XhoI and XbaI (H.A.). The pTNT cloning vector (Promega, Madison, WI #L5610) was also digested with XhoI and XbaI (H.A.). The BII-BI fragments, now with overhangs were ligated into the pTNT cloning vector (Figure 37)(H.A. and E.K.). The resulting vectors were used by H.A. to perform in vitro transcription/splicing assays. For use in mammalian cells, the BII-BI fragments would have to be cloned into a vector containing a promoter responsive to mammalian cells. The pCMVTNT vector (Promega, #L5620) was used (Figure 38 & 39). The BII-BI fragments inside the pTNT vector were digested with XhoI and XbaI as was the pCMVTNT vector. The two were ligated. pCMVTNT BII-BI ss3 was tested by transfecting it into L6 skeletal muscle cells. However, using the CMV_FP1 and CMVRP1 primers (sequence listed below), many products were observed (Figure 40) due to the presence of a chimeric intron and T7-SP6-
β-globin exon within the pCMVTNT vector (Figure 41). To bypass this obstacle, pCMVTNT BII-BI plasmids were cut with BbsI and XhoI (Figure 40). The BII-BI fragments inside the pCMVTNT vector were PCRed out by using BbsI_CMV_F and ss1-ss4 reverse primers (sequence listed below). This PCR fragment was digested with BbsI and XbaI and ligated to the digested pCMVTNT BII-BI vector (Figure 42). Of the resultant clones pCMVTNT BII-BI ss1 and ss4 (minus the chimeric intron) were tested. Both the resultant ss1 and ss4 clones were transfected into L6 skeletal muscle cells and resulted in appropriate splice products (Figure 43 & 44) using CMV_FP1 & B1R3 primers (sequence listed below). The ss4 clone was the most desired clone because it had the most intronic sequence thereby allowing for more potential SR protein binding analysis. Because the ss4 splice products were in low abundance, the decision was made to use the more responsive ss1 plasmid and reclone the additional splice sites into the ss1 clone (Figure 45). pCMVTNT ss4 was PCRed using BbsI_CMV_F & ss4 antisense primers to release the BII half of the ss4 fragment. Before this experiment could proceed, an additional BamHI located on the pCMVTNT ss1, had to be eliminated. This was done by starting from scratch and digesting the original pCMVTNT vector with BamHI. This was blunted by Klenow fill-in and blunt ligated. The ss1 fragment was recloned from the pTNT ss1 vector as before (Figures 45). Now the PCR product and the pCMVTNT ss1 (-Chimeric intron) were digested with BbsI and BamHI. The two were ligated. The resultant vector, pCMVTNT ss4 (-CI, -BamHI), was proven to be functional.

To study the relationship between the PKCβ promoter and gene splicing, the CMV promoter was replaced with the human PKCβ promoter (Figure 46). To do this, PCR was
performed using bprom_5 and bprom_AS primers (sequence listed below) on Construct 5 PKCβ promoter Luciferase vector [121]. The resultant PCR product contained 2243bp of the human PKCβ promoter including the transcriptional and translational start site. The PCR product and pCMVTNT ss4 (-CI, -BamHI) were digested with BsrGI and PstI, then ligated. The resultant vector was named Bprom 2243 BII-BI ss4 (Figure 46). A subsequent truncated version was made to study a putative PPRE. To do this Bprom BII-BI ss4 was digested with BsrGI and SacI restriction enzymes, then subsequently blunted and relegated. The resultant clone was named Bprom 1143 BII-BI ss4 (Figure 47). Both Bprom clones were verified by restriction digestion and sequencing (Moffitt Cancer Center Sequencing Core). The splice products of Bprom 2243 BII-BI ss4 were compared to that of the vector with the CMV promoter by sequencing and were found to be identical (Figure 48).

**Mutation of Putative PKCβ Minigene PPRE**

Mutation of the putative PPRE within the minigene PKCβ promoter was accomplished using the QuikChange Lightning Site Directed Mutagenesis kit #210515 (Stratagene, La Jolla, CA) according to manufacturer manual. The putative PPRE was a DR2 (direct repeat with 2 base pairs separating). The putative PKCβ promoter DR2 was 5’ – AGCTCATCAGTTC – 3’. This DR2 PPRE was -1848bp upstream of the PKCβ transcript start sequence as defined by Vega Human TransView. The mutagenic forward primer named DR2_60_sense had the sequence of 5’ – AAG GCA GGA TGT GGG CTG TGA GCT TCC TGT GGT – 3’ (red font denoting mutated nucleotides). The mutagenic reverse primer was named DR2_60_anti with a sequence of 5’ – TGC TGA AGT AGA TTT CCT TTG AAC TGA TGA
CAC AAG GGA AGC TCA CAG CCC ACA TCC TGC CTT – 3’. The resultant clone was named Bprom 2243 DR2 mut BII-BI ss4.

**RT-PCR**

RNA was extracted using RNA Bee (Tel Test Inc., Friendswood, TX), according to manufacturer’s protocol. Reverse Transcriptase was performed using Omniscript RT kit (Qiagen, Valencia, CA, #205113) according to manufacturer’s protocol. PCR was performed using rTaq PCR kit (#R001A, Taqara Bio Inc., Japan). Primers used for PCR reactions were as follows:

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Species</th>
<th>Primer Direction</th>
<th>5’ – 3’ Primer Sequence</th>
<th>Overhang</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCβII &amp; PKCβI (endogenous)</td>
<td>Rat</td>
<td>Forward</td>
<td>GGC GTA TCC CAA GTC CAT GTC TA</td>
<td></td>
</tr>
<tr>
<td>PKCβII &amp; PKCβI (endogenous)</td>
<td>Rat</td>
<td>Reverse</td>
<td>GGG TTA GTA TAC GAG AAG CCA GC</td>
<td></td>
</tr>
<tr>
<td>PKCβII only (endogenous)</td>
<td>Rat</td>
<td>Forward</td>
<td>ATG AAA CTG ACC GAT TTT AAC TTC CTG</td>
<td></td>
</tr>
<tr>
<td>PKCβII only (endogenous)</td>
<td>Rat</td>
<td>Reverse</td>
<td>CGG AGG TCT ACA GAT CTA CTT AGC TCT</td>
<td></td>
</tr>
<tr>
<td>PGC1α</td>
<td>Rat</td>
<td>Forward</td>
<td>CCC ACG ACT CCT CCT CAT AAA GC</td>
<td></td>
</tr>
<tr>
<td>PGC1α</td>
<td>Rat</td>
<td>Reverse</td>
<td>GGC GCT CTT CAA TTG CTT TCT GC</td>
<td></td>
</tr>
<tr>
<td>PPARγ</td>
<td>Rat</td>
<td>Forward</td>
<td>GGC CCA CCA ACT TCG GAA TCA GC</td>
<td></td>
</tr>
<tr>
<td>PPARγ</td>
<td>Rat</td>
<td>Reverse</td>
<td>CCG CCA ACA GCT TCT CCT TCT CG</td>
<td></td>
</tr>
<tr>
<td>SRp40</td>
<td>Rat</td>
<td>Forward</td>
<td>CGC AGA CCT CGA AAT GAT AGA CG</td>
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</tr>
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<td>SRp40</td>
<td>Rat</td>
<td>Reverse</td>
<td>CGC CAC CCA CTT GAA GGA TAC TAC C</td>
<td></td>
</tr>
<tr>
<td>PKCβ promoter</td>
<td>Human</td>
<td>Forward</td>
<td>TTC TGT ACA GTT TAA CAG TAT CTG GAA C</td>
<td></td>
</tr>
<tr>
<td>PKCβ promoter</td>
<td>Human</td>
<td>Reverse</td>
<td>TTA CTG CAG GGG CTG TCA CTC GCC CAG CTG CTG</td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>Forward</td>
<td>CGT GCA GTG CTT CAG CCG CTA</td>
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<td>Type</td>
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</tr>
<tr>
<td>GFP</td>
<td></td>
<td>Reverse</td>
<td>CC GGG CGG TCA CGA ACT CCA GC</td>
<td></td>
</tr>
<tr>
<td>GATA 2</td>
<td>Mouse</td>
<td>Forward</td>
<td>CGG GGC TCT CCT GGT GTC TCT TAC</td>
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<tr>
<td>GATA 2</td>
<td>Mouse</td>
<td>Reverse</td>
<td>GGC GGG CAC ATA GGA GGG ATA GC</td>
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<tr>
<td>RUNX1</td>
<td>Mouse</td>
<td>Forward</td>
<td>CGG CCT CTC GCT ACC ACA CTT AC</td>
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<tr>
<td>RUNX1</td>
<td>Mouse</td>
<td>Reverse</td>
<td>TCA GCT CAG TAG GGC CGC CAC AC</td>
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<tr>
<td>HnrnpG</td>
<td>Mouse</td>
<td>Forward</td>
<td>CCG GGA AAC CAA CAA ATC AAG AG</td>
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</tr>
<tr>
<td>HnrnpG</td>
<td>Mouse</td>
<td>Reverse</td>
<td>CGT GGT GGA CCC CCG TAG CTA TC</td>
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</tr>
<tr>
<td>MZF1</td>
<td>Mouse</td>
<td>Forward</td>
<td>GCT TTG CCC ATG CCC TTG TAC TAC</td>
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</tr>
<tr>
<td>MZF1</td>
<td>Mouse</td>
<td>Reverse</td>
<td>CGC CAC ACA CGA ACG GTC TCT C</td>
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</tr>
<tr>
<td>Nkx2-5</td>
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<tr>
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<td>Mouse</td>
<td>Reverse</td>
<td>TGG GGT AGG GGT AGG CGT TGT AG</td>
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</tr>
<tr>
<td>Prrx2</td>
<td>Mouse</td>
<td>Forward</td>
<td>GCT CGC CGT GTC AAC CTC AGT G</td>
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</tr>
<tr>
<td>Prrx2</td>
<td>Mouse</td>
<td>Reverse</td>
<td>GAT GGA AAG GCA CGG GAG AG</td>
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<tr>
<td>AP2α</td>
<td>Mouse</td>
<td>Forward</td>
<td>CCC CGC CCT ACC AGC CTA TCT AC</td>
<td></td>
</tr>
<tr>
<td>AP2α</td>
<td>Mouse</td>
<td>Reverse</td>
<td>TTC CGC CAC CGT GAC CTT GTA C</td>
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</tr>
<tr>
<td>GATA 1</td>
<td>Mouse</td>
<td>Forward</td>
<td>CGC CCA CTG ACC ATG AGG AAA G</td>
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<tr>
<td>GATA 1</td>
<td>Mouse</td>
<td>Reverse</td>
<td>CGG GTG GGG AAG AGG GTT GTA G</td>
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<td>GATA 3</td>
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<td>CGG GTC TGG ATG CTC TCT TTC TTC</td>
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<td>SpiB</td>
<td>Mouse</td>
<td>Forward</td>
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</tr>
<tr>
<td>SpiB</td>
<td>Mouse</td>
<td>Reverse</td>
<td>CGG CCA GGG CTA CAC AGA GAA AC</td>
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<tr>
<td>Spi1</td>
<td>Mouse</td>
<td>Forward</td>
<td>TTC CAG TTC TCG TCC AAG CAC AAG</td>
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</tr>
<tr>
<td>Spi1</td>
<td>Mouse</td>
<td>Reverse</td>
<td>GGG CGA CGG GTT AAT GCT ATG G</td>
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<tr>
<td>Gene</td>
<td>Species</td>
<td>Primer Type</td>
<td>Forward/Reverse Sequence</td>
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<td>-------------</td>
<td>-----------------------------------------------------------------------------------------</td>
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<tr>
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<td>Forward</td>
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<tr>
<td>GAPDH</td>
<td>Mouse</td>
<td>Reverse</td>
<td>GGC TGC AGG AGA AGA AAA TG</td>
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<tr>
<td>GLUT4</td>
<td>Mouse</td>
<td>Forward</td>
<td>CGG TTC CTC ATT GGC GCC TAC TC</td>
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</tr>
<tr>
<td>GLUT4</td>
<td>Mouse</td>
<td>Reverse</td>
<td>CGA CTC GAA GAT GCT GGT TGA ATA G</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Rat</td>
<td>Forward</td>
<td>GTG GGC CGC TCT AGG CAC CAA</td>
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</tr>
<tr>
<td>β-actin</td>
<td>Rat</td>
<td>Reverse</td>
<td>CTC TTT GAT GTC ACG CAC GAT TTC</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Mouse</td>
<td>Forward</td>
<td>AGC CAC AAA CCA AAG AGA GGG AC</td>
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<tr>
<td>β-actin</td>
<td>Mouse</td>
<td>Reverse</td>
<td>GTC AAT CAC CTT ACC TGG CTT AC</td>
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</tr>
<tr>
<td>PKCβ</td>
<td>Mouse</td>
<td>Forward</td>
<td>GGA AAG GTA CCA CAA CTA AAA CAG</td>
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</tr>
<tr>
<td>PKCβ</td>
<td>Mouse</td>
<td>Reverse</td>
<td>GTG TTA GCT TGC TTG AGG GTG TC</td>
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<tr>
<td>PKCβ</td>
<td>Mouse</td>
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<td>GTG TTG CCT GCA TTG GAG AGT AC</td>
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</tr>
<tr>
<td>PKCβ</td>
<td>Mouse</td>
<td>Reverse</td>
<td>CAC AAA CCA CAA AGC AGA ATA GC</td>
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</tr>
<tr>
<td>PKCβ</td>
<td>Mouse</td>
<td>Forward</td>
<td>GAC CGA ATG AGA CAG TGC ACA AG</td>
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</tr>
<tr>
<td>PKCβ</td>
<td>Mouse</td>
<td>Reverse</td>
<td>CCT GGT GTT GAG TTT AGC ATC TG</td>
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<tr>
<td>PKCβ</td>
<td>Mouse</td>
<td>Forward</td>
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</tr>
<tr>
<td>PKCβ</td>
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<td>Reverse</td>
<td>CCA GCA ATG ACC AAA CGC CTA AG</td>
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<tr>
<td>PKCβ C4B2</td>
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Minigene primers

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<tr>
<th></th>
<th>Species</th>
<th>Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BII sense</td>
<td>Rat</td>
<td>Forward</td>
<td>GGG CTC GAG GAA AAA CCA CAC CCG GTT CC</td>
</tr>
<tr>
<td>Ss1 antisense</td>
<td>Rat</td>
<td>Reverse</td>
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</tr>
<tr>
<td>Ss2 antisense</td>
<td>Rat</td>
<td>Reverse</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>Rat</td>
<td>Reverse</td>
<td>CAT TTG GCA CTG TTA GAT GAT CAA AT</td>
</tr>
<tr>
<td>Bbs_CMV_F</td>
<td>pCMV</td>
<td>Forward</td>
<td>GCG AAG ACT CTT GCG AAA AAC CAC ACC CGG TTC C</td>
</tr>
<tr>
<td>BII sense</td>
<td>Rat</td>
<td>Forward</td>
<td>CTC GGA TCC ACA GGC GTT GTC ATT GAG TT</td>
</tr>
<tr>
<td>BII antisense</td>
<td>Rat</td>
<td>Reverse</td>
<td>CAT TAA TGT GTA GGT GAA TGT CTA GAC CA</td>
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<tr>
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<td>Human</td>
<td>Forward</td>
<td>TTC TGT ACA GTT TAA CAG TAT CAG GAA</td>
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<td>Human</td>
<td>Reverse</td>
<td>TTA CTG CAG GGG CTG</td>
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<tr>
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<td>pCMV</td>
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<td>pCMV</td>
<td>Forward</td>
<td>CAC CTG CAG AAG TTG GTC GTT AGG CAC TGG G</td>
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<tr>
<td>B1R3</td>
<td>Rat</td>
<td>Reverse</td>
<td>AGT TTG TCA GTG GGA GTC AG</td>
</tr>
</tbody>
</table>

**Silver Staining**

6% polyacrylamide (using 40% Acrylamide/Bis cat# 1610146 from Bio-Rad Laboratories, Hercules, CA) gel was prepared. Samples were run on gel and put in 10% ethanol (Fisher) for 3 minutes. The ethanol was replaced with 1% nitric acid (Fisher) for 3 minutes. Gel was then rinsed three times with water and soaked in 0.1% silver nitrate (Sigma) for 10 minutes. After a quick wash with water, gel was developed using 6% sodium carbonate (Fisher) with 0.2% formaldehyde 37% solution (Fisher). Reaction was terminated with addition of 10N glacial acetic acid (Fisher).
Agarose Gel

1% agarose gel (Fisher Scientific, Agarose Molecular Biology Grade cat# BP1356) was made with ethidium bromide to detect nucleic acids.

