PPARγ-INDEPENDENT MECHANISMS OF SRC KINASE ACTIVATION AND EGFR TRANSACTIVATION IN RESPONSE TO THIAZOLIDINEDIONES

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ABSTRACT

BRIAN J. DEWAR: PPARγ-independent mechanisms of Src kinase activation and EGFR transactivation in response to thiazolidinediones
(Under the direction of Lee M. Graves, Ph.D.)

The thiazolidinediones (TZDs), a drug family used in the improvement of type II diabetes, are synthetic ligands for the peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone receptor that mediates the expression of genes important in glucose homeostasis, energy metabolism, and cell proliferation and differentiation. TZDs also mediate, independently of PPARγ, activation of the mitogen-activated protein kinases (MAPKs) and the signal transduction pathways contributing to their activation have only recently begun to be explored. Interestingly, TZDs mediate the activation of different MAPK subfamilies; however, the mechanisms responsible for this differential activation are not fully understood. The goal of this work was examine mechanisms regulating signal transduction pathways contributing to the activation of MAPKs in response to TZDs.

Here we demonstrate that two TZDs, ciglitazone and troglitazone, activated the non-receptor tyrosine kinase Src by affecting known regulatory tyrosine phosphorylation in the protein. Activation of Src involved protein tyrosine phosphatase (PTPase)-mediated dephosphorylation of a specific tyrosine residue known to negatively regulate Src's kinase activity. Disruption of lipid rafts, distinct plasma membrane structures that function to localize signaling proteins, prevented dephosphorylation of this site; however, no significant amount of Src or PTPase activity was observed in lipid rafts before and after TZD treatment.

A PTPase, putatively identified as phosphatidylinositol polyphosphate-5 phosphatase, was shown to be constitutively associated with Src protein. In contrast to the activation of Src by both TZDs, epidermal growth factor receptor (EGFR) transactivation and extracellular signal-regulated kinase (Erk) activation were only observed with ciglitazone. Both TZDs mediated a transient increase in cytosolic calcium concentrations, suggestive of endoplasmic reticulum (ER) store release; however, only ciglitazone induced a secondary calcium influx, indicative of capacitative calcium entry (CCE). Ciglitazone-induced EGFR transactivation and Erk activation were prevented by either removal of extracellular calcium or by 2-aminoethyl diphenyl-borinate (2-APB), a known CCE inhibitor. Collectively, these studies provide additional information of the PPARγ-independent signaling pathways affected by TZDs contributing to the activation MAPKs. Ultimately, this knowledge may help us better understand both the pharmacological actions and potential toxic side effects associated with these compounds.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

Ad	adenovirus
ACN	acetonitrile
ADAM	a disintegrin and metalloproteinase
AM	acetoxymethyl
2-AP	2-aminopurine
2-APB	2-aminoethyl diphenyl-borinate
Ang II	angiotensin II
ATP	adenosine triphosphate
BAPTA	1,2 bis(o-aminophenyoxy)ethae-N,N,N',N'-tetraacetic acid
CaMK II	calcium/ calmodulin-dependent protein kinase II
Cbp	CSK binding protein
CCE	capacitative calcium entry
Ciglitazone	5-[4-(1-methyl-cyclohexylmethoxy)-benzyl]thiazolindine-2,4-dione
Δ2-Ciglitazone	5-[4-(1-methyl-cyclohexylmethoxy)-benzylidene]thiazolindine-2,4-dione
I_{crac}	calcium-release-activated Ca^{2+} current
CRACM	CRAC modulators
CRNK	calcium-dependent tyrosine kinase-related non-kinase
CSK	C-terminal Src kinase
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF2α	eukaryotic initiation factor 2 α