Western Blot Analysis

Cell lysates were combined with 2X Laemmli Buffer (Bio-Rad Laboratories) with additional SDS up to 8%. Lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gel proteins were electrophoretically transferred to Hybond-C Extra nitrocellulose membranes (Amersham, Piscataway, NJ). Membranes were blocked with Tris-buffered saline (Bio-Rad), 0.05% Tween 20 (Bio-Rad) containing 5% nonfat dried milk, and then incubated with primary and secondary antibodies. The only exception was when probing for PKCβII, where pig gelatin (Bio-Rad) was used for the blocking (3%), primary (1%) and secondary (1%). Detection was performed using SuperSignal West Pico Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL). Antibodies used are as follows: PKCγ C-19, PKCζ C-20, PKCδ C-17, PKCβI C-16, pPKCβII/δ Ser660, pPKCζ Thr 410, Akt1/2/3 H-136, pAkt1/2/3 Thr308, GLUT4 sc1608 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), PPARγ 81B8, pIRβ Tyr 1150/1151, PKCα #2056, phospho mTOR Serine 2481, PGC1α 3G6, PU.1 9G7, pAkt Ser473 #4058, Adiponectin C45B10 (Cell Signaling, Boston, MA), GLUT4 C-Terminus 07-1404 (Millipore, Billerica, MA), β-actin A5441, anti-flag M2 (Sigma). PKCβII (NH2–(GC) EGFSFVNSEFLKPEVKS-COOH), SRp40 (NH2-(GC) EVTFADAHRLPKLNE-COOH), and SRp55 NH2-(GC) GERVIVEHARGP RRDRD-COOH) were raised by BioSynthesis Inc. (Lewisville, TX) and purified using Nab Protein A Plus Spin Kit (Pierce #89948). Film bands were quantified using UnScan software (Silk Scientific, Orem, UT).
Co-immunoprecipitation

3T3-L1 adipocytes were harvested with Cell Lysis Buffer (Cell Signaling #9803) with added protease inhibitors (SigmaFast Protease Inhibitor Tablet, Sigma). 500μg cell lysate was pre-cleared with 50μL Protein A Magnetic Beads #S1425S (New England Biolabs, NEB, Ipswich, MA) for 1 hour 4°C with rotation. This step eliminated non-specific binding of protein to magnetic beads. Lysate was separated from beads using Magnetic Separation Rack (#S1506S, NEB). 5μg PKCβII antibody (sc-210, Santa Cruz) or GLUT4 (sc-7936, Santa Cruz) was incubated with pre-cleared lysate O/N 4°C with rotation. Lysates were now incubated with 50μL Protein A Magnetic Beads for 1 hour 4°C. Magnetic field was applied to separate beads from unbound lysate. Beads were washed three times with cell lysis buffer. 75μL 2X Laemml Buffer (Bio-Rad) with DTT was added to the beads. Samples were boiled for 10 minutes and loaded onto a 10% SDS PAGE gel.

Real-time PCR

Real-Time PCR reactions were performed using TaqMan Universal PCR MasterMix (Applied Biosystems (AB) Inc., Foster City, CA, #4304437) according to manufacturer’s protocol. Vic-labeled mouse β-actin was the endogenous control (AB, #4352341E). PKCβII primers and probe were custom made from AB. Forward primer 5’- GGAGATTTCAGCCACCTTATAACCA - 3’. Reverse primer 5’- GGTGGATGGC GGGTGAAAA - 3’. Fam-labeled probe (PKCβ2/PKCβ1 junction) 5’- TTCGCCCACAAGCTT - 3’. Real-Time PCR was analyzed on the ABI PRISM 7900HT Sequence Detection System (AB).
Glucose Uptake

[^3]H2-deoxyglucose uptake into differentiated 3T3-L1 cells was measured in six-well plates [411]. Briefly, cells were serum starved with DMEM high glucose for 4 h. Media was then changed to 1mL Krebs-Ringer HEPES (121mM NaCl; 4.9mM KCl; 1.2mM MgSO₄; 0.33mM CaCl₂; 12mM HEPES, pH 7.4) ± CGP53353 (Novartis, Basel, Switzerland) for 30 min ± 100nM pig insulin (Sigma) for 15 min.[^3]H2-deoxyglucose (1μCi per well) was added for another 4 min. The reaction was terminated by addition of KRH with 25mM D-glucose (Sigma) containing 10μM cytochalasin B (Sigma). Cells were washed, lysed with 0.1% sodium dodecyl sulfate, and radioactivity was counted using a Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter. Carrier-specific uptake was obtained by subtracting nonspecific diffusion of[^3]H2-deoxyglucose into the cells in the presence of 10μM cytochalasin B. Counts were normalized to protein concentration using the BCA protein assay (Pierce).

Subcellular Fractionation

Subcellular fractionation was performed essentially by the method of Elmendorf et al. [412]. Three 25cm plates of cells were used per condition in order to generate enough protein yield for the PM fractions. After treatment, cells were rinsed with HEPES-EDTA-sucrose (HES) buffer. All subsequence steps were performed at 4°C. Each plate was scraped in 2mL HES buffer with 1X protease (Sigma) and 1X phosphatase (Sigma) inhibitors. This was followed by dounce homogenizing with 50 strokes using a Potter-Elvehjem grinder. The homogenate was centrifuged at 19,000 g for 20 min. The resulting supernatant was centrifuged at 41,000 g for 20 min. The high-density microsomal fraction (HDM) pellet was resuspended in HES buffer. The
supernatant was centrifuged at 180,000 g for 75 min, yielding the low-density microsomal (LDM) fraction pellet. The pellet from the initial 19,000 g spin was layered onto 1.12M sucrose cushion in HES buffer and centrifuged 100,000 g for 60 min. This yielded a white band at the interface [plasma membrane (PM) fraction] and pellet consisting of nuclei/mitochondria (M/N fraction). The PM fraction was resuspended in HES buffer and pelleted at 40,000 g for 20 min. Protein levels in each fraction were quantified using the BCA protein assay (Pierce).

Plasma Membrane Sheet Assay

3T3-L1 cells were differentiated to day 8 on BD BioCoat Collagen Type I 8-well CultureSlides. PM sheets were obtained using a modified protocol of Olson et al. [140]. Cells were swelled with three rinses of hypotonic buffer (23mM KCl, 10mM HEPES, 2mM MgCl₂, 1mM EGTA [pH 7.5]). Swollen cells were sonicated and washed two times with sonication buffer (70mM KCl, 30mM HEPES, 5mM MgCl₂, 3mM EGTA, 1mM dithiothreitol, 0.1mM PMSF [pH7.5]). PM sheets were fixed with 2% formaldehyde (70mM KCl, 30mM HEPES [pH7.5], 5mM MgCl₂, and 3mM EGTA) at room temperature 20 min. Fixed membranes were quenched 15 min at 25°C in 100mM glycine-PBS (pH7.5). PM sheets were rinsed three times PBS and blocked 5% donkey serum (Sigma) 4°C O/N. They were then incubated with GLUT4 C-20 (Santa Cruz #1608) 1 hour, washed three times in PBS and incubated with donkey anti goat Alexa Fluor 488 secondary antibody (Molecular Probes). PM sheets were washed three times in PBS and incubated 15 min with Deep Red Cell Mask Plasma Membrane Stain (Molecular Probes) as an internal marker of membrane amount. Stained PM sheets were
visualized with Leica SP2 laser confocal microscope and analyzed using Leica Confocal Software (Leica Microsystems Wetzlar GmbH, Germany).

**GLUT4 Exofacial Loop Translocation/Fusion Immunofluorescence Assay**

3T3-L1 cells were differentiated to day 8 to day 12 on BD BioCoat Collagen Type I 8-well CultureSlides. This experiment was modeled after that of Yashamoti et al. [413]. After treatment, cells were washed quickly with PBS buffer and fixed with 4% formaldehyde 15 minutes room temperature. Cells were washed three times with PBS and incubated O/N 4°C with goat GLUT4 N-20 antibody (sc-1606, Santa Cruz) diluted in 3% donkey serum. Cells were washed three times with PBS and incubated 45 minutes with donkey anti-goat Alexa Fluor 594 (Molecular Probes) diluted in 3% donkey serum. Cells were washed three times with PBS, dried completely and mixed with Vectashield mounting media with DAPI (Vector Laboratories, Inc., Burlingame, CA) just before coverglass application. Cells were imaged and analyzed as mentioned in the plasma membrane sheet assay. Experiment was also attempted by incubating live cells with primary antibody immediately after treatment for 30 minutes (with or without Cell Mask Plasma Membrane Stain, Molecular Probes), fixing 15 minutes after treatment and then incubating with secondary. This was done because even without detergent, GLUT4 antibody was penetrating the plasma membrane. However, even with these modification, GLUT4 antibody was still able to penetrate the plasma membrane.

**Immunofluorescence Measure of pAkt Ser473**

3T3-L1 cells were differentiated to day 8 through day 12 on BD BioCoat Collagen Type I 8-well CultureSlides. After treatment, cells were washed quickly with PBS buffer and fixed with 4% formaldehyde 15 minutes room temperature. Cells were
then blocked 60 min 1% BSA blocking solution with 0.05% saponin. Cells were then incubated with pAkt Serine 473 Antibody (#4058, Cell Signaling) in blocking buffer 4°C O/N. After washing three times with PBS, cells were incubated with combination of anti-rabbit Alexa Fluor 568 Secondary Antibody (#A11011, Molecular Probes), TO-PRO-3 nucleic acid stain (Molecular Probes) and RNase A (#19101, Qiagen) 45 minutes, room temperature in the dark. The RNase A digested the RNA so only the DNA (thus the nucleus) will show. Cells were washed five times for five minutes with PBS. Vectashield mounting media without DAPI (Vector Labs) was applied. Approximately 10 arbitrary fields were pictured per condition. Experiment was repeated four times. Images in figure were processed using Image J software. Z-stack series from each field were stacked and shown as Z projection with maximum intensity.
RESULTS

TZDs and Alternative Splicing (A) Hela Cells

TZDs are a class of drugs used for the treatment of non-insulin dependent diabetes mellitus. The molecular target of these drugs is PPARγ. The main tissue target is adipose, which has robust PPARγ expression. PPARγ activation in this cell type leads to overall increased insulin sensitivity in peripheral insulin sensitive tissues. Insulin resistance occurs despite qualitatively and quantitatively normal insulin receptors. Therefore, the mechanism for increased glucose disposal lies downstream of the insulin receptor [414]. Initial experiments focused on determining whether there was a link between TZD treatment and increased alternative splicing of PKCβII, which had been shown to be involved in mediating insulin-stimulated glucose uptake in skeletal muscle cells [36; 40; 113]. The HeLa cervical cancer cell line was first tested. Cells were grown to confluency and serum starved for 6 hours. Cells were either treated with 50nM of bovine insulin or 1μM Pioglitazone for 24 hours. Pioglitazone was able to mimic the effects of insulin (30 min) on PKCβ alternative splicing. Relative to control, both insulin and Pioglitazone treated cells were able to regulate PKCβ such that PKCβI mRNA decreased concomitant with increased PKCβII mRNA expression (Figure 23). This was also novel in that it showed that HeLa cells expressed insulin receptors and that they were insulin responsive.
TZDs and Alternative Splicing (B) Vascular Smooth Muscle Cells

TZDs increase insulin sensitivity in insulin-resistant patients and animal models. However, they also beget other benefits that may not be mutually exclusive to one another. These benefits include lower blood glucose, decreased circulating free fatty acids and triglycerides, lower blood pressure, reduced inflammatory markers, and reduced atherosclerosis [313]. PPARγ is expressed in vascular endothelial cells and vascular smooth muscle cells (VSMCs). Proliferation and migration of VSMCs are essential in the progression of atherosclerosis. Inflammation is considered a key stimulator of this proliferation and migration. PPARγ activation can suppress the expression of TNFα-induced expression of inflammatory genes such as VCAM-1, MCP-1 and fractalkine via inhibition of NFκB [415]. Yamamoto et al. has shown that PKCβII expression is associated with decreased VSMC proliferation [41]. A possible link between TZDs and PKCβII mediated effects on VSMCs was examined. Figure 24 shows A10 rat VSMCs treated with 1μM Pioglitazone for 26 hours increased the mRNA expression of PKCβII while decreasing the mRNA expression of PKCβI. However, A10 cells proved difficult to culture and synchronize. The focus turned to L6 rat skeletal muscle cells which represent the main insulin responsive tissue responsible for glucose disposal. These cells divide and differentiate rapidly making them an ideal cell line.

TZDs and Alternative Splicing (C) L6 Skeletal Muscle Cells

Skeletal muscle represents a major insulin-responsive tissue where most glucose disposal occurs. TZDs result in improved whole-body glucose disposal which includes skeletal muscle [416]. However, it is unclear whether this effect on skeletal muscle is a result of indirect actions such as lowering FFAs or TZDs can actually exert effects on
skeletal muscle signaling. Thus, a possible link between TZDs and skeletal muscle signaling was explored. Using L6 cells, 1μM Pioglitazone for 24 hours was able to mimic 100nM bovine insulin for 30 minutes in terms of PKCβII mRNA expression (Figure 25). When Pioglitazone and insulin were combined, there was a synergistic increase in PKCβII mRNA expression. Protein expression of PKCβII revealed a similar trend whereby 1μM Pioglitazone was able to mimic 45 or 60 minutes of 100nM bovine insulin (Figure 26). SRp40 overexpression was also able to increase PKCβII expression. Three SRp40 constructs were used. After this experiment, only the construct donated by Dr. Rebecca Taub was used. This data is in concurrence with previous Cooper lab data showing SRp40 enhancement of PKCβII alternative splicing [37; 38; 211]. RT-PCR detection of PKCβI using primers for both PKCβI and PKCβII was sporadic and not reliable.

**PKCβII mRNA Regulated by Overexpression of PPARγ, PGC1α and SRp40**

Since the main target of TZDs is PPARγ, overexpression of PPARγ was next assessed along with overexpression of PGC1α and SRp40. PGC1α expression favors the slow twitch, oxidative muscle fiber type [372] and this fiber type has increased insulin-stimulated glucose uptake [417]. Possible regulation of PKCβ via PGC1α would explain the enhanced ability for glucose uptake muscles obtain during TZD treatment. As shown in Figure 27, overexpression of PPARγ, PGC1α and SRp40 and combinations of the three resulted in increased mRNA expression of PKCβII compared to control. The combination of all three constructs may have been too much and thus created an imbalance. This is maybe why there is a slight decrease in PKCβII levels compared with combinations of two constructs.
Functionality of PGC1α C-terminal Domain

PGC1α upregulation of PKCβII mRNA necessitated the question of which domain was critical in mediating this effect. The C-terminal domain was thought to be the critical effector because it provided the bridge between nuclear receptors and the mediator complex as well as serving as a landing pad for splicing factors [361]. In fact, SRp40 has been confirmed to bind PGC1α [362]. Figure 28 shows that overexpression of CTD mutant PGC1α led to a decrease in PKCβII mRNA expression (compared with control) versus wild-type PGC1α which resulted in an expected increase in PKCβII mRNA in L6 cells.

TZDs Influence PKCβ Expression Level and Alternative Splicing

Since PGC1α is able to participate in both transcription and splicing, it seemed reasonable that TZD treatment may work in a similar mechanism in relation to the PKCβ gene. L6 cells were treated with either 1μM Pioglitazone or Rosiglitazone for 24 hours. Both TZDs were able to increase the protein expression of PKCβII while keeping PKCβI relatively constant (Figure 29). This suggested TZDs could influence transcription and splicing preferentially selecting the PKCβII isoform. Rosiglitazone seemed to perform even better than Pioglitazone in terms of PKCβII induction.