ER	endoplasmic reticulum
Erk	extracellular signal-regulated kinase
ET-1	endothelin-1
FAK	focal adhesion kinase
FBS	fetal bovin serum
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GDP	guanosine diphosphate
GM6001	N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide
GPI	glycolsylphosphatidylinositol
GTP	guanosine triphosphate
HB-EGF	heparin binding-epidermal growth factor
IB	immunoblot
Ins	inositol
IP_3	inositol 1,4,5-trisphosphate
JNK	c-jun N-terminal kinase
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated extracellular signal-regulated kinase
MEM	minimum essential medium
Met	methionine
MKK	mitogen-activated protein kinase kinase
MKKK	mitogen-activated protein kinase kinase kinase
MMPs	matrix metalloproteinases
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide

Orai	see CRACM1
PBS	phosphate buffered saline
PD153035	4-[(3-bromophenyl)amino]-6,7-dimethoxyquinazoline
PDGF	platelet-derived growth factor
PERK	protein kinase R-like endoplasmic reticulum kinase
PI_3K	phosphatidylinositol- 3-kinase
PKC	protein kinase C
PKR	double stranded ribonucleic acid-activated protein kinase
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PP2	4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
PP3	4-amino-7-phenylpyrazol[3,4-d]pyrimidine
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
PTB	phosphotyrosine-binding
PtdIns	phosphatidylinositol
PTPase	protein tyrosine phosphatase
Pyk2	proline-rich tyrosine kinase
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
Rosiglitazone	5-{4-[2-(methyl-pyridin-2-yl-amino)-ethoxy]-benzyl}thiazolidine-2,4-dione
RTK	receptor tyrosine kinase
RXR	retinoic acid receptor
SAM	sterile alpha motif
SAP	SLAM-associated protein

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
SERCA	sarcoplasmic/ endoplasmic reticulum Ca^{2+} ATPase
SFKs	Src Family Kinases
SH2	Src homology 2
SH3	Src homology 3
SLAM	signaling lymphocyte activation molecule
Sos	son of sevenless
STAT	signal transducers and activators of transcription
STIM	stromal interacting molecule
TBS	Tris-buffered saline
TBST	Tris-buffered saline + Tween 20
TPA	12-o-tetradecanoylphorbol-13-acetate
Troglitazone	5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-ylmethoxy)-benzyl] thiazolindine]2,4-dione
Δ2-troglitazone	5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-ylmethoxy)-benzylidene] thiazolindine-2,4-dione
Tyr	tyrosine
TZD	thiazolidinedione
U0126	1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene
UV	ultraviolet

CHAPTER I

GENERAL INTRODUCTION

A. SIGNIFICANCE

Thiazolidinediones (TZDs) are a class of drug that was developed nearly 30 years ago with the synthesis of ciglitazone, an analogue of the anti-hyperlipidemic drug clofibrate (Saltiel and Olefsky, 1996). In addition to its ability to lower circulating blood lipid levels, ciglitazone also possessed anti-hyperglycemic effects. From this initial compound, additional TZDs were developed for treatment of type II diabetes, a condition where sufficient insulin is produced, but is ineffective in mediating glucose uptake in extrahepatic tissues such as muscle and adipose tissue. During their development, it was found that TZDs were ligands for the peroxisome proliferator-activated receptor γ (PPARγ), a specific isoform in the PPAR nuclear hormone receptor family of transcription factors (Ibrahimi et al., 1994; Lehmann et al., 1995).

In addition to the research showing that TZDs mediate PPARγ-dependent gene transcription by acting as PPARγ ligands, there is evidence indicating that TZDs also elicit cellular effects independent of PPARγ activation; these have also been described as "non-genomic" effects. TZDs can affect many target tissues, including skeletal muscle, liver and adipose tissues, as well as pancreatic β-cells; however expression of PPARγ within these targets varies widely, being high in only adipose tissue. The PPARγ-independent effects of TZDs has also been shown to extend to their ability to activate members of the mitogen-activated protein kinases (MAPKs), a well-characterized kinase family involved in a number of cellular processes, including promotion of cell proliferation and differentiation, mediation of responses to cell stress, and the induction of apoptosis and known themselves to function as transcriptional regulators (Gardner et al., 2003; Lennon et al., 2002; Rokos and Ledwith, 1997). The upstream signal transduction pathways contributing to the activation of MAPKs

by TZDs have only recently begun to be explored (Gardner et al., 2005a). Work from our lab has demonstrated distinct differences in the ability of two different TZDs to activate specific signaling pathways leading to MAPK activation. The reasons for these differential effects are unknown. The focus of this study was to examine signaling mechanisms contributing to the differential activation of MAPKs in response to TZDs; this differential activation of MAPKs could contribute to the pharmacological mechanism of action or explain potential toxic side effects of the TZDs.

B. PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS

PPARs were first identified in the early 1990's with the discovery of PPARα, a receptor that was activated in response to a diverse array of rodent hepatocarcinogens (Issemann and Green, 1990). At this time, these hepatocarcinogens were known for their ability to induce proliferation of the subcellular organelle the peroxisome, whose primary function was oxidation of long chain fatty acids and subsequent detoxification of hydrogen peroxide, a metabolic byproduct. Two additional PPARs have been identified, PPARγ and PPARβ/δ, since the cloning of PPARα. Three separate genes encode the PPAR family members; PPARγ has been further characterized into three isoforms, $PPAR\gamma_1$, $PPAR\gamma_2$, and $PPAR\gamma_3$ due to differential RNA splicing and alternative promoter use (Fajas et al., 1997; Fajas et al., 1998).

PPARs contain two functional domains common to all nuclear receptors (Figure 1.1A). The N-terminal region of the protein contains two zinc fingers that make up the DNA-binding domain, which functions to recognize specific hormone response elements in promoter regions of target genes (Berg, 1989). The C-terminal region allows for ligand binding, important in receptor activation and other protein-protein interactions (Klug and

Schwabe, 1995). These signature domains indicated that PPARs functioned as nuclear hormone receptors, a super family of intracellular ligand-activated transcription factors that includes receptors for progesterone, vitamin D3, estrogen and thyroid hormone (Issemann and Green, 1990). Following agonist binding, heterodimers are formed between PPARs and the retinoic acid receptor (RXR); this leads to recruitment of additional cofactor proteins leading to transcription initiation (Kliewer et al., 1992; Xu et al., 1999a). These cofactor proteins or "coactivators" either possess or are able to recruit histone acetyltransferase activity to the transcription start site to allow for DNA unwinding from the tightly packed chromatin structure. Unwinding exposes specific peroxisome proliferator response elements (PPREs) in the promoter region of PPARγ-dependent target genes and allows association of the active PPAR/ RXR transcription complex (Tugwood et al., 1992). The PPRE contains a specific sequence consisting of a direct repeat of two hexameric nucleotide sequences separated by a single base and acts as the essential determinant of PPAR/ RXR binding specificity (Figure 1.1B).

PPARs are susceptible to activation by a diverse array of agonists due to the unusually large ligand-binding domain, a unique feature compared to other nuclear hormone receptors (Nolte et al., 1998; Xu et al., 1999a). A variety of endogenous fatty acids and eicosanoids have been shown to serve as physiological ligands for PPARs demonstrating the importance of these receptors as nutrient sensors and regulators in lipid biology (Clarke et al., 1999). Indeed, genes regulated by PPARs are involved in lipid metabolism, glucose uptake and homeostasis, cellular differentiation, eicosanoid signaling and inflammation.

1. PPARγ

Of the receptors in this family, PPARγ is one of the most intensely studied. Among the three different isoforms of PPARγ there appears to be no significant functional difference, however expression is highly tissue specific. PPARγ_1, like PPARα, is expressed in mostly metabolic tissues, including heart, skeletal muscle, colon, small and large intestines, kidney, pancreas, and spleen. PPARγ_2 is expressed almost exclusively expressed in adipose tissue (Fajas et al., 1997). PPARγ is distinct in the PPAR subfamily of nuclear hormone receptors in that its main physiological function is the regulation adipocyte proliferation and differentiation (Masugi et al., 1999; Tontonoz et al., 1994b). However additional work has demonstrated that PPARγ is involved in other physiological processes. That PPARγ was involved in glycemic control was not known until the insulin-sensitizing agents TZDs were demonstrated to be high-affinity PPARγ ligands (Lehmann et al., 1995). Additionally, growth inhibitory actions of PPARγ have also been observed, most notably in colon cancers (Sarraf et al., 1998), but also in breast and lipsosarcoma cancer cells (Demetri et al., 1999; Mueller et al., 1998). In addition to the TZDs, synthetic PPARγ ligands, the eicosanoid, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 is thought to serve as endogenous ligand of PPARγ (Kliewer et al., 1997).

2. Thiazolidinediones - PPARγ Synthetic Ligands

Following their development nearly 15 years ago, several observations were made in the 1990s that fortuitously led to the discovery of the primary molecular site of action of the anti-diabetic agents TZDs (Figure 1.2). In addition to the induction of adipocyte differentiation, TZDs increased the expression of specific adipocyte genes, one of which was the fatty acid-

binding protein *aP2* (Kletzien et al., 1992). Concomitantly, other work reported that the *aP2* gene contained a PPARγ response element regulating its expression (Tontonoz et al., 1994a). These observations led to research that determined that TZDs were high-affinity ligands for PPARγ (Ibrahimi et al., 1994; Lehmann et al., 1995). Based on their capabilities as anti-diabetic agents, a correlation was demonstrated between their ability to bind and activate PPARγ and their *in vivo* insulin-sensitizing effects.

TZDs entered the clinical market with the introduction of troglitazone (Rezulin®; Pfizer) in March of 1997. Although ciglitazone, another TZD family member demonstrated effective insulin-sensitizing effects and lowered circulating glucose levels, its clinical applications were abandoned due to liver toxicity (Saltiel and Olefsky, 1996). By November of that same year, 135 cases of hepatotoxicity and six deaths had been reported in patients being given troglitazone for type II diabetes (Watkins and Whitcomb, 1998). In the U.S., nearly 2 million patients had been treated with troglitazone from 1997 to 2000, when the drug was removed from the market due to persistent problems with liver failure that were characterized as idiosyncratic (Faich and Moseley, 2001). Currently, the TZDs rosiglitazone (Avandia®; GlaxoSmithKline) and pioglitazone (Actor®; Lilly) are available in the U.S. for the treatment of type II diabetes. Both these compounds are at least 100-fold more potent ligands for PPARγ than troglitazone, suggesting that a lower dose may account for the lack of toxicity observed with either rosiglitazone or pioglitazone (Scheen, 2001; Willson et al., 1996). It is clear that TZDs are effective ligands of the PPARγ and all possess anti-hyperglycemic effects, however differing chemical structures and affinities for PPARγ lead to TZD-specific effects and could explain differences in toxicity observed with one compound and not others. Moreover, while rosiglitazone does not appear to mediate

idiosyncratic hepatotoxicity commonly associated with troglitazone, recent evidence suggests that rosiglitazone may increase the risk of heart disease (Nissen and Wolski, 2007).

C. PPAR-INDPENDENT SIGNALING

PPARs are expressed widely in various tissues of the body, are activated by a diverse array of natural and synthetic ligands, and regulate many important genes that are critical to maintaining normal cell function. Evidence has accumulated showing that many PPAR ligands also possess PPAR-independent effects. While the ability of PPAR ligands to mediate receptor-independent effects conflicts with their classical mechanisms of action, this has also been described for other nuclear hormone receptor agonists. For example, rapid changes in signaling pathways have been demonstrated in response to progesterone, estrogen, aldosterone, thyroid hormone, and vitamin D_3 and these non-genomic effects are thought to play a role in their biological mechanisms of action (Losel and Wehling, 2003). Some of the first evidence suggesting that PPAR agonists could mediate effects independent of activation of their target receptor was work demonstrating that the carcinogenicity of PPARα agonists did not always correlate with peroxisome proliferation (Marsman et al., 1988), an event known to be PPARα-dependent (Lee et al., 1995b). These PPAR-independent effects similarly extend to the TZDs, specific PPARγ agonists. TZDs affect many tissues in the body, including muscle liver and adipose tissue, however the expression levels of PPARγ varies widely in many of these target tissues, being highest only in adipose tissue (Evans et al., 2004). Other works have demonstrated that the growth inhibitory effects of TZDs do not always correlate with PPARγ expression (Baek et al., 2003). This is clearly seen in experiments demonstrating that inhibition of cell growth was not affected in PPARγ$^{-/-}$ mouse embryo fibroblasts in response to TZD treatment (Palakurthi et al., 2001).