Hypothesis #1: TZDs Co-transcriptionally Regulate PKCβ Gene Expression

Data obtained up to this point led to the hypothesis that TZDs were able to co-transcriptionally regulate the PKCβ gene, thereby mimicking the insulin signaling cascade (Figure 30). This would result in increased transcription as well as increased alternative splicing that would favor the PKCβII isoform. This phenomenon would come to fruition via TZD-mediated activation of PPARγ. PGC1α would bind PPARγ. Even
though PPARγ does not have to be active to bind PGC1α, PPARγ activation would be required for PKCβ promoter activation. PPARγ-bound PGC1α C-terminal domain would serve as a landing pad for splicing factors that have a positive regulatory effect on PKCβII exon inclusion. These splicing factors would eventually be dropped off to the CTD of RNA polymerase II. Selective SR protein recruitment (e.g. SRp40) would result in RNA polymerase II pausing at the weak PKCβII 3′ splice site (intrinsic sequence just before PKCβII exon). This pause would result in enhanced recognition of the weak splice site and thus greater PKCβII exon inclusion. TZD treatment could regulate factors downstream of the insulin signaling pathway, bypassing upstream insulin signaling that may be dysfunctional in the insulin-resistant or diabetic state.

**Role of Overexpressed PPARγ on PKCβ Protein Levels**

The ability of PPARγ overexpression to induce PKCβ co-transcriptional splicing was assessed. 1 or 3μg PPARγ cDNA construct was transfected into L6 cells for 72 hours. Figure 31 shows that PPARγ overexpression (regardless of dosage) was able to increase protein expression of both PKCβI and PKCβII isoforms. However, this is not the same mechanism of control as compared with TZD treatment. PPARγ expression appears to exert more of a transcriptional effect on the PKCβ gene since PKCβI is increased too. The ligand-dependent AF2 transcriptional activator domain appears to be necessary for the increase in PKCβII protein levels. Overexpression of an AF2 mutated PPARγ construct resulted in an increase in PKCβI protein expression but no increase in PKCβII protein expression (Figure 32).
Role of Overexpressed PGC1α on PKCβ Protein Levels

PGC1α overexpression was utilized to determine whether it could more closely mimic the effects of TZD treatment in terms of PKCβ co-transcriptional splicing in L6 cells. As shown in Figure 33, PGC1α overexpression (both 2 and 4μg) mimicked TZD treatment and resulted in increased PKCβII protein expression while PKCβI protein expression was little changed. Establishing PGC1α overexpression was difficult. The flag antibody did not work for this tagged protein and the endogenous PGC1α antibody could barely be detected.

Effect of siRNA Knockdown of PPARγ, PGC1α and SRp40 on PKCβII mRNA

A knockdown strategy using 24 hour transfected siRNA was next employed to determine whether endogenous levels of PPARγ, PGC1α or SRp40 are influential in generating PKCβII. In Figure 34, knockdown of PPARγ and PGC1α resulted in reduced PKCβII mRNA levels. In the case of PPARγ knockdown, PKCβII mRNA levels went further down with 24 hour treated 1μM Rosiglitazone. However, this may be due to experimental design. Since the drug was added near the time of transfection, the transfection reagent may have reacted with Rosiglitazone causing a change in L6 signaling. Rosiglitazone treatment could not rescue PKCβII mRNA levels in PGC1α knockdown cells. Figure 35 shows that SRp40 is necessary for PKCβII alternative splicing. This had been predicted based on earlier Cooper lab data but had not been shown with SRp40 siRNA. Again, Rosiglitazone treatment was unable to rescue PKCβII mRNA levels most likely because of the same experimental design flaws described above. Even Rosiglitazone with scrambled siRNA had no effect on PKCβII, suggesting inhibition of the drug by the transfection reagent.
Generation of Heterologous PKCβ Promoter-driven PKCβ Minigene

To determine whether PPARγ had a direct effect (via binding) on the PKCβ promoter, a heterologous PKCβ minigene was created as outlined in the Experimental Procedures section and illustrated in Figures 36-48. This ultimately resulted in a minigene that had the BI and BII exons with sufficient flanking intronic sequences. In addition, the minigene was under the control of 2243bp of the human PKCβ promoter. Figure 48 shows that this PKCβ minigene was functional since transcriptional products mimicked those of the CMV promoter construct. The BI and BII mRNA products were confirmed by sequencing. The minigene was also shown to be responsive to 2nM phorbol ester treatment (Figure 49). TPA activated the promoter and increased both BI and BII products. GFP expression is used to assess transfection efficiency because its expression is not governed by TPA treatment.

Effect of TZD on PKCβ Minigene

TZD treatment results in co-transcriptional splicing of endogenous PKCβ. To assess if this effect was related to a direct effect on the PKCβ promoter, the minigene was transfected for 48 hours and concomitantly the cells were treated with 1μM Pioglitazone for 24 hours as seen in Figure 50. Pioglitazone was able to directly affect the PKCβ promoter and increase alternative splicing. The result is dramatically increased BII minigene product.

PPARγ-mediated PKCβ Transcriptional Regulation: Direct vs. Indirect?

Logical progression led to co-transfection of the minigene and PPARγ to assess whether PPARγ may be able to bind to a putative PPRE on the PKCβ promoter. The minigene construct without the PKCβ promoter co-transfected with PPARγ had no effect
on either the BI or BII minigene products. Conversely, the minigene construct with the PKCβ promoter co-transfected with PPARγ had a dramatic effect on both minigene products (Figure 51). The only caveat of this experiment is that vector control was able to increase the BI minigene product. This may be due to unintended effects of the control vector on the PKCβ promoter. Still, PPARγ overexpression was able to elicit expression of the BII minigene product. Compared with vector control, PPARγ decreased minigene BI expression. This data indicates that PPARγ affects the PKCβ promoter and regulates alternative splicing as well as transcription.

To test whether there was a direct binding between PPARγ and the PKCβ promoter, the truncated PKCβ promoter minigene construct was utilized. Within the upstream region of the PKCβ promoter insert, there is a putative DR2 PPRE as described in Experimental Procedures. From bioinformatic analysis (Nuclear Hormone Receptor Scan software), this putative PPRE was the most promising of the 2243 PKCβ promoter sequence. The truncated PKCβ promoter construct lacked this DR2 PPRE. PPARγ was co-transfected with both PKCβ promoter constructs. Figure 52 revealed that PPARγ was not acting through this putative DR2 PPRE. Either PPARγ was acting on a more distal PPRE or PPARγ was affecting the PKCβ promoter indirectly through regulation of other transcription factor or coactivators.

Role of TZDs in 3T3-L1 PKCβII Expression

It was determined that the mouse 3T3-L1 pre-adipocyte cells might be a better system for examining effects of TZDs on PKCβ co-transcriptional splicing in 3T3-L1 mouse pre-adipocyte cells. This cell model seemed a good candidate because during differentiation into adipocytes, they express high levels of PPARγ which is the target of
TZDs [418; 419; 420]. In addition, 3T3-L1 cells synthesize PPARγ agonists such as 15d-PGJ2 [421] and a yet to be identified potent ligand that materializes at the onset of differentiation, lasts 48 hours, then disappears [422]. Figure 53 a-d demonstrated 3T3-L1 differentiation (day 0 to day 6) using phase contrast microscopy. Figure 53 e-h depicted accumulation of lipid droplets (red) from day 4 to day 6 via Oil Red O staining. Oil Red O is a fat soluble dye (lysochrome) that stains triglycerides. The purpose of this stain was to confirm differentiation protocol before proceeding to further experimentation.

The first experiment in 3T3-L1 cells focused on establishing a link between TZD treatment and PKCβ co-transcriptional splicing based on the experiments performed in L6 skeletal muscle cells. As depicted in Figure 54, differentiating 3T3-L1 cells at day 4 and 6 were chosen for TZD treatment. It was known that by day 4, copious amounts of PPARγ were expressed. Because culture conditions had not yet been completely established, both low and high glucose media were used. 1μM rosiglitazone for 24 hours activated PPARγ. The evidence of this is that the level of PPARγ (both 1 and 2) decrease with Rosiglitazone treatment. PPARγ activation via TZDs increased PPARγ proteasomal degradation. This effect is not necessarily dependent on transcriptional activation but is dependent on the AF2 domain [423]. PPARγ activation had no discernable effect on either PKCβI or PKCβII protein levels on day 4 or day 6. However, there was a dramatic change in the expression of PKCβ isoforms during the span of these two days. From day 4 to day 6, PKCβII expression increased over 6.6 fold while PKCβI expression was reduced roughly 1.5 fold. This data was consistent with a report stating that the expression of PKCβ (no isoform distinction) increased at least 10 fold from pre-
adipocytes to adipocytes [419]. This appeared to be a novel finding which represented
developmentally regulated PKCβ co-transcriptional splicing.

**Discovery of Novel Differentiation-regulated PKCβ Alternative Splicing in 3T3-L1 Cells**

The experiment was repeated but included more days of differentiation. Figure 55 shows that again, 1μM Rosiglitazone failed to change the ratio of PKCβ isoforms during differentiation even though PPARγ was being activated. But it was clear that PKCβII was heavily induced during differentiation while PKCβI was concomitantly decreased. PPARγ and GLUT4 both served as markers of adipocyte differentiation. This experiment was again repeated (without TZD treatment) but probed for additional proteins of interest. Figure 56 spans from day 0 to day 12. PKCβ was regulated as before. PKCβII expression peaks at day 8 and then expression plateaus. PKCδ has a similar expression pattern as PKCβII with very low levels at day 0, peaks at day 8 and then plateaus. Longer exposures of the PKCδ blot revealed possible additional splice variants. Seven splice variants have been reported in mice testis [424]. From the 20 sec exposure, four putative PKCδ splice variants were visible. From the 15 min exposure, the remaining three putative PKCδ splice variants were visible. Phospho PKCβII/δ antibody probing was very interesting. This antibody detects the phospho epitope of PKCβII S660 and PKCδ S662. The band displayed was almost certainly that of phospho PKCδ. PKCβ was not expressed on day 0 and should not be phosphorylated regardless of the day in the absence of external stimuli such as insulin. PKCδ has been reported to undergo biphasic serum-mediated phosphorylation in fibroblasts [425]. PKCα peaked at day 2 then tappers off. This was consistant with the literature which states that PKCα is
downregulated during adipogenesis [426]. PKCγ also has a similar expression pattern to that of PKCβII. However, its expression was very low in the early days with a dramatic jump from day 6 to day 8. After day 8, there was a precipitous fall in PKCγ expression. Initially, PKCγ was reported to be only in the brain and spinal cord [11]. However, a recent paper has described PKCγ expression in 3T3-F442A cells [48]. We believe this to be the first report of PKCγ expression in 3T3-L1 cells. PKCζ expression steadily increased during adipogenesis peaking at day 6, after which is precipitously drops. This pattern is similar to that described by Ways et al. [426]. PPARγ, GLUT4 and adiponectin are used as markers of differentiation. β-actin indicates relatively equal loading.

**Real-Time PCR Confirms PKCβII Adipogenesis Protein Expression Patterns**

Real-Time PCR following the mRNA expression pattern of PKCβII during adipogenesis revealed a similar expression profile to protein with a peak at day 6 then plateauing (Figure 57). Real-Time PCR was also performed using primers specific for PKCβI but this was unsuccessful. Hence, PKC isozyme-specific expression, with peaks of PKCβII, PKCγ and PKCδ, were a hallmark of adipocyte differentiation.

**Use of Distal PKCβII polyA Tail During 3T3-L1 Differentiation**

The PKCβII mRNA transcript has two possible polyA tails to use as reported [427] and illustrated in Figure 5. To determine which polyA tail was used by adipocytic PKCβII, RT-PCR was performed with indicated primers (Figure 58) that distinguish polyA tail usage. RNA was collected from both day 0 and day 8 3T3-L1 cells. Only the PKCβII transcript containing the distal polyA tail showed up. GAPDH is a housekeeping gene used as a loading control and PPARγ indicates adipocyte differentiation.
Hypothesis #2: PKCβII Can Regulate GLUT4 Expression

Previous Cooper lab data had shown that knockdown of PKCβII in L6 skeletal muscle cells resulted in reduced GLUT4 protein expression levels (unpublished). In 3T3-L1 cells, PKCβII and GLUT4 expression appear at roughly the same time. It was thought that PKCβII may regulate GLUT4 expression.

To test this hypothesis, knockdown of PKCβII would be necessary. Transfection of one of two PKCβII specific silencer select siRNA was attempted using siRNA transfection reagents from Ambion (siPORT NeoFX), Mirus Bio (TransIT-TKO) and Panomics (DeliverX Plus). In the case of DeliverX Plus, this reagent is supposedly tailored for differentiated 3T3-L1 adipocytes. However, none of the above reagents gave any significant knockdown of PKCβII (Figure 59). Further research into the literature revealed that differentiated 3T3-L1 adipocytes are notoriously difficult to transfect if not impossible [428; 429]. Electroporation is also not feasible for this cell line with very low efficiency and low cell survival. Lentiviral transduction has been reported to have success [430]. However, there is only one available construct for the PKCβII. Due to this uncertainty, this aim was put on hold.

Regulation of PKCβ Promoter During 3T3-L1 Differentiation

Differentially regulated PKCβ alternative splicing suggests an important role for PKCβII in adipogenesis and adipocyte function. Therefore, the factors that regulate this differential alternative splicing necessitated further inspection. The mouse PKCβ promoter was examined for putative transcription factors that also had expression patterns similar or opposite (for negative regulators) to those of PKCβII during adipogenesis. Figure 60 shows RT-PCR of RNA samples from day 0 to day 6 for factors
that had their respective consensus binding sites on the PKCβ promoter. AP2, also known as fatty acid-binding protein 4 (FABP4), is responsible for fatty acid transport in adipocyte. It is a marker of terminal adipocyte differentiation. It can also influence transcription. Interestingly, FABP4 can cooperate with PPARγ to regulate gene expression [431]. GATA1 is a transcription factor that can inhibit PU.1 transcriptional activity [432]. GATA2 (assayed for later) and GATA3 are transcription factors known to inhibit adipocyte differentiation [433]. Surprisingly, GATA3 expression goes up even though it is supposed to inhibit differentiation. hnRNP G can influence alternative splicing [434] and is downregulated at least ten fold from the pre-adipocyte to adipocyte stage [419]. RUNX1 is a transcription factor that was reveal by a chromatin immunoprecipitation screen to bind the human PKCβ promoter [435]. MZF-1 is a transcription factor belonging to the Kruppel family of zinc finger proteins [436].

Figure 61 shows RT-PCR and protein expression of PU.1. PU.1 was considered the most promising transcription factor due its many putative consensus binding sites blanketing the PKCβ promoter as well as its confirmed role in bridging transcription and splicing [405]. PU.1 has been reported to inhibit the differentiation of 3T3-L1 cells in concert with GATA2. However, this was done using overexpression systems which may indicate threshold dynamics. In this same paper, PU.1 mRNA and protein expression were shown to increase over the course of differentiation which is consistent with data shown in Figure 61 [437]. Also shown in Figure 61 is the protein expression of SRp40, a splicing factor that could possibly contribute to PKCβ alternative splicing during adipogenesis based on its role in L6 skeletal muscle cells [37; 38; 211]. SRp55, another
splicing factor that is regulated by insulin [38], may influence PKCβ alternative splicing (unpublished).

Using the Chromatin Immunoprecipitation assay, PU.1 is shown to bind the 3T3-L1 PKCβ promoter at various spots at both day 0 and day 8. SRp40 seems to also display affinity for the PKCβ promoter (Figure 62). This led to a third hypothesis.

**Hypothesis #3: Developmental Regulation of PKCβ Splicing by PU.1**

PU.1 would be able to bind the PKCβ promoter during the course of 3T3-L1 adipocyte differentiation and influence the developmental regulation of PKCβ alternative splicing. It would accomplish this by recruiting coactivators (while simultaneously displacing corepressors), which would then bind splicing factors such as SRp40 that would ultimately result in the PKCβII exon inclusion.