At the molecular level, both PPARα and γ agonists activated members of the MAPK family at times too rapid for PPAR-dependent gene transcription and protein synthesis (Lennon et al., 2002; Mounho and Thrall, 1999; Rokos and Ledwith, 1997; Teruel et al., 2003). The ability of PPAR agonists to affect MAPK activation has been described in many different cells types (Table 1.1) (Gardner et al., 2005a). As MAPKs themselves are well known regulators of gene transcription (Johnson and Lapadat, 2002), the ability of TZDs to mediate their MAPK activation provides a possible mechanism explaining how these PPARγ agonists are able to induce cellular effects in a PPARγ-independent manner. Indeed, recent studies using specific inhibitors of various MAPKs dissociated transcriptional changes induced by PPAR agonists into distinct MAPK- or PPAR-dependent events (Baek et al., 2003).

D. MECHANISMS OF MAPK ACTIVATION BY TZDs

While the ability of TZDs to activate MAPKs has been well established, few studies had investigated the signal transduction pathways contributing to MAPK activation. Consequently, work from our lab has shown that similar to other published reports, the TZDs ciglitazone and troglitazone effectively activated several MAPK family members in the rat liver epithelial cell line GN4 (Gardner et al., 2003). Moreover, these data have provided evidence describing two separate signaling pathways contributing to either the activation of Erk or p38 MAPK (Gardner et al., 2005a) (Figure1.3).

1. Epidermal growth factor receptor (EGFR) Transactivation

Using GN4 rat liver epithelial cells, we demonstrated that nafenopin (a PPARα ligand), ciglitazone and to a lesser extent troglitazone (PPARγ ligands), rapidly increased

Erk phosphorylation (Gardner et al., 2003). Inhibition of EGFR kinase activity prevented Erk phosphorylation in response to PPAR ligands, demonstrating a role of this receptor family in this pathway. These results were consistent with other works demonstrating a link between the EGFR and MAPK signaling in response to other PPARα ligands (James and Roberts, 1994; Orellana et al., 1993; Pauley et al., 2002). Further investigation showed that both nafenopin and ciglitazone were capable of inducing EGFR transactivation (i.e., phosphorylation of the receptor in the absence of ligand). Later work demonstrated that both Erk and EGFR transactivation in response to ciglitazone was independent of PPARγ activation (Gardner et al., 2005b). Interestingly, troglitazone, which only minimally induced Erk phosphorylation, did not increase phosphorylation of the EGFR. Several mechanisms required for EGFR transactivation were examined and only inhibition of the non-receptor tyrosine kinase Src prevented EGFR transactivation in response to PPAR ligands. Neither protein kinase C (PKC) nor matrix metalloproteinase (MMP)-dependent proteolytic cleavage of diffusible epidermal growth factor (EGF)-like ligands, mechanisms previously described to be involved in EGFR transactivation, were found to be involved in PPAR ligand-induced EGFR transactivation (Shah and Catt, 2002; Zwick et al., 1999a). Subsequent to EGFR transactivation, expression of dominant-negative Ras, as well as inhibition of MEK1/2 with U0126, blocked nafenopin and ciglitazone-induced Erk phosphorylation. These data collectively described the signaling pathway depicted in Figure 1.3, where Src-dependent EGFR transactivation results in Ras activation, which in turn leads to successive phosphorylations in the canonical RAF, MEK, Erk pathway, resulting ultimately in Erk activation.

2. Endoplasmic Reticulum Stress and Calcium

In addition to Erk activation, nafenopin and both ciglitazone and troglitazone were shown to rapidly activate p38 MAPK (Gardner et al., 2003). Interestingly, neither inhibition of Src nor EGFR kinase activity affected the ability of these agents to induce p38 phosphorylation. These results suggested that the two signal transduction pathways contributing to MAPK activation in response to PPAR agonist stimulation were independent of each other (Figure 1.3). This was supported further by evidence showing that inhibition of key signaling effectors involved in p38 activation, calcium/ calmodulin-dependent protein kinase II (CaMK II) and mitogen-activated protein kinase kinase (MKK) 3/6, did not affect EGFR and Erk phosphorylation (Gardner et al., 2005a). Initial examination into the mechanism of p38 activation demonstrated that this event was calcium-dependent. Subsequent experiments revealed that in addition to calcium-dependent CaMK II activation, classical markers of endoplasmic reticulum (ER) stress were activated and contributed to p38 activation following treatment of cells with PPAR ligands. Specifically, double stranded ribonucleic acid-activated protein kinase (PRK), protein kinase R-like endoplasmic reticulum kinase (PERK), and eukaryotic initiation factor 2 α (eIF2α) were all activated in response to TZD treatment. Interestingly, inhibition of CaMK II blocked both PKR and p38 activation, suggesting that PKR was a downstream effecter of CaMK II; however, future studies will be required to characterize the specific role of CaMK II in PKR activation. These data suggested that TZDs could affect the ER directly to cause calcium release, important in activation of CaMK II, but also provided the mechanistic evidence that induction of ER stress and p38 phosphorylation are tightly-coupled signaling events. Moreover, the involvement of

calcium in mediating other signaling events influenced by TZD treatment should be investigated.

E. SRC FAMILY KINASES

Nearly 100 years ago it was demonstrated that the Rous Sarcoma Virus was the causative agent inducing tumors in chickens (Rous, 1911). This was not fully appreciated until over 6 decades later when the Src protein was discovered (Brugge and Erikson, 1977). Interestingly, the viral Src (v-Src) gene has a normal progenitor gene expressed in human, chickens and other animals, which was named c-Src, the first proto-oncogene (Stehelin et al., 1976). Src was later shown to be the first tyrosine kinase (Hunter and Sefton, 1980). There are now 11 Src-related, membrane associated, non-receptor tyrosine kinases that comprise the Src Family Kinases (SFKs), including Src, Blk, Brk, Frk, Fyn, Hck, Lck, Lyn, Srm and Yes in humans. Of these Src, Yes and Fyn are expressed rather ubiquitously, while other members are more cell-type specific. In general, SFKs are activated and participate in signal transduction pathways that promote cell proliferation, survival, motility and invasiveness (Thomas and Brugge, 1997).

1. Structure

SFKs possess several domains that are important in the kinases' function and regulation. From the N-terminus, the secondary structure of the protein consists of a 14-carbon myristol group attached to the SH4 domain; this is followed by a unique region, the SH3 and SH2 domains, the SH2-kinase linker region, the protein kinase domain and lastly, the C-terminal tail (Brown and Cooper, 1996). The kinase domain of SFKs is bilobular, with a small amino-terminal lobe important in anchoring and orienting ATP and the larger carboxy-terminal