The next step would have been to knockdown PU.1 during differentiation and assess PKCβ mRNA and protein levels. However, due to the extreme difficulties in transfecting differentiated 3T3-L1 cells (as mentioned above), this aim was postponed.

**Linking PKCβII with 3T3-L1 Adipocyte Insulin-stimulated Glucose Transport**

At the same time, the possibility of PKCβII regulation of glucose uptake in 3T3-L1 adipocytes, was being explored. Previous studies show that PKCβII was critical in mediating glucose uptake in skeletal muscle cells, another insulin-sensitive tissue [40]. CGP53353 is an inhibitor of cPKCs with greatest affinity for PKCβII (see Table 7). It was anticipated that the inhibitor might provide evidence of a role for PKCβII in 3T3-L1 adipocyte ISGT. Figure 63a is a dose curve showing 50μM CGP53353 attained an 85% inhibition of ISGT compared to insulin alone in 3T3-L1 adipocytes day 8 to day 12. Figure 63b shows this 50μM dose within a complete experiment with control and drug
control. CGP53353 inhibited ISGT over 6 fold compared to insulin alone. Drug alone had no effect on basal glucose uptake levels compared with control. In addition to this, LY379196, another PKCβ selective inhibitor, was used. Figure 64a is a dose curve showing that LY379196 (with insulin) can inhibit ISGT at 50μM. Next, both 25 and 50μM LY379196 were able to significantly inhibit ISGT (3 fold and ~4.3 fold respectively) with no effect on basal glucose uptake (Figure 64b).

**CGP53353 Specificity**

A kinase assay via western blot was next pursued to affirm that CGP53353 was specifically inhibiting PKCβII and not other critical insulin signaling effectors. Figure 65 shows that insulin caused phosphorylation of both PKCβII S660 and insulin receptor Y1150/1151. Only PKCβII S660 phosphorylation is inhibited with drug. The antibody targeting PKCβII also targets phospho PKCδ S662 (thick lower band). Phospho PKCδ migrates at 78kDa on the gel whereas phospho PKCβII migrates at 80kDa on the gel. Large gels were run longer in order to obtain reasonable separation between the two bands. The phosphorylation status of PKCδ, PKCζ and the insulin receptor were unaffected by the drug. Total PKCβII levels remained constant. This led to the last hypothesis.

**Hypothesis #4: PKCβII Regulates 3T3-L1 Adipocyte ISGT in Part via GLUT4 Trafficking or GLUT4 Fusion**

PKCβII's ability to regulate ISGT in adipocytes was likely due to an effect on GLUT4 translocation or late-stage GLUT4 fusion. The rationale behind this was a report demonstrating the ability of PKCβII to phosphorylate Akt at S473 [438]. Fully active Akt is required for late stage GLUT4 translocation [184]. Also, in 3T3-L1 adipocytes,
PLD1 mediates GLUT4 fusion to the PM [141]. PKCβII (not PKCβI) has been shown to bind and activate PLD1. PLD1 activity caused PKCβII (not PKCβI) to translocate to a juxtanuclear subset of recycling endosomes (presumably where GLUT4 is located) [439].

In skeletal muscle cells, PLD1 membrane localization is regulated via insulin-stimulated PKCβII [113].

**Subcellular Fractionation Points to a Role for PKCβII in GLUT4 Translocation**

To test whether GLUT4 translocation was being affected by CGP53353 inhibition, subcellular fractionation assays were performed. As shown in Figure 66, insulin caused a redistribution of GLUT4 from the low-density microsomes (LDM) to the plasma membrane (PM). Without insulin stimulation, GLUT4 remained in the LDM. Addition of CGP53353 dramatically blocked insulin-stimulated GLUT4 translocation to the PM. Cytoplasmic fractions were also western blotted and probed for β-actin to show that protein concentrations measured by the BCA protein assay were accurate. It also shows that cell death was not responsible for the trends observed. Numerous other proteins were tested as internal controls of protein loading. For the PM fraction, insulin receptor, TNFα Receptor 1 & 2, APMAP (adipocyte plasma membrane associated protein) were tested. For the LDM fraction, IRS1 levels were tested. However, insulin and/or CGP53353 treatment affected the expression and/or stability of these proteins and thus they could not be used as controls. In addition to cell surface GLUT4, phosphorylated GLUT4 was assessed by probing the PM fraction for pGLUT4 S488 (Santa Cruz). Earlier reports indicated that PM GLUT4 C-terminal phosphoserine is reduced in insulin-stimulated rat adipocytes. This has led to speculation that phosphorylation of the GLUT4 C-terminus may actually inhibit its intrinsic activity.

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We were interested to see if PKCβII inhibition altered GLUT4’s phosphorylation status and likely its folding/binding partners. However, we were unable to detect any phospho GLUT4. This was also the case in whole cell lysate. Other methods are needed to increase antibody sensitivity.

**PM Sheet Assay Affirms a Role for PKCβII in GLUT4 Translocation**

A second method was sought to definitively link PKCβII to GLUT4 translocation. The PM sheet assay (Figure 67) reveals a similar trend to that of subcellular fractionation in 3T3-L1 adipocytes day 8 to day 12. Insulin treatment illuminated GLUT4 staining (Figure 67b). However, this staining was abrogated (~3.8 fold) when treated with CGP53353 (Figure 67d). Staining with Deep Red Cell Mask Plasma Membrane Stain shows relatively equal PM protein content (Figure 67e-h). The merger of insulin-stimulated GLUT4 can be seen with PM proteins (Figure 67j). Hence, PKCβII is likely regulating adipocyte ISGT through regulation of GLUT4 translocation.

In addition to regulating GLUT4 translocation, PKCβII was believed to regulate GLUT4 fusion to the PM. This was because PKCβII can bind and activate PLD1 which is responsible for insulin-stimulated GLUT4 fusion to the PM [19; 141]. Immunofluorescence was used to measure GLUT4 that had fused to the PM. When GLUT4 is fused to the membrane, there is a large exofacial N-terminal loop that protrudes into the extracellular space (Figure 11). Santa Cruz Biotechnology has an antibody that recognizes this exofacial loop. The protocol for this experiment was based on that performed by Fulcher et al. [413] (Experimental Procedures). Formaldehyde fixed cells were incubated with the antibody with no permeabilizing detergents. However, the antibody was still able to penetrate the PM and stain the entire cell.
Despite this, there was a subtle trend. Insulin was often able to aggregate GLUT4 near the nucleus, possibly the perinuclear region where GLUT4 resides before translocating to the PM in an insulin-dependent manner (Figure 68b). CGP53353 treatment prevented this formation resulting in a more dispersed localization around the nucleus (Figure 68d). PKCβII (and possibly other cPKCs) may have a role in basal state and insulin-stimulated GLUT4 intracellular trafficking. Bioinformatic analysis of GLUT4 protein sequence revealed a putative PKC phosphorylation site beside the C-terminal insulin-responsive motif (IRM), LXXLPDEX(D/E). The GLUT4 IRM has been shown to be crucial for insulin-stimulated GLUT4 redistribution to the PM [441]. Another attempt to use immuno-fluorescence was made by first incubating the live pre-formaldehyde fixed cells with the GLUT4 antibody followed by fixation (fixation was also attempted after secondary antibody). The antibody was still able to penetrate the PM. Primary or secondary antibodies that are bulkier must be made in order to perform this assay. The GLUT4 exofacial loop can be glycosylated (Figure 11). N-glycosylation at this site (possibly mediated by Golgin-160) may be necessary for ISGT [442]. It is possible that the antibody was made to recognize only the unglycosylated loop. This would mean a new antibody that recognizes that glycosylated loop would have to be raised and developed.

**PKCβII Regulation of Akt Activity**

As mentioned earlier, activated Akt is required for GLUT4 translocation [184]. PKCβII has been shown to regulate Akt S473 (S473) phosphorylation in a cell and stimulus-specific manner [438]. To that extent, we investigated whether PKCβII could regulate Akt activity in 3T3-L1 adipocytes. Insulin treatment induced the dramatic
appearance of T308 and S473 phosphorylation (Figure 69). CGP53353 treatment decreased S473 phosphorylation over 17 fold. Phosphorylation of T308 remained relatively constant with CGP53353 treatment. This was consistent with the literature where T308 is regulated by PDK1 (via insulin) and S473 is regulated by a separate PDK2 (possibly PKCβII) and that S473 phosphorylation is not dependent on T308 phosphorylation [174; 175; 229]. It is important to note that PKCβII may be regulating phosphorylation of mouse Akt1 at S473, Akt2 at S474, Akt3 at S472 or any combination thereof since this antibody recognizes the phosphorylated hydrophobic motif of all three Akt isoforms. From here on, Akt phosphorylation at the hydrophobic motif will be referred to as Akt S473 since the antibody was labeled as phospho Akt S473.

**Immunofluorescence Confirms Role for PKCβII in Phosphorylation of Akt S473**

To confirm PKCβII as a possible PDK2 that regulates Akt S473 phosphorylation, immunofluorescence was performed on 3T3-L1 adipocytes from day 8 to day 12. Insulin stimulated dramatic staining of phospho Akt S473 (Figure 70b) as expected. PM staining as well as intracellular aggregation staining near the nucleus was apparent. Treatment with CGP53353 eliminated staining of phospho Akt S473 (Figure 70d). DAPI staining for nuclei could not be used because the short wavelengths caused high background fluorescence of CGP53353 drug. TO-PRO3 was used to avoid this problem with an emission spectrum around 633nm. TO-PRO3 stains nucleic acid, DNA and RNA. In order to select for DNA (nucleus), incubation of TO-PRO3 was performed along with RNaseA treatment (Figure 70e-h).
PKCβII Downstream of mTORC2 but Upstream of Akt

The mTORC2 complex was shown by Sarbassov et al. to regulate phosphorylation of Akt S473 [174]. Phosphorylation of mTOR kinase at S2481 distinguishes activated mTORC2 from mTORC1 [61]. However, it is known that mTORC2 is also responsible for phosphorylation of PKCβII/PKCα at the turn motif which allows the kinases to undergo autophosphorylation at the hydrophobic motif [54; 55]. This suggested that PKCβII is activated by mTORC2 and then goes on phosphorylate Akt S473, thus fully activating Akt. Figure 71 shows insulin stimulated mTORC2 activation via phosphorylation of mTOR S2481. Insulin has been shown to similarly activate mTORC2 in HEK293 cells [61]. CGP53353 treatment did not alter the phosphorylation status of mTOR S2481. This indicated that PKCβII was likely downstream of mTORC2.

Integrating the role of mTORC2 represented a more mechanistic overview of how PKCβII was exerting its effects. Before mTORC2 was pursued, another promising putative PKCβII substrate was examined. PLD1 is critical in 3T3-L1 adipocyte ISGT by enabling fusion of GLUT4 to the PM [141]. PKCβII is capable of phosphorylating and activating PLD1 [19]. Using two commercial antibodies for both PLD1 and phospho PLD1, detection was attempted by western blotting whole cell lysates, western blotting PM and cytosolic fractions and immunofluorescence. An in vitro PLD1 activity assay (Amplex Red, Invitrogen) was also performed. None of these experiments could detect PLD1 or its activity. This could have been due to low abundance of PLD1 or low PLD1 antigenicity or low stoichiometric activity.
Insulin-stimulated Binding of PKCβII to mTORC2

Data from Figure 71 showed that PKCβIII (using Santa Cruz antibody for IP which is different than the custom made PKCβIII antibody used in Figures 56 & 65) was likely being activated by mTORC2 before having the capability to phosphorylate Akt S473. Co-immunoprecipitation showed that insulin stimulated direct binding between PKCβII and mTORC2 (Figure 72 lane 3). This binding was dramatically reduced by CGP53353-mediated inhibition of PKCβII (lane 5). Immunoblotting for PKCβIII with the same antibody used for co-immunoprecipitation was unsuccessful. Several additional antibodies were utilized without success including a mouse PKCβII antibody (Sigma P2584) and a mouse PKC antibody (PKC A-9 Santa Cruz #17804) that recognizes all PKC isoforms. Perhaps PKCβII is extremely sensitive to degradation or antigenicity is severely reduced upon cell lysis.

Insulin-stimulated Binding of PKCβII to Akt

Figure 73 provides preliminary evidence that PKCβII is able to bind Akt. PKCβII co-immunoprecipitates with Akt that is phosphorylated at S473 suggesting that PKCβII could be the kinase that directly phosphorylates Akt at S473. The membrane was reprobed with PKCβII. This was unsuccessful due to reasons discussed. The conditions for the reverse co-immunoprecipitation are being worked out.