lobe, which is responsible for substrate binding (Xu et al., 1999b). The catalytic site of Src resides in the cleft between the two lobes. The two kinase lobes can move relative to each other, thereby opening or closing the catalytic cleft (Williams et al., 1997). The movement of the kinase domain lobes is achieved by intermolecular interactions of the SFKs SH2 and SH3 domains. The SH3 domain is important for protein-protein interactions and binds the consensus proline-rich sequence RPLPPLP (Weng et al., 1995), though this binding target sequence varies among the different SFKs. A proline-rich sequence exists within the SH2-kinase linker region and the intramolecular interaction between this and the SH3 domain helps maintain the kinase in a closed and inactivate state. More importantly, the SH2 domain of SFKs bind tyrosine phosphorylated residues, both within SFK themselves and in other proteins (Moran et al., 1990; Waksman et al., 1992). Specifically, the SH2 domain binds tyrosine 527 (Tyr^{527}) in the C-terminal tail region when it is phosphorylated. Similar to the proline-rich protein/ SH3 domain interaction, this phospho-Tyr^{527}/ SH2 intramolecular interaction closes the catalytic cleft and maintains the kinase in an inactive conformation (Xu et al., 1999b) (Figure 1.4).

2. Mechanisms of Activation

a. Phosphorylation of SFKs

As SFKs are important to a number of cellular processes and are known proto-oncogenes, the kinase activity of this protein family is tightly regulated. Critical to the regulation of SFKs activity is the Tyr^{527} site, six residues from the C-terminus. The SFKs own SH2 domain binds this residue when it is phosphorylated and this interaction maintains the kinase domain in a "closed" and inactive conformation. Mutation of this site to an unphosphorylatable (Tyr^{527}Phe) residue produces a more active enzyme compared to wild-

type Src and induces cell growth in culture as well as tumors in vivo (Kmiecik and Shalloway, 1987). Interestingly, the C-terminal tail region is missing in v-Src and thus the lack of the inhibitory phosphotyrosine confers a constitutively active kinase. Under normal conditions approximately 90-95% of SFKs are phosphorylated on Tyr^{527}; dephosphorylation of Tyr^{527} results in an intermolecular conformational change, trans-autophosphorylation of Tyr^{416} within the kinase domain and enhanced kinase activity (Zheng et al., 2000). While phosphorylation of Tyr^{416} enhances the activity of SFKs, mutation of this site to phenylalanine does not prevent kinase activity or block transforming properties (Snyder et al., 1983). Thus, phosphorylation, particularly the Tyr^{527} site in the C-terminal tall region plays a critical role in regulating the activities of SFKs.

b. Mediators of SFK phosphorylation

The phosphorylation of the C-terminal Tyr^{527} site within SKFs is mediated by the cytoplasmic C-terminal Src kinase (CSK) (Okada and Nakagawa, 1988). CSK contains a bi-lobular kinase domain similar to SFKs and also possesses both SH2 and SH3 domains; however, the three dimensional structure and orientation of the SH2 and SH3 domains of CSK is different from SFKs (Ogawa et al., 2002). Moreover, CSK lacks the C-terminal tail region that SFKs possess, and therefore does not have the corresponding Tyr^{527} inhibitory phosphorylation site. Taken together, these differences help maintain the catalytic kinase domain of CSK in an unconstrained and active conformation. Therefore, CSK is thought to principally exist as an uninhibited kinase that sustains the phosphorylation of the key regulatory site in SFKs, thereby maintaining them in inactive state. Theoretically, a decrease or inhibition of CSK activity could modulate an increase in SFK activity.

In addition to CSK, the adaptor protein CSK-binding protein (Cbp), also termed phosphoprotein-associated with GEMs (PAG), has been shown to contribute to the regulation of SFKs (Takeuchi et al., 2000). Cbp is a transmembrane protein with a short extracellular domain, a transmembrane domain with a palmitoylation motif and a large cytoplasmic domain and is expressed in most tissues (Brdicka et al., 2000). Cbp is localized to discreet regions of the plasma membrane called lipid rafts. Interestingly, treatment of cells with polyunsaturated fatty acids results in displacement of other palmitoylated proteins from lipid rafts but does not affect Cbp, suggesting that other structures are important to Cbp raft localization (Zeyda et al., 2002). Important to Cbp's regulation of SFKs is the cytoplasmic domain, which contains several tyrosine residues that can serve as potential SFKs substrates. Work has shown that CSK (via its SH2 domain) binds phosphorylated Tyr^{317} in the cytoplasmic domain of Cbp (Kawabuchi et al., 2000). Thus Cbp functions as an adapter protein that translocates CSK to the plasma membrane, where CSK can then interact with and phosphorylate SFKs to inhibit their activity.