Co-immunoprecipitation Between PKCβII and GLUT4

Co-immunoprecipitation was performed to assess possible binding of PKCβII to GLUT4. As mentioned earlier, bioinformatic analysis indicated multiple PKC substrate sites spanning murine GLUT4. Akt had been shown to bind GLUT4-containing vesicles and then phosphorylate their component proteins in response to insulin [182]. It was
hypothesized that PKCβII could act in a similar fashion. Figure 74 shows that IP with PKCβII resulted in increased GLUT4 band intensity compared to rabbit IgG control. However, probing for PKCβII (after IPing with PKCβII) yielded nothing, which was expected. IP with GLUT4 antibody was able to pick up PKCβII and this was stimulation independent. GLUT4 band intensity for this was weak but still slightly higher than IgG control. Whole cell lysate probing for GLUT4 using the specified antibody was unsuccessful. This was unexpected for GLUT4 and is probably due to using an antibody different than the one used on other blots. Repeating this assay yielded similar results. This experiment still leaves open the possibility that PKCβII is able to directly influence insulin-stimulated GLUT4 trafficking, possibly through phosphorylation of vesicle component proteins in addition to regulating Akt activity.
Figure 23. TZD Pioglitazone mimics insulin’s effect of increased PKCβII exon inclusion in HeLa cells. HeLa cells were grown as described and serum starved for 6 hours. Cells were treated with either 50nM bovine Insulin (15 or 30 minutes) or 1μM Pioglitazone 22 hours. Total RNA was extracted followed by RT-PCR using the endogenous PKCβII-PKCβI primers (compatible with rat). Samples were run on a PAGE gel and silver stained. Experiment was repeated twice.
Figure 24. Pioglitazone increases PKCβII exon inclusion in A10 vascular smooth muscle cells. A10 cells were grown as described and serum starved for 48 hours. 26 hours prior to harvesting, cells were treated ± 1μM Pioglitazone 26 hours. Total RNA was extracted followed by RT-PCR using the endogenous PKCβII-PKCβI primers or β-actin primers. Samples were run on a PAGE gel and silver stained.
Figure 25. Pioglitazone combined with insulin synergistically increases PKCβII exon inclusion in L6 skeletal muscle cells. L6 skeletal muscle cells were grown as described and serum starved 6 hours prior to treatment with 100nM bovine insulin (15 or 30 minutes). For wells treated with 1μM Pioglitazone 24 hours, treatment began 24 hours before harvesting. Pioglitazone was re-added to appropriate wells during serum starvation. (a) Total RNA was extracted followed by RT-PCR using the endogenous PKCβII-PKCβI primers (compatible with rat). Samples were run on a PAGE gel and silver stained. (b) Graphical representation of PKCβII mRNA expression in arbitrary scan units derived from pixels. Experiment was repeated twice.
Figure 26. Pioglitazone treatment and SRp40 overexpression mimic insulin’s upregulation of PKCβII protein levels.  (a) L6 skeletal muscle cells were grown as described and serum starved for 6 hours. Cells were either treated with 100nM bovine insulin (15, 30, 45 or 60 minutes), treated with 1μM Pioglitazone 24 hours (re-added after serum starvation) or transfected with 1.6μg SRp40 (constructs). Protein was harvested and western blot performed.  (b) Graphical representation of PKCβII protein levels in arbitrary units.
Figure 27. Overexpression of PPARγ, PGC-1α and SRp40 individually or in combination are able to increase PKCβII protein expression. (a) L6 skeletal muscle cells were transfected with 2ug of either PPARγ, PGC-1α or SRp40 (name construct for SRp40) for 48 hours. Total RNA was extracted followed by RT-PCR using PKCβII only primers or β-actin primers. Samples were run on a PAGE gel and silver stained. (b) Graphical representation of PKCβII protein levels in arbitrary units.
Figure 28. CTD of PGC1α is necessary for PKCβII exon inclusion. L6 cells were transiently transfected with transfection reagent alone, 2μg PPARγ, PGC1-α, or PGCΔCTD for 48 hours as indicated above. (a) Total RNA was extracted followed by RT-PCR using endogenous PKCβII specific primers. PCR for β-actin serves as an internal control. (b) Graphical representation of PKCβII mRNA expression in arbitrary units.
Figure 29. TZDs Rosiglitazone and Pioglitazone stimulate PKCβ co-transcriptional splicing. (a) L6 skeletal muscle cells were treated with either DMSO vehicle control, 1μM Rosiglitazone or 1μM Pioglitazone for 24 hours. Protein was harvested and western blot was performed. Experiment was repeated two times. (b) Graphical representation of PKCβII/PKCβI protein levels in arbitrary units.
Figure 30. Hypothetical model of TZD mechanism. Pioglitazone would activate PPARγ, allowing it to bind to the PKCβ PPRE. PPARγ would recruit PGC1α which would then serve as a landing pad for SRp40 and other splicing factors, thereby influencing the alternative splicing of PKCβ.
Figure 31. PPARγ overexpression increases both PKCβI and PKCβII protein levels. (a) L6 skeletal muscle cells were transfected with either 3μg vector control, 1μg PPARγ, or 3μg PPARγ for 72 hours. Whole cell lysate was run on gel for western blot detection. Experiment was repeated two times. (b) Graphical representation of PKCβII/Actin protein levels in arbitrary units. (c) Graphical representation of PKCβI/Actin protein levels in arbitrary units.
Figure 32. Ligand-binding domain is necessary for PPARγ mediated upregulation of PKCβII protein levels. 
(a) L6 skeletal muscle cells were transfected with either 2μg vector control, PPARγ or mutant PPARγE499Q for 72hrs. Protein was harvested and western blot was performed. PPARγE499Q is a mutant in the AF2 domain. It binds PPARγ ligands with comparable affinity as wild-type, but no ligand-dependent transcriptional activation. (b) Graphical representation of PKCβII/Actin protein levels in arbitrary units. (c) Graphical representation of PKCβI/Actin protein levels in arbitrary units.
Figure 33. PGC1α overexpression mimics TZD stimulation of PKCβII co-transcriptional splicing. (a) L6 skeletal muscle cells were transfected 72 hours with 4μg vector control, 2 or 4μg PGC1α. Whole cell lysate was run on gel for western blot detection. Experiment was repeated two times. (b) Graphical representation of PKCβII/PKCβI protein levels in arbitrary units.
Figure 34. PPARγ or PGC1α knockdown reduces basal PKCβII mRNA. L6 skeletal muscle cells were transfected with 10nM of either scrambled siRNA, PPARγ siRNA or PGC1α siRNA for 24 hours and concomitantly treated ± 1μM Rosiglitazone for 24 hours. (a) Total RNA was harvested followed by RT-PCR using specified primers. PKCβII was detected using primers that detected endogenous PKCβII & PKCβI. (b) Graphical representation of PKCβII protein expression levels in arbitrary units.
Figure 35. SRp40 knockdown results in lower basal PKCβII protein levels. L6 skeletal muscle cells were transfected with 10nM scrambled siRNA or SRp40 siRNA for 24 hours and concomitantly treated ± 1μM Rosiglitazone for 24 hours. (a) Total RNA was harvested followed by RT-PCR. PKCβII was detected using primers that detected endogenous PKCβII & PKCβI. (b) Graphical representation of PKCβII protein expression levels in arbitrary units.
Figure 36. PCR on rat genomic DNA. PCR yielded the PKCβII and PKCβI exons with their respective flanking intronic sequences.
Figure 37. The BII-BI fragment digested and inserted into the pTNT cloning vector.
Figure 38. pCMVTNT vector used for mammalian cell expression.
Figure 39. BII-BI fragments cloned into pCMVTNT vector for mammalian cell expression.
**Figure 40. Multiple minigene products.** L6 skeletal muscle cells were transfected for 48 hours 0.7ug GFP or 2ug CMVTNT BII-BI ss3 (Mss3) and treated ± 100nM bovine insulin for indicated time points. RNA was extracted followed by RT-PCR using CMV_FP1 and CMV_RP1 primers. Product prediction to the right represents best guess. BII* represents a possible BII product with an additional splice site(s) or has a cryptic splice site.

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- **GFP** indicates the presence of GFP mRNA.
- **Ins** indicates the presence of insulin mRNA.
- **Mss3** indicates the presence of Mss3 mRNA.
Figure 41. Chimeric intron removed. Chimeric intron removed from the CMVNT BII-BI clones via digestion.
Figure 42. PCR generates BII-BI fragment with different overhangs. PCR was performed on CMVTNT BII-BI clones to lift out the BII-BI fragments with BbsI restriction site on the 5′ end and a XbaI restriction site on the 3′ end. These were then inserted into the CMVTNT vectors missing their chimeric introns.
Figure 43. Predicted splicing events from CMVTNT BII-BI ss1 clone (-CI). Two main hypothetical products, BII (top right) and BI (bottom right) are shown from CMVTNT BII-BI ss1 clone without chimeric intron (-CI).
Figure 44. Products observed after transfection of CMVTNT BII-BI clones. L6 skeletal muscle cells were transfected with 0.7ug GFP ± 1.5ug pCMVTNT (-CI) BII-BI ss1 (Mss1) or pCMVTNT (-CI) BII-BI ss4 (Mss4). In addition each well was serum starved and treated with 100nM bovine insulin 60 min. Total RNA was extracted followed by RT-PCR using CMV_FP1& B1R3 primers. 54°C or 52°C was used as the annealing temperature.
Figure 45. Cloning ss4 fragment into ss1 vector. BamHI site was removed from the original pCMVTNT vector. BII-BI ss1 was re-cloned into the pCMVTNT vector, again losing the chimeric intron. Now, the ss4 fragment was lifted out via PCR and inserted into the pCMVTNT BII-BI ss1 vector.
Figure 46. Replacing CMV promoter with human PKCβ promoter. Human PKCβ promoter was lifted out from another a Luciferase vector using PCR and then cloned into the pCMVTNT (-BamHI, -CI) BII-BI ss4 vector.
Figure 47. Truncation of full length PKCβ promoter. The full length PKCβ promoter was truncated via digestion and blunt ligation.
Figure 48. PKCβ promoter-driven expression of BII and BI minigene products. L6 skeletal muscle cells were transfected with either 2μg CMVTNT (-)BamHI BII-BI ss4 or βprom 2243 BII-BI ss4 for 48 hours. RNA was harvested and RT-PCR was performed for both PKC beta minigene products using CMV_FP1 and CMV_RP1 primers. Product legend is shown to the right. Product identity was confirmed by sequencing.
Figure 49. TPA induces transcription of PKCβ promoter minigene. L6 skeletal muscle cells were transfected with GFP expression vector ± βprom 2243 BII-BI ss4 vector for 48 hours. Additionally, one plate was treated for 24hrs with 2nM TPA (phorbol ester) dissolved in ethanol. (a) Total RNA was extracted followed by RT-PCR using T7flgFP5 & CMV_RP1 primers. PCR for GFP shows transfection efficiency. (b) Graphical representation of BII expression in arbitrary units. (c) Graphical representation of BI expression in arbitrary units.
Figure 50. TZD induces co-transcriptional splicing of minigene. L6 skeletal muscle cells transfected with βprom 2243 BI-BI ss4 vector for 48 hours and were treated with either DMSO solvent control (24hrs) or 1µM Pioglitazone (24hrs). (a) RNA was harvested followed by RT-PCR using CMV_FP1 & B1R3 primers. GFP was a measure of transfection efficiency. (b) Graphical representation of BI expression in arbitrary units. (c) Graphical representation of BI expression in arbitrary units.
**Figure 51.** PKCβ promoter responsive to PPARγ overexpression. L6 skeletal muscle cells transfected 48 hours with 0.5μg GFP ± either 2.5μg CMVTNT BII-BI ss4 or 2.5μg Bprom 2243 BII-BI ss4 ± 1μg PPARγ or 1μg vector control. RNA was harvested followed by RT-PCR using CMV_FP1 & B1R3 primers.
**Figure 52.** PPARγ does not target putative DR2 on PKCβ promoter of minigene. L6 skeletal muscle cells were transfected with GFP, ± βprom 2243 BII-BI ss4 or βprom 1143 BII-BI ss4 ± 2μg PPARγ. (a) Total RNA was extracted followed by RT-PCR using CMV_FP1 & B1R3 primers. GFP shows transfection efficiency. (b) Graphical representation of BII expression in arbitrary units. (c) Graphical representation of BI expression in arbitrary units.
Figure 53. 3T3-L1 differentiation. (a-d) Phase contrast of 3T3-L1 differentiating cells from day 0 to day 6. (e-h) Oil red O staining of 3T3-L1 differentiating cells from day 0 to day 6.
Figure 54. PKCβ splicing is developmentally regulated. 3T3L1 pre-adipocyte cells were cultured in DMEM high (4.5g/L) or low (1g/L) glucose for 4 or 6 days and treated ± 24 hours with 1μM Rosiglitazone. (a) Whole cell lysate was extracted followed by Western blot analysis. (b) Graphical representation of PKCβII protein expression in arbitrary units. (c) Graphical representation of PKCβI protein expression in arbitrary units.
Figure 55: Rosiglitazone has no effect on developmentally regulated PKCβ splicing. 3T3L1 pre-adipocyte cells were cultured in DMEM low glucose (1g/L) for 0 to 8 days treated ± 24hrs with 1µM Rosiglitazone. Whole cell lysate was extracted followed by Western blot analysis.
Figure 56. 3T3-L1 PKCβ (and other PKC isoform) protein expression during differentiation. (a) 3T3-L1 pre-adipocytes were differentiated from day 0 through day 12. Whole cell lysates (50μg protein) from each day were run on an SDS-PAGE gel and probed with the indicated antibodies via western blotting. Experiments were repeated three times. (b) Graphical representation shows PKCβII/actin, where * represents a statistically significant increase in PKCβII protein level as compared with day 0 using arbitrary units. (c) Graphical representation of PKCβI/actin, where * represents a statistically significant decrease in PKCβI protein level as compared with day 0 using arbitrary units. Unpaired t-test (p<0.05) was performed using Prizm5 (GraphPad Software, Inc., LaJolla, CA, USA).
Figure 57. Differentiating 3T3-L1 PKCβII mRNA expression mimics protein expression. 3T3-L1 pre-adipocytes were differentiated from day 0 through day 10. 2μg from each day was used for reverse transcription. Real Time analysis was performed using arbitrary units as described in Materials and Methods. * represents a statistical significance in terms of fold change of PKCβII/actin mRNA compared to day 0 using unpaired t-test, p<0.05. Experiment was repeated three times.
Figure 58. Differentiating 3T3-L1 adipocytes use distal polyA tail for PKCβII alternative splicing. RNA of 3T3-L1 pre-adipocytes (Day 0) and 3T3-L1 adipocytes (Day 8) were extracted. RT-PCR from 2μg RNA was performed using indicated primers. C4B2 indicates the forward primer spanning the C4 exon – PKCβII exon junction. B2i indicates the reverse primer binding on the intronic sequence downstream of the PKCβII exon but upstream of the proximal polyA tail. B1 indicates the reverse primer that binds on the PKCβI exon.
**Figure 59. Differentiated 3T3-L1 adipocytes not transflectable.** Day 5 3T3-L1 adipocytes were transfected with 10nM siRNA targeting PKCβII and harvested on Day 8. Lysates were run on SDS-PAGE gel and immunoblotted with indicated antibodies.
Figure 60. 3T3-L1 transcription factor mRNA expression during adipogenesis. 3T3-L1 cell RNA was harvested on the indicated day of differentiation. 2μg RNA was used for RT-PCR using primers for the gene indicated on the right side.
Figure 61. PU.1 expression through adipocyte differentiation. Day 0 through Day 10 adipocytes were harvested for RT-PCR. Day 0 through Day 12 adipocytes were harvested for Western Blot analysis. 2μg RNA was used for RT-PCR with primers for the product specified. 50μg protein was loaded on SDS-PAGE and probed with indicated antibodies.
Figure 62. ChIP of mouse PKCβ promoter. Day 0 pre-adipocytes and Day 8 adipocytes were harvested for ChIP analysis as mentioned in Materials and Methods. Antibodies corresponding to the names given at the top of the figure were used to assess transcription factor binding. Left Y axis numbers represent the forward and reverse primer positions relative to the PKCβ1 transcript start as defined by Vega Mouse TransView (Mouse Genome Informatics, MGI). All PCRs were performed with the same amount of cycles, primers and template.
Figure 63. PKCβII inhibition via CGP53353 attenuates adipocyte glucose uptake. Day 8 to day 12 3T3-L1 adipocytes were serum starved for 4 hrs, ± 30 min 50μM CGP53353, ± 15 min 100nM insulin. Glucose uptake assay was performed as described in Methods and Materials. (a) Dose curve shows the percentage decrease in glucose uptake when comparing insulin vs. drug with insulin. (b) Graphical representation of 50μM treatment where * represents a statistically significant decrease (using arbitrary units) in glucose uptake comparing insulin vs. drug with insulin, using unpaired t-test, p<0.05. Experiments were repeated three times.
Figure 64. PKCβ inhibition via LY379196 attenuates adipocyte glucose uptake. Day 8 3T3-L1 adipocytes were serum starved for 4 hrs, ± 30 min 25 or 50μM LY379196, ± 15 min 100nM insulin. Glucose uptake assay was performed as described in Methods and Materials. (a) Dose curve shows the effect of increasing drug concentrations on glucose uptake comparing insulin vs. drug with insulin. (b) Effect of 25 or 50μM drug concentrations on glucose uptake. In both graphs, * represents a statistically significant decrease in glucose uptake (using arbitrary units) comparing insulin vs. drug with insulin, using unpaired t-test, p<0.05. Experiments were repeated three times.
Figure 65. CGP53353 specifically targets PKCβII phosphorylation. Day 8 3T3-L1 adipocytes were serum starved for 4 hrs, ± 30 min 50μM CGP53353, ± 15 min 100nM insulin. (a) Whole cell lysates were run on an SDS-PAGE gel and probed with respective antibodies. (b) Graphical representation shows the ratio of pPKCβII/pPKCδ, where * is a statistically significant decrease comparing insulin vs. drug with insulin using unpaired t-test, p<0.05. Experiments were repeated three times.
Figure 66. GLUT4 translocation is blocked by PKCβII inhibition. Day 8 3T3-L1 adipocytes were serum starved for 4 hrs, ± 30 min 50µM CGP53353, ± 100nM insulin 15 min. Subcellular fractionation was performed as described. 5µg for each sample of each fraction (a,b,c) was loaded onto a SDS-PAGE gel and immunoblotted. Graphical representation of PM GLUT4 (d) and LDM GLUT4 (e), where * represents a statistically significant change (using arbitrary units) comparing insulin vs. drug with insulin using an unpaired t-test, p<0.05. Experiments were repeated on three occasions with similar results.
Figure 67. PM sheet assay confirms PKCβII role in GLUT4 translocation. Day 8 to day 12 3T3-L1 adipocytes were serum starved for 4 hrs, ± 30 min 50µM CGP53353, ± 100nM insulin 15 min. PM sheets were obtained as described in Experimental Procedures. (a-d) Green staining represents GLUT4. (e-h) Red staining represents PM. (i-l) Colocalization (yellow) is the merger of GLUT4 and PM staining. Five to ten arbitrary fields per condition were obtained, the average being shown. Images shown here are the representative single optical section from z-series sections taken at average 0.2 µm step. (m) Graphical representation shows a statistically significant decrease (using arbitrary units) in GLUT4 over PM staining comparing insulin vs. drug with insulin using an unpaired t-test (*, p<0.05). Experiment was repeated three times.
Figure 68. PKCβII may affect organization of GLUT4. Day 8 – Day 12 3T3-L1 adipocytes were treated ± 50uM CGP53353 30 min ± 100nM pig insulin 15 min. Cells were fixed and stained as described. Red staining represents N terminal GLUT4. Blue (DAPI) staining represents the nucleus.
Figure 69. Effect of PKCβII inhibition on Akt phosphorylation. Day 8 3T3-L1 adipocytes were serum starved for 4 hrs, ± 30 min 50µM CGP53353, ± 100nM insulin 15 min. (a) Whole cell lysates were run on an SDS-PAGE gel and probed with respective antibodies. (b) Graphical depiction of pAkt/total Akt, where black bars represent pAkt Serine 473 / total Akt and white bars represent pAkt Threonine 308 / total Akt. * represents a statistically significant inhibition (using arbitrary units) of phosphorylation of Akt at Serine 473 comparing insulin vs. drug with insulin using unpaired t-test, p<0.05. Experiments were repeated three times.
Day 8 – Day 12 3T3-L1 adipocytes were treated ± 50μM CGP53353 30 min ± 100nM pig insulin 15 min. Cells were fixed and stained as described. (a-d) Red staining represents pAkt 473. (e-h) Blue staining indicates DNA. (i-l) Colors were merged.

**Figure 70.** PKCβII inhibition abolishes phosphorylation of Akt Serine 473 and its subcellular locations. Day 8 – Day 12 3T3-L1 adipocytes were treated ± 50μM CGP53353 30 min ± 100nM pig insulin 15 min. Cells were fixed and stained as described. (a-d) Red staining represents pAkt 473. (e-h) Blue staining indicates DNA. (i-l) Colors were merged.
Figure 71. CGP53353 treatment does not alter mTORC2 activation. Day 8 3T3-L1 adipocytes were treated ± 50μM CGP53353 30 min ± 100nM pig insulin 15 min. (a) Lysate were run on a SDS-PAGE gel and western blotted with indicated antibodies. (b) Graphical representation of two independent experiments (measured using arbitrary units) using unpaired t-test, p<0.05.
Figure 72. Insulin-dependent binding of PKCβII with activated mTORC2. Day 8 3T3-L1 adipocytes were treated ± 50uM CGP53353 30 min ± 100nM pig insulin 15 min. Lysate was harvested under non-denaturing conditions and co-immunoprecipitated with antibody listed. Co-immunoprecipitated proteins were combined with Laemmli’s buffer and boiled. Samples were run on a SDS-PAGE gel and western blotted with indicated antibody.
Figure 73. Insulin-dependent binding of PKCβII with phospho Akt S473. Day 8 3T3-L1 adipocytes were treated ± 50μM CGP53353 30 min ± 100nM pig insulin 15 min. Lysate was harvested under non-denaturing conditions and co-immunoprecipitated with antibody listed. Co-immunoprecipitated proteins were combined with Laemmli’s buffer and boiled. Samples were run on a SDS-PAGE gel and western blotted with indicated antibody.
**Figure 74.** Co-IP of GLUT4 and PKCβII. Day 8 3T3-L1 adipocytes were treated ± 50μM CGP53353 30 min ± 100nM pig insulin 15 min. Lysate was harvested under non-denaturing conditions and co-immunoprecipitated with antibodies listed. Co-immunoprecipitated proteins were combined with Laemmli’s buffer and boiled. Samples were run on a SDS-PAGE gel and western blotted with indicated antibodies.
DISCUSSION

The major findings of my research are: 1) TZDs may be able to regulate co-transcriptional splicing of PKCβ in rat L6 skeletal muscle cells; 2) PGC1α also seems to be able to mimic TZD’s effect on PKCβ co-transcriptional splicing; 3) the PGC1α C-terminal domain is necessary for bridging PKCβ transcription and splicing; 4) 3T3-L1 cells developmentally regulate PKCβ expression where PKCβII increases during adipogenesis and PKCβI decreases during adipogenesis; 5) PU.1 binds to the PKCβ promoter in 3T3-L1 adipocytes and may be responsible for developmental switching between the two isoforms; 6) PKCβII is critical for ISGT by regulating GLUT4 translocation; and 7) PKCβII (being downstream of activated mTORC2) regulates GLUT4 translocation by directly phosphorylating and fully activating Akt at Serine 473.

Thiazolidinediones have been used since 1997 to treat hyperglycemia in type 2 diabetes. Currently, Pioglitazone and Rosiglitazone are the only compounds licensed for type 2 diabetic patients. [443]. TZDs work by decreasing insulin resistance. To elicit their insulin-sensitizing effects, TZDs directly activate PPARγ. They seem to do this by primarily working on adipose tissue even though the major effect on insulin-sensitization occurs in skeletal muscle. This is in part due to altered free fatty acid supply. By activating adipocyte PPARγ, TZDs promote differentiation of adipose tissue that is smaller and more responsive to insulin, pre-dominantly subcutaneously. In addition, TZDs reduce the output of inflammatory TNFα and FFAs while increasing output of
adiponectin which increases peripheral insulin-sensitivity [338]. TZDs have also shown benefit to the vasculature. Rat VSMCs undergo reduced migration and proliferation in response to TZD treatment [444]. Yamamoto et al. showed that PKCβ isoforms have opposing functions in A10 VSMCs. PKCβI was associated with accelerated cell doubling time and increased S phase cell population whereas PKCβII was associated with attenuation of cell doubling time and delayed entry into S phase [41]. Patel et al. showed that acute hyperglycemia, which aggravates cardiovascular tissue injury, post-transcriptionally destabilizes PKCβII mRNA [445; 446]. Here, we show that TZD treatment is able to regulate alternative splicing, favoring the PKCβII isoform (Figure 24). PPARγ expression in VSMCs is associated with growth inhibition and differentiation through a GATA-6 dependent transcriptional mechanism [447]. Whether PPARγ is mediating the observed effects on VSMC PKCβ alternative splicing, remains to be seen.

L6 cells represent a good model to study insulin signaling because skeletal muscle accounts for the majority of post-prandial glucose disposal [420]. 24 hour treatment of 1µM Pioglitazone mimicked insulin's effect on PKCβII exon inclusion, even synergizing when combined with insulin stimulation (Figure 25). Western blotting confirmed that 24 hours 1µM Pioglitazone was able to stimulate PKCβII protein expression to a similar extent as insulin (Figure 26). This is significant because the mechanism, in which TZD benefits skeletal muscle signaling, as it pertains to glucose disposal, remains unknown [448]. PKCβII is a critical regulator of L6 ISGT [40; 113]. TZD treatment could possibly bypass a defective upstream insulin signaling pathway to upregulate PKCβII expression. Since TZD treatment is supposedly ineffective at lowering serum glucose
levels in the absence of insulin [414], TZDs could enhance L6 insulin sensitivity by priming cells with PKCβII. This may lower the threshold of insulin signaling required for full PKCβII activation and thus ISGT. It is even possible that some alternatively spliced PKCβII, as a result of TZD stimulation, could become activated by another pathway in the absence of insulin and therefore clear serum glucose in the basal state. Non-insulin stimulated PKCζ (another PKC isoform involved in GLUT4 translocation) activation and basal glucose uptake were increased in adipocytes with Rosiglitazone treatment [449]. Rosiglitazone has been shown to enhance acute 5′AMP-Activated Protein Kinase mediated glucose uptake (insulin independent) in muscle and adipose tissue of high-fat fed rats [450]. Contraction-stimulated muscle glucose uptake is normal in type 2 diabetics and signals through the AMPK pathway. AMPK (stimulated by muscle contraction as well as hypoxia, ischaemia, heat shock, decreased pH, glycolysis inhibition, and by uncouplers of oxidative phosphorylation) can stimulate skeletal muscle glucose uptake in the absence of insulin and through a PI3K independent pathway. aPKCs have been shown to have a role in AICAR (aminomidazole carboxamide ribonucleotide)-stimulated glucose uptake in both L6 cells and in isolated rat muscle [451]. PKCα has been ruled out as a possible mediator of contraction-mediated glucose uptake in mouse skeletal muscle cells. However, due to the fact that cPKC inhibitors inhibit contraction-stimulated glucose uptake [452], other cPKC isoforms such as PKCβII can not be ruled out. It is possible that TZDs could synergize with skeletal muscle contraction-mediated glucose uptake in part by having PKCβII bioavailability which could be a downstream target of AMPK.
Overexpression of PPARγ, PGC1α and SRp40 all resulted in increased PKCβII mRNA and protein levels (Figures 26, 27, 31, 33). Knockdown using siRNA further stressed the importance of these factors in PKCβII exon inclusion (Figure 34-35). Overexpression of the PPARγ E499Q mutant resulted in reduced PKCβII protein expression with no effect on PKCβI levels (Figure 32). This PPARγ mutant can bind ligands with affinity equal to wild-type but can not undergo ligand-dependent transcriptional activation. However, PKCβI expression is unaffected which suggests that basal transcriptional activity in this case is independent of PPARγ ligands. The most plausible explanation for this is that the overexpressed PPARγ is becoming transcriptionally active via its AF1 domain. PPARγ activation depends on the conformation of its C-terminal AF2 helix. Ligands such as TZDs lock the AF2 in an active conformation. The active AF2 conformation forms a charge-clamp pocket that interacts with the LXXLL motif of coactivators. On the other hand, PPARγ in known to have high basal ligand-independent activity [453]. Fibroblast overexpression of PPARγ without a functional ligand binding domain was still able to drive adipogenesis. It is possible that when PPARγ was overexpressed, there is a threshold that when crossed may relieve the requirement for ligand activation [454]. There is no evidence of PPARγ ligands being produced in cultured skeletal muscle cells; although it can not be ruled out (different medium could make a difference). Even in adipose tissue, which abundantly expresses PPARγ, endogenous ligands remain poorly characterized [455]. The structure of ligand-free PPARγ assumes both active and inactive conformations. The active conformation may be favored by increased amounts of PPARγ coactivators, such as PGC1α, which can bind PPARγ in the absence of ligands [453]. To explain why the
PPARγ E499Q mutant prevented PKCβII expression even though the ligand binding is likely not needed for transcriptional activation of the PKCβ promoter, the mutation must affect the ability of coactivators to bind PPARγ. Coactivators such as SRC-1/NCoA-1, CBP/p300, pCAF, TRAP220 and PGC1α can activate PPARγ in a ligand-dependent as well as ligand-independent manner. These coactivators dock on PPARγ on the AF2 or helix 12 region of the LBD and this binding shows sequence conservation [456]. Without exogenous ligands, skeletal muscle cell PPARγ is required for normal rates of fatty acid uptake [457] suggesting that forced PPARγ expression will be active. It is possible that either the mutant PPARγ disrupts the sequence recognition of the coactivator or it alters the folding/conformation of PPARγ such that it can not bind certain coactivators or oscillate between the active-inactive conformations. In either case, this would prevent it from binding coactivators that might potentially recruit splicing factors to influence PKCβ alternative splicing.

Hypothesis #1 asserted that PPARγ would influence PKCβ alternative splicing by directly binding to a PPRE located on the PKCβ promoter. In order to test this, we devised a heterologous PKCβ minigene transcriptionally regulated by 2243bp of the human PKCβ promoter (Figures 36-48). Initial experiments showed promising results whereby PPARγ was able to influence both BI and BII exon inclusion when the plasmid had the PKCβ promoter as opposed to the CMV promoter (Figure 51). However, deletion of the putative DR2 proved to have no effect on overexpressed PPARγ’s influence on the PKCβ promoter (Figure 52). It is possible that other putative PPREs that were overlooked might have been better candidates. This is unlikely because the other candidates were inverted repeats or everted repeats which are not standard PPREs.
PPREs have been known to be located far away from the transcriptional start site [455]. The PKCβ promoter insert may have been too short and would therefore not harbor the actual PPRE. To make matters more complicated, PPARγ exhibits promiscuity in binding to the PPRE as opposed to RXRα which can not tolerate deviation from the consensus sequence. The first three bases of the 5’ half site appear to be more critical than the last three for PPARγ recognition [296]. The 2243bp of the human PKCβ promoter insert may contain more putative PPREs (possibly even DR1s) if the emphasis was put on only the 3’ half site.

PPARγ is still possibly a major player in regulating the PKCβ promoter in skeletal muscle cells. Despite being detected by RT-PCR (Figure 34), PPARγ expression is notoriously low in skeletal muscle. In addition, PPARγ does not account for all skeletal muscle benefits via TZD treatment. Muscle-specific PPARγ deficient mice that are insulin resistant still respond to TZD treatment [457]. The coactivators and other activators present in skeletal muscle may alter the stoichiometry of PPARγ such that very little is needed. An interesting point discussed by Lefterova et al. is that in some genes, PPARγ is constitutively associated with coactivators, leading to high levels of transcription [455]. Overexpressed PPARγ in skeletal muscle cells may be constitutively bound to a coactivator on the PKCβ promoter causing constitutive co-transcriptional splicing.

One experiment that would have given a more complete picture is PPARγ overexpression combined with TZD treatment. The combined TZD treatment would ensure PPARγ activation. However, one caveat with this is that PPARγ may not be activated in the same manner. TZD is considered a full agonist [300]. For argument’s
sake, we'll ascribe PPARγ overexpression as a partial agonist. A partial agonist is a compound that at saturating concentrations produces activity below that of saturating concentrations of a full agonist. It is important to note that partial agonists do not increase the interaction of PPARγ with corepressors. Depending on the level of activation, PPARγ will differentially bind coactivators. By causing PPARγ to become selective in terms of the coactivators it recruits, partial agonists will allow the induction of some but not all PPARγ target genes. This is the concept behind “selective PPARγ modulators” (SPPARγM). This is the idea that future PPARγ agonists will modulate the metabolic genes necessary and sufficient for insulin sensitization while not affecting genes involved in fat accretion and other side effects (e.g. edema) associated with TZD treatment [300]. PA-082 was a novel partial agonist of PPARγ which selectively recruited PGC1α. This drug was able to prevent Rosiglitazone-driven triglyceride accumulation in mouse stem cells. At the same time, PA-082 was also able to induce mRNAs of insulin signaling components and adipogenic differentiation pathways [458].

The point of this is that TZD activation of overexpressed PPARγ may have caused PPARγ to associate with competing coactivators which may have hindered its ability to stimulate PKCβ alternative splicing. Overexpressed PPARγ on its own may be able to bind coactivators, without competition, that promote PKCβ alternative splicing.