In addition to CSK and Cbp, protein tyrosine phosphatases (PTPases) are important regulators of SFKs and several have been demonstrated to mediate dephosphorylation of the Tyr^{527} site. These phosphatases include the cytoplasmic PTP1B, SH2 domain-containing tyrosine phosphatase 1 (Shp1) and Shp2 and the transmembrane localized CD45, PTPα, PTPε, and PTPλ. The mechanism by which this diverse array of proteins regulates Src dephosphorylation varies widely; however many are themselves tyrosine-phosphorylated proteins. Tyrosine phosphorylation within the PTPase functions to displace the phosphorylated Tyr^{527} from the Src's SH2 domain, thereby allowing access of the PTPase to the Tyr^{527} site. PTPα is phosphorylated on Tyr^{789} in its C-terminus and this dislodges

phospho-Src Tyr^{527} from the intramolecular SH2 interaction allowing the catalytic domain of PTPα to mediate Tyr^{527} dephosphorylation (den Hertog et al., 1994; Zheng et al., 2000). In addition, many PTPases possess their own SH2 domain, allowing for interaction and targeting of tyrosine phosphorylated proteins, like Src. Other works highlight that localization of the PTPase is important for regulation. PTP1B is localized predominantly to the ER, with its phosphatase domain oriented towards the cytoplasm (Frangioni et al., 1992). Calcium-mediated calpain activation cleaves PTP1B at a site upstream from the ER targeting sequence, producing a cytosolic form of the protein (Frangioni et al., 1993). This potentially allows movement of PTP1B to the plasma membrane where it can interact with and mediate Src activation. Lastly, some PTPases, like Shp2, mediate Src dephosphorylation by an indirect mechanism. Shp2 has been shown to mediate the dephosphorylation of Cbp, rather than Src. Phosphorylated Cbp attracts CSK to membrane-associated Src, but Shp2 mediated Cbp dephosphorylation would abolish the CSK binding site and interaction (Zhang et al., 2004). Thus CSK mediated SrcTyr^{527} phosphorylation and inhibition is lost.

c. Alternative mechanisms of activation

Alternative to the classical phosphorylation-dependent mediated mechanism of activation, SFKs have been shown to be activated by protein-protein interactions. This occurs when the SFKs SH2 and/or SH3 domains bind their cognate motifs in other proteins. This was first observed with SFKs SH2 domain. Work showed that platelet-derived growth factor (PDGF) treatment of fibroblasts led to a co-association of phosphorylated PDGF receptors and three SFK members, Src, Yes and Fyn (Kypta et al., 1990); this binding was accompanied by an increase in activation of the SFKs. Further work demonstrated that PDGF receptor phosphopeptides could mediate Src activation (Alonso et al., 1995). This led

to the conclusions that tyrosine-phosphorylated sequences in other proteins can compete for binding of SFKs SH2, thereby displacing the SH2-phospho-Tyr^{527} intermolecular interaction. This displacement subsequently exposes the phospho-Tyr^{527} site to PTPases thereby facilitating PTPase-dependent dephosphorylation. Additionally, SFKs have been shown to be activated by displacement of their SH3 domain. The HIV protein Nef contains a proline rich sequence that confers tight binding to SH3 domains; *in vitro* Nef mediated activation of the SFK Hck (Lee et al., 1995a; Moarefi et al., 1997). Nef-mediated SH3 domain displacement leading to Hck activation did not require dephosphorylation of the C-terminal Tyr^{527} site, suggesting that SH3 domain displacement was sufficient enough to mediate SFK activation. Additionally, more recent work has shown SH3 domain displacement of Fyn with signaling lymphocyte activation molecule (SLAM)-associated protein (SAP), an adaptor protein associated with SLAM-family receptors (Latour et al., 2001). This binding disrupts the autoinhibitory interaction of Fyn SH3 domain leading to enhanced