The benefits of TZDs in skeletal muscle are largely independent of PPARγ [443]. A much more tantalizing prospect is TZD mediating its skeletal muscle effect through PGC1α. PGC1α overexpression is able to closely mimic TZD treatment in terms of its effects on PKCβ co-transcriptional splicing in muscle. 1μM Rosiglitazone and Pioglitazone for 24 hours were able to increase overall transcription yet select the PKCβII
isoform through alternative splicing (Figure 29). PGC1α was able to elicit a similar trend (Figure 31), keeping PKCβI constant while increasing PKCβII exon inclusion. Although PGC1α protein expression was very difficult to detect by western blotting, it is said to be expressed heavily in skeletal muscle [459]. This problem with detection could be due to the L6 cell line. PGC1α protein expression in rat skeletal muscles is highly correlated with mitochondrial density and oxidative capacity. Muscles that express PGC1α switch from a type IIb (fast twitch) muscle fiber type to a fast twitch type IIa and type I (slow twitch) which contain more mitochondria and exhibit higher rates of oxidative metabolism [376; 459]. In addition, PGC1α expression increases muscle glycogen stores via increased glucose transport (also involves increased GLUT4 expression), suppression of glycolytic flux and by inhibition of glycogen degradation pathway (down-regulation of glycogen phosphorylase levels and activity). Muscles with forced expression of PGC1α (similar to exercise-induced expression of PGC1α) energize by fatty acid oxidation instead of glycolysis [357; 373]. Of interest here is the fact that PGC1α controls glucose uptake. This could provide an explanation as to why PGC1α would regulate PKCβ co-transcriptional splicing. PKCβII is crucial in L6 insulin signaling. C-terminal deletion PKCβII mutant expression as well as pharmacological inhibition using PKCβII-specific CGP53353 (1μM) revealed that PKCβII was crucial for insulin-stimulated glucose uptake [40]. PKCβII mediates these effects through MARCKS phosphorylation, increased membrane PLD1 and cytoskeletal remodeling [113]. If TZD stimulation in skeletal muscle cells were exerting their effect through PGC1α, it could do so either through increased expression (coactivation) or increased activity/disabling repression. The former has been demonstrated in mouse primary brown adipocytes and 3T3-L1
adipocytes. This regulation occurs via PPARγ-mediated transcriptional activation on the PGC1α promoter PPRE [356]. Even though PPARγ in L6 cells is expressed in small quantities, it is still possible that TZD-mediated PPARγ transcriptional activation could induce increased PGC1α expression. Or TZD stimulation in skeletal muscle may activate other transcription factors (which may even be coactivated by PGC1α) that will lead to increased PGC1α protein levels. TZDs may also alter the activity of PGC1α. p38 stress-activated MAPK phosphorylates PGC1α at three residues (T262, S265, and T298) in the negative regulatory domain. These phosphorylations lead to dissociation of repressor-bound p160MBP. Ultimately, PGC1α becomes more stable with increased half-life as well as possessing increased transcriptional activity [353; 460]. TZDs are capable of activating p38 independent of PPARγ in rat GN4 liver epithelial cells [461]. Glucose uptake in insulin resistant skeletal muscle cells in restored by TZD treatment in part by activation of p38 [462]. PGC1α activity can be regulated by means other than phosphorylation. Methylation via PRMT1 (protein arginine methyltransferase 1) has been shown to positively regulate PGC1α transcriptional activity [463]. Acetylation (nicotinamide-induced) has been reported to negatively regulate PGC1α transcriptional activity (albeit in liver cells) [464]. TZD-induced PGC1α activation may affect co-transcriptional splicing of PKCβ. It is important to mention that the PGC1α and TZD effect on splicing needs to be confirmed showing RNA data from both PKCβI and PKCβII isoforms. Until then, it is possible that both treatments increase only PKCβII mRNA translation. In that scenario both PKCβ transcripts could be transcriptionally upregulated while splicing remained unaffected. Although unlikely, it is even possible
that the treatments aid in selectively stabilizing the PKC\(\beta\)II mRNA versus the PKC\(\beta\)I mRNA.

PGC1\(\alpha\)’s cited involvement in alternative splicing is another reason why it is an attractive PKC\(\beta\) modulator. Monsalve et al. evinced the role of PGC1\(\alpha\) CTD in bridging transcription and splicing. The CTD contains two RS domains and an RRM domain (Figure 22). The RRM is required for induction of target genes. In the basal state, PGC1\(\alpha\) co-localizes with SR proteins at nuclear speckles. PGC1\(\alpha\), through its RS domain is able to bind SR proteins, most prominently SRp40. Upon activation (phosphorylation), both PGC1\(\alpha\) and SR proteins alter their intracellular location within the nucleus to sites of active mRNA synthesis. These proteins end up being part of the hyperphosphorylated RNA polymerase II complex. It was shown that PGC1\(\alpha\) (via CTD deletion) was able to modulate splicing of a fibronectin minigene [362]. The CTD of PGC1\(\alpha\) is critical in mediating its effect on PKC\(\beta\) alternative splicing (Figure 28). Co-overexpression of PGC1\(\alpha\) and the PKC\(\beta\) minigene would provide more insight as to PGC1\(\alpha\)’s role in PKC\(\beta\) alternative splicing. SRp40 overexpression in another Cooper lab PKC\(\beta\) minigene (BII exon only) stimulates insulin-induced exon inclusion [37]. In vivo, insulin induces Akt-mediated SRp40 phosphorylation which stimulates PKC\(\beta\)II exon inclusion (assayed by RT-PCR) by direct binding to a SRp40-binding motif [38; 211]. Here, we further demonstrate that SRp40 overexpression leads to increased PKC\(\beta\)II protein expression (Figure 26) and knocking down SRp40 decreases PKC\(\beta\)II exon inclusion (Figure 35). It should be noted that the degree of SRp40 knockdown would more closely mirror the knockdown of PKC\(\beta\)II if the number of SRp40 PCR cycles were reduced. As far as hypothesis #1 is concerned (Figure 30), it is possible that TZD
treatment activates PGC1α which recruits SRp40 (would likely have to be phosphorylated) via PGC1α’s CTD. SRp40 would then associate with the RNAPII CTD and influence splice site selection of the nascent PKCβ pre-mRNA. However, the transcription factor that would initially bind PGC1α to the promoter is even less certain. With respect to the minigene, it is possible that not enough SRp40 was available even if PPARγ was bound to the minigene PKCβ promoter or even if PGC1α was able to coactivate the minigene PKCβ promoter. It is also possible that some sort of stimulation that promoted endogenous SRp40 hyperphosphorylation may be needed for translocation and coactivator docking. Overexpression of SRp40 may induce its phosphorylation by increasing the probability of interacting with an SR kinase such as Akt or Clk or possibly increasing splicing of certain SR kinase genes (personal speculation). This could explain why combined overexpression with either PPARγ or PGC1α resulted in higher endogenous PKCβII mRNA levels than when overexpressed alone (Figure 27).

Work on skeletal muscle signaling has revealed PGC1α as the most promising potential target of TZDs. Both PGC1α and PKCβII expression are associated with enhanced skeletal muscle ISGT. Future experiments would focus on establishing whether PGC1α is able to associate with the endogenous PKCβ promoter in skeletal tissue (via ChIP assay). Assessing PGC1α’s activity levels with TZD treatment would also give insight into whether its coactivation ability was enhanced as well as its ability to bind SR proteins (e.g. phosphorylated RS domain). Knocking out PGC1α expression (via siRNA transient transfection) and then treating with TZDs would reveal whether PGC1α is necessary for the TZD effect on PKCβ co-transcriptional splicing.
Additionally, performing a ChIP assay with the PGC1α antibody would confirm indirect binding of PGC1α to the PKCβ promoter.

3T3-L1 pre-adipocytes were next used to assess possible TZD-mediated PKCβ co-transcriptional splicing. When differentiated properly, 3T3-L1 cells become adipocytes. Differentiation technique was verified by phase contrast microscopy as well as oil red O staining. A conscious effect was made to avoid DMSO in dissolving the differentiation compounds Dexamethasone and IBMX (insulin was dissolved in water). This is because the action of DMSO is associated with increased expression of PKCβ, α, γ isoenzymes and alternative splicing [121; 465]. For reasons that are apparent in the results section, use of DMSO could have called into question the observed effects of differentiation-regulated PKCβ alternative splicing (as well as differentiation-regulated transcription of PKCα and γ). Ethanol was instead used to dissolve the two compounds mentioned.

Glucose concentrations in the media were also examined. Most papers use high glucose media to culture and differentiate 3T3-L1 cells. But because we were looking at PKCβII expression, this presented a potential problem. Patel et al. had reported that hyperglycemia can post-transcriptionally destabilize PKCβII mRNA (but not PKCβI mRNA) in A10 VSMCs and primary human aorta cultures [466]. This is the reason why both low and high glucose DMEM media was tested initially. If high glucose destabilized PKCβII expression, then low glucose media would be opted for. However, neither media had an effect on 3T3-L1 adipocyte PKCβII expression during development (Figure 54-55). Therefore, we chose to stick with the mainstream culture media.
The 3T3-L1 cell line is an ideal model for studying fat cell development and signaling since results in 3T3-L1 adipocytes have repeatedly been confirmed in mouse models [467]. During differentiation, many genes are programmed to initiate or cease. cDNA microarray analysis of 3T3-L1 cells show PKCβ expression at least ten fold higher in 3T3-L1 adipocytes versus 3T3-L1 fibroblasts [419]. This suggested a role for PKCβ in adipogenesis and other adipocyte metabolic functions. Our results confirm that PKCβ is regulated during 3T3-L1 differentiation (Figure 56-57). However, Guo et al. did not address which PKCβ isoform was upregulated over ten fold. We believe this to be the first example of differentiation-regulated PKCβ alternative splicing, whereby PKCβI starts off as the predominant 3T3-L1 pre-adipocyte isoform and is later replaced during differentiation with the PKCβII isoform. This form of regulation is almost certainly disparate from the type of PKCβ alternative splicing that occurs in L6 skeletal muscle cells within 15 min of insulin treatment [39]. The fact that PKCβII protein expression peaks at day 8, the same time when robust lipid droplets can be observed under the microscope, suggests that PKCβII could be involved in adipogenesis. This could explain why protein and mRNA levels of PKCβII dip after peaking. PKCβII may be critical for terminal differentiation. Once this occurs, PKCβII may be needed in lesser amounts. As far as the mRNA of day 8 and day 10 falling below day 4, this could be explained by the fact that a single PKCβII mRNA strand may be more frequently translated after terminal differentiation. As to why PKCβII mRNA peaks at day 6 (Figure 56) and PKCβII protein peaks at day 8 (Figure 57), this could be explained by differences in technique sensitivities or other factors such as microRNAs regulating protein translation. Figure 58 indicates that the distal polyA tail (located downstream of the PKCβI exon) is used for
both PKCβI and PKCβII mRNA expression. This may add stability to the mRNA transcript. The expression pattern of PKCα and ζ (Figure 56) were congruent with expression patterns reported by McGowan et al. [426]. PKCζ’s expression pattern is a non-sequitur for a kinase confirmed to play a major role in 3T3-L1 adipocyte insulin signaling [127; 129; 133; 145; 153; 283]. It is possible that heightened PKCζ expression is needed for adipogenesis and then returns to a level that can still support insulin-stimulated GLUT4 translocation. PKCδ (with all its possible isoforms) may also function in promoting adipogenesis based on its expression pattern. PKCδ’s function in skeletal muscle primary cultures suggests that it could provide a negative feedback to insulin receptor tyrosine kinase activity [468]. The expression pattern of PKCγ, to our knowledge, has not been reported in this cell line. It too could be involved in differentiation but its expression rises and falls so precipitously from day 6 to day 8 and then from day 8 to day 10 respectively, that it may only be needed for the terminal phase of adipogenesis. PPARγ2 and PPARγ1 (which can compensate if PPARγ2 is deficient) are essential for adipogenesis [455] and their presence confirms proper adipogenetic signaling. Adiponectin is also a marker of adipocytes. GLUT4 protein expression (Figure 56) signals the beginning of insulin responsiveness. PKCβII and GLUT4 protein expression are very similar. Based on previous data from the Cooper lab in which PKCβII knockdown resulted in reduced GLUT4 protein expression, it was hypothesized (#2) that PKCβII could affect GLUT4 expression/stability.

Our inability to transfect 3T3-L1 adipocytes prevented this hypothesis from going any further. Factors that could influence differentiation-regulated PKCβ alternative splicing were next examined. As mentioned in the Results section, PU.1 was the lead
candidate because it had consensus binding sites covering the PKCβ promoter as well as being a factor that could regulate alternative splicing. PU.1 interacts with proteins containing RNA binding motifs. One of these proteins is TLS/FUS, whose C-terminal domain contains three RNA recognition motifs (RRMs) and two arginine glycine-rich regions (RGGs). The RGG2-3 domain is capable of recruiting SR proteins [469]. We hypothesized that if PU.1 bound the PKCβ promoter during differentiation, it could select the SR proteins, and therefore the PKCβ isoform, based on coactivator recruitment. ChIP assays revealed that PU.1 is likely associated with the PKCβ promoter. It was bound to several spots on the promoter, although usually stronger on day 0 than on day 8. Several possibilities exist as to PU.1’s function on the promoter. First, it could be a stimulator or repressor of PKCβ alternative splicing depending on which site it occupies. Second, even though PU.1 overexpression has been shown to inhibit overall 3T3-L1 differentiation, it could be transcriptionally repressive with respect to most adipogenic genes (allowing for adipogenesis) but active with respect to PKCβ. This would explain why 3T3-L1 adipocytes continue to express PU.1 (Figure 61) even though it has anti-adipogenic properties when overexpressed [437]. Also, as mentioned in the Results section, overexpression of PU.1 in 3T3-L1 must be interpreted with caution as this may have caused PU.1 to cross a threshold expression level to where it became anti-adipogenic. PU.1 may behave like PPARγ. By this I mean that the binding of PU.1 to the PKCβ promoter (whether day 0 or day 8) may be the same for a given site but its activation may change due to differentiatial coactivator recruitment or post-translational modifications. This change in activity may not be possible at day 0 due to the lack of a certain factor that can only be expressed when the proper adipogenic signals are present.
To our surprise, SRp40 precipitated with the PKCβ promoter. There were noticeable differences between day 0 and day 8 depending on the region. Regardless, association with the PKCβ promoter upstream of the transcriptional start site poses the possibility that SRp40 is recruited via transcription factors and coactivators during transcription. According to the literature, this is possible as long as SRp40 is serine phosphorylated prior to arrival [470]. GATA2, like PU.1, is also a known anti-adipogenic protein. Its expression could not be verified using RT-PCR, however, it did appear in the ChIP assay showing binding preferences based on whether it was day 0 or day 8. Of interest, from -4384 to -4183, GATA2 was bound strongly on day 0 and not day 8. Perhaps, PKCβ needs this site to be free of GATA2 in order to accomplish alternative splicing. GATA1 and AML1 did not bind in this assay, even though AML1 did bind in a previous assay. AML1/RUNX1 has been confirmed to bind the human PKCβ promoter in U937 cells [435]. Perhaps the AML1 antibody had lost potency or minute changes in cell culture conditions altered intracellular signaling to the point where AML1 could no longer bind the PKCβ promoter.

Due to difficulties in transfecting 3T3-L1 adipocytes, PU.1 could not be knocked down so we could not test directly whether PU.1 is needed for PKCβ alternative splicing (hypothesis #3). Despite this, we moved on to test the physiological relevance of PKCβII with respect to ISGT. Establishing a connection between PKCβII and ISGT would be very significant. Adipose tissue accounts for only a fraction of glucose disposal after a meal (~10-15%) with muscle accounting for much of the remainder. However, global glucose homeostasis depends on adipocytes. This is not only based upon increased or decreased adiposity but on the GLUT4 signaling inside the adipocyte [420]. Adipocyte
GLUT4 signaling has effects on circulating serum adiponectin, an adipokine crucial for peripheral insulin sensitivity [471]. Adipose–specific overexpression of GLUT4 has been reported to reverse insulin resistance and diabetes in mice lacking muscular GLUT4 [472]. Adipose-selective targeting of the GLUT4 gene in mice impairs insulin action in muscle and liver [473]. Regulation of adipocyte GLUT4 affects not only adipocyte glucose uptake but global glucose homeostasis. The fact that PKCβII is critical in skeletal muscle ISGT begs the question as to whether it holds a similar role in adipocytes. Several lines of independent experimentation support the notion that insulin-dependent GLUT4 translocation is similar or identical in skeletal muscle and adipocytes [474; 475]. In both cases, insulin-dependent GLUT4 translocation is PI3K dependent [476].

To establish this connection, experiments were modeled after Chalfant et al. using PKCβII pharmacologic inhibition and [3H]2-deoxyglucose uptake [40]. 50μM CGP53353 was able to significantly inhibit adipocyte ISGT (Figure 63). 25μM LY379196 was also able to significantly inhibit adipocyte ISGT (Figure 64). Use of both inhibitors has culminated in a narrow list of possible glucose uptake effectors. According to the IC₅₀ spectrum (in nM) of CGP53353, 50μM will inhibit PKCβII (IC₅₀=0.41), PKCβI (IC₅₀=3.8), PKCα (IC₅₀=1.9), PKCγ (IC₅₀=22) and EGFR (IC₅₀=0.7) [126]. Beyond 50μM, CGP53353 will not inhibit any other PKC isoform including PKCζ which is considered the predominant PKC isoform involved in 3T3-L1 adipocyte ISGT [127]. Glucose uptake assays were performed on day 8 or later. At this point there is virtually no PKCβI protein expression relative to day 0 (Figure 56). It is unlikely that it plays a significant role in glucose uptake. In addition, there is no documented role for PKCβI in insulin-stimulated glucose uptake in any cell line [40; 477]. CGP53353 at 20μM is able
to inhibit ISGT by roughly 45%. Combined with the reality that intracellular concentrations of the drug are likely much lower than 20μM, PKCγ can be ruled out as a major drug target. Use of the LY379196 did not help to further eliminate cPKC isoforms. However, the fact that 25μM was able to significantly inhibit ISGT confirmed that there may be a novel PKC isoform involved in ISGT other than PKCζ (IC50=48μM). LY379196 also helped to eliminate EGFR from possible CGP53353 effectors since LY379196 does not inhibit this protein [125]. EGFR is downregulated during 3T3-L1 differentiation and was not considered to be a factor in ISGT [478]. PKCδ (IC50=0.7) would also be inhibited by LY379196. However, inhibition of PKCδ activity does not inhibit glucose transport in 3T3-L1 adipocytes [479]. Both drugs inhibit PKCα with IC50s close to that of PKCβII and for this reason, PKCα can not be completely excluded.

On the other hand, PKCα has been shown to negatively regulate the insulin receptor tyrosine kinase activity by interacting with IRS1 [480]. Angiotensin II inhibits insulin-induced Akt activation through PKCα in VSMCs [481]. Hence, it would explain why 3T3-L1 cells downregulate PKCα during the course of adipogenesis and it is thus unlikely that CGP53353 is mediating its effects through PKCα [426]. This leaves PKCβII as the best candidate.

Hypothesis #4 asserted that PKCβII would regulate adipocyte ISGT through GLUT4 translocation or GLUT4 fusion. In L6 cells, Chappell et al. showed that CGP53353 at 1μM, a dose specific for PKCβII, was able to prevent GLUT4 fusion by isolating PM fractions [113]. This result hinted at the possibility that PKCβII regulated vesicle fusion but it was also assumed that this effect was due to inhibition of GLUT4 translocation. It is worth while to address the issue that the role of PKCβII in ISGT is
controversial. Several papers have been published for and against a role for PKCβII in ISGT regulation. The use of C-terminal mutant PKCβII and CGP53353 in L6 cells has already been described [40]. In addition, LY379196 (at 3 X 10^8 mol/l) was able to inhibit ISGT in 6 day-old cultured myotubes [482]. Interestingly, the same inhibitor was used to make the case that cPKCs could not regulate L6 ISGT [483]. However, the same group also showed that PKCα, PKCβ and PKCζ were activated by insulin in 3T3-L1 cells [484]. This would suggest that PKCβ has some role in insulin signaling. Systemic knockout of mouse PKCβ was reported to have no effect on glucose homeostasis in both fat and skeletal muscle tissue [485]. This supposedly ruled out PKCβII as a player in fat or muscle GLUT4 trafficking. However, caution must be exercised when drawing conclusions from systemic knockouts. Systemic knockout of GLUT4 has only mild perturbations in glucose homeostasis even though it is the *bona fide* transporter in insulin-sensitive tissues. Tissue selective targeting of GLUT4, however, has dramatic effects on ISGT [486]. Adipocyte-specific inducible knockout of PKCβII (future project) needs to be performed to definitively determine the role of PKCβII in adipocyte ISGT. However, work done here lays a foundation providing preliminary evidence that indeed, PKCβII (or at the very least cPKC) is involved in GLUT4 translocation (possibly fusion) and adipogenesis.

First, specificity of CGP53353 was determined using western blot analysis (Figure 65). Phosphorylation of insulin receptor, PKCδ and PKCζ were not altered. It was important to rule out PKCζ as this PKC isoform is involved in GLUT4 translocation [127; 153]. PKCζ activation involves PDK1 mediated Thr410 phosphorylation. Based on the literature we assumed this would be insulin-stimulated [487]. As is obvious, every
condition demonstrated pPKCζ Thr410. Activation of PKCζ has been reported through other stimuli including glucose in rat adipocytes [488]. The major activation pathway of PKCζ is PI3K-manufactured PIP3. PDK1 binds with high affinity to PIP3 in order to phosphorylate PKCζ. PKCζ isolated from bovine kidney is phosphorylated via PI3K-mediated PIP3 in response to growth factors [489]. Interestingly, Alessi et al. have shown that PDK1 isolated from unstimulated or insulin-stimulated cells possess the same activity towards the Akt target and others. In 293 cells, insulin stimulation did not change the phosphorylation status of PDK1. It is proposed that PDK1 could be constitutively active and that it is the substrate that needs to undergo a conversion in order to become phosphorylated [490]. In this same cell line, ceramide was shown to activate PKCζ in vitro [491]. 3T3-L1 adipocyte ceramide is associated with non-insulin dependent glucose uptake. Ceramide can stimulate PI3K in 3T3-L1 cells [492]. Perhaps PKCζ also has a role in basal glucose uptake and this necessitates its constitutive phosphorylation. Phosphorylation of PKCβ S660 (hydrophobic motif) was dramatically reduced with drug treatment with total PKCβ protein levels remaining constant. We also attempted to assess the phosphorylation status of PKCα (even though it is almost certainly inhibited as well) by using a Cell Signaling pPKCα/βII (T638/641) antibody. Only one band materialized and since we did not possess a peptide inhibitor at the time, this data could not be interpreted. Antibodies for pPKCγ were not practical as they cross-react with pPKCβII. pPKCβII (T641) (Abcam) was also probed for but there was too much background.

The dosage used for CGP53353 also needs to be addressed. Differentiating 3T3-L1 adipocytes develop impermeable cell membranes. During differentiation, more than
90% of the adipocyte population have reduced junctional permeability [493]. This is almost certainly the reason why pharmacologic inhibition can only be achieved by spiking the dosage. Multiple investigators have used high drug concentrations in 3T3L1 adipocytes to block intracellular signaling. For example, PD-98059 at 50μM was used to inhibit MEK activity and 100 μM naringenin to inhibit PI3K in 3T3-L1 adipocytes [494]. LY294002 at 50μM was used to inhibit PI3K activity [495]. SP600125 at 50μM was used to inhibition JNK activity and LY294002 at 100μM was used to inhibit PI3K activity [496]. These are concentrations at least 10-fold higher than what is required in skeletal muscle to block signaling.

CGP53353 administration abrogated GLUT4 translocation as evidenced by subcellular fractionation and PM sheet assays (Figure 66-67). This implicates PKCβII as a critical regulator of adipocyte ISGT. In addition, PKCβII may also have a role in “priming” GLUT4 for translocation. Figure 68 (b & d) shows that insulin is able to concentrate a pool of GLUT4, near what could possibly be the TGN. CGP53353 treatment causes GLUT4 to become more dispersed around the nucleus. This may implicate PKCβII in insulin-stimulated GLUT4 trafficking leading up to translocation. This data lends credence to part of Model 2 (Intro) concerning GLUT4 trafficking which states that insulin causes more GLUT4 to accumulate at a non-cycling pool near the TGN, which will translocate to the PM directly. Co-immunoprecipitation suggests that PKCβII may be able to influence GLUT4 directly or act on the GSVs to direct trafficking (Figure 73).

There are at least two discrete signaling pathways involved in insulin-regulated GLUT4 translocation in muscle and fat cells. The first involves PI3K and the second
involves the proto-oncogene c-Cbl. The two targets of PI3K that have been identified are serine/threonine kinase Akt and PKCζ. PI3K activates Akt by generating phosphoinositides in the inner leaflet of the plasma membrane. Akt docks to this through a pleckstrin homology domain bringing it in close proximity with PDK1 [127]. The mechanism of PKCζ activation is not known although it may involve dissociation from 14-3-3 proteins among other things [19]. Recent reviews describe PI3K signaling diverging into two post-PDK pathways [133; 145; 254; 283; 497]. One pathway diverges to an atypical PKC pathway shown to be crucial for activation of glucose transport in both muscle and fat cells. The other Akt dependent pathway leads to AS160 phosphorylation. Five of six AS160 consensus Akt phosphorylation sites are phosphorylated in response to insulin [498]. These phosphorylations render AS160's GAP domain inactive making it unable to negatively regulate Rab proteins. This allows Rab-dependent GLUT4 translocation to occur [497]. Using western blot analysis and immunofluorescent staining, CGP53353 selectively inhibited Akt phosphorylation at S473 while phosphorylation at T308 remained undisturbed (Figure 69-70). Immunofluorescent staining shows strong insulin-dependent recruitment of Akt to the PM as well as intracellular deposits that could be part of the GSV trafficking itinerary (Figure 70). This data suggests that PKCβII could be upstream of Akt during insulin signaling. This is not unusual since it has been reported that PKCβII mediates Akt S473 phosphorylation in a cell type and stimulus-specific event [438]. The importance of Akt in ISGT can not be overstated. James et al. reported that in 3T3-L1 adipocytes, fully active Akt is involved in a late stage insulin-induced GLUT4 translocation to the PM. This latter process involves docking and fusion of GLUT4 vesicles with the PM [499].

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Constitutively active Akt stimulated glucose uptake and GLUT4 translocation in 3T3-L1 adipocytes [180]. A kinase inactive, phosphorylation deficient Akt resulted in almost complete loss of L6 cell surface GLUT4 [185]. A similar strategy was used to verify Akt’s role in promoting insulin-stimulated cell surface GLUT4 in rat adipocytes [500]. More direct influence of Akt on GLUT4 has come from studies showing that insulin increases association of Akt2 with GSVs and this leads to phosphorylation of GSV component proteins [181; 182]. The functional consequence of this interaction, by use of fusion constructs with Akt and GLUT4, results in insulin-stimulated GLUT4 translocation to the PM [501]. PKCβII, through Akt could direct GLUT4 traffic. If Akt were to mimic GLUT4 localization, then PKCβII indirectly causes more GLUT4 to be available near the TGN for direct translocation to the PM (Figure 68).

The mTORC2 complex is responsible for Akt S473 phosphorylation and thus full activation in 3T3-L1 adipocytes. Phosphorylation of S473 results in the interaction between the hydrophobic motif and the N-terminal lobe leading to activation [174; 175; 229]. mTORC2 can be distinguished from mTORC1 based on residue phosphorylation of mTOR serine/threonine kinase. mTOR phosphorylation at S2481 defines the activate mTORC2 complex [61]. To resolve the apparent discrepancy that PKCβII was needed for S473 phosphorylation (in our model) and yet mTORC2 was the kinase responsible for this (throughout most of the literature), we used CGP53353 inhibition to assess its effect on mTORC2 activity. Figure 71 depicts an insignificant change in mTORC2 S2481 phosphorylation (and thus activity) when treated with CGP53353. This suggested few possibilities. One was that PKCβII was downstream of mTORC2 and it was really PKCβII that was phosphorylating Akt S473 in this cell line. Another possibility was that
PKCβII associated with the mTORC2 complex and was needed for substrate specificity. These assertions are reasonable given that PKCβII is phosphorylated at the turn motif by mTORC2 in MEF cells [54]. In addition, the phosphorylation of Akt via mTORC2 has not been shown to be direct [502]. Through co-immunoprecipitation, we provide evidence that the turn motif phosphorylation of PKCβII is associated with direct binding of mTOR (part of mTORC2) (Figure 72). This association increases with insulin treatment compared to control (lane 3 vs. lane 2) and is dramatically reduced with CGP53353 drug treatment (lane 3 vs. lane 5). PKCβII would then go on to autophosphorylate itself and subsequently phosphorylate Akt. Preliminary data is shown that suggests that this interaction between PKCβII and Akt is possible (Figure 73).

There is ample circumstantial evidence connecting PKCβII to GLUT4 translocation and ISGT alluded to throughout the introduction. PKCβII can be localized to the pericontron to play a role in hormone responses by controlling the trafficking of recycling endosomes containing Rabs. PKCβII can translocate to the pericentron in a biphasic DAG response that depends on PLD-derived DAG [19]. 3T3-L1 adipocytes undergo this biphasic DAG reponse [479]. PKCβII (not PKCβI) can activate PLD (involved in GLUT4 fusion) [19]. It is important to note that it is the PLD1 isoform which is activated by cPKCs in response to insulin. The PLD2 isoform has high basal activity and is not stimulated to the same extent by PKC family members, ARF (ADP-ribosylation factor) and Rho [503]. 3T3-L1 adipocytes express both PLD isoforms which are confined to LDM as detected by Millar et al. Within the LDM fraction, PLD2 is associated with intracellular membranes whereas PLD1 is associated with IRS1 and PI3K [504]. Other reports have PLD1 at the perinuclear vesicles and PLD2 at the PM
Subcellular localization of PLD needs further investigation. In HEK293 cells, PKCα and PLCα are both involved in insulin-stimulated PLD1 and PLD2 activation [503]. Human pulmonary artery endothelial cells (HPAEC) require PKCε for PLD2 activation which then goes on to activate PKCζ [506]. In human colon cancer cells, a PKC/Ras/ERK/NFκB-pathway is responsible for PLD1 (not PLD2) activation [507]. Lastly, in the human-airway epithelial cell line (CFNPE9o), Src and PKCδ are needed for PLD1 activation [508]. Clearly, many factors converge on PLD isoforms to regulate their activity as well as localization. Cell-type and agonist-type likely dictate which factors are utilized. Therefore, if PKCβII was needed for PLD1 activation in 3T3-L1 adipocytes, other factors could compensate by either activating PLD1 or the cell could switch to PLD2.

PKCβII binds to and is activated by F-actin where it has been shown to phosphorylate many cytoskeletal-associated proteins in vitro including adducin, MARCKs, troponin, and vimentin. PKCβII association with F-actin resulted in a 5-fold increase in F-actin phosphorylation, indicating F-actin is a PKCβII substrate [105]. Disruption of F-actin in 3T3-L1 cells, via cytochalasin D, depolymerizes actin thus inhibiting GLUT4 translocation and ISGT. The dynamic rearrangements of F-actin and the actin-based cytoskeleton play a critical role in insulin-stimulated GSV translocation to the PM [283].

As mentioned earlier, PKCβII can phosphorylate Akt S473 in a cell and stimulus dependent manner [438]. Data presented leads to a final model whereby the insulin signaling cascade in 3T3-L1 adipocytes results in mTORC2 activation. This leads to direct mTORC2-mediated PKCβII phosphorylation and activation. PKCβII will then
phosphorylate and fully activate Akt (S473). Fully active Akt perpetuates the insulin signaling cascade eventually culminating in GLUT4 translocation and ISGT. Active PKCβII may also act on GLUT4 or GSV components to positively regulate ISGT. Figure 75 represents a proposed model of 3T3-L1 adipocyte insulin signaling that is dependent on PKCβII activity. Future directions would include studying other potential contributors (e.g. AKAP and/or mTORC2 components) in the binding between mTORC2 and PKCβII. Development of an inducible construct that specifically targets the PKCβII exon would allow assessment of its role during adipogenesis as well as confirm PKCβII’s role in GLUT4 translocation and ISGT.
Figure 75. Hypothetical model for PKCβII-regulated ISGT via Akt activation in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes are stimulated by insulin. Subsequent insulin receptor tyrosine kinase activity recruits IRS leading to PIIK generated PIP, which sequesters both PDK1 and Akt through their PH domains. Preceding PM localization, Akt is phosphorylated at Threonine 450 by a mechanism that depends on mTORC2. Whether this is a direct phosphorylation by mTORC2 or involves PKCβII is uncertain. Once PM bound, PDK1 phosphorylates Akt at Threonine 308 as well as PKCβII at Threonine 500. Activated mTORC2 (which may require Ras-mediated phosphorylation) includes mTOR, Rictor, Protor, Sin1, mLST8, phosphatidic acid (PA) and the TSC1/TSC2 complex. Since PKCβII can bind and activate PLD1 in select cells, the possibility exists that PKCβII is involved in a positive feedback loop for enhanced insulin-stimulated mTORC2 activation by indirectly generating PA. However, in spite of PKCβII inhibition, mTORC2 may still be able to assemble due to other positive regulators of PLD1. High basal PLD2 activity could also compensate. Serine 2481 phosphorylation of mTOR during insulin stimulation distinguishes mTORC2 activity allowing it to directly phosphorylate PKCβII at Threonine 641 leading to PKCβII autophosphorylation at Serine 660. After PM translocation and pseudosubstrate release, fully active PKCβII phosphorylates Akt at Serine 473. Akt is now fully active and able to elicit GLUT4 translocation to the PM culminating in glucose import. PKCβII may also be able to act on GLUT4, or components of the GLUT4 storage vesicles, directly.
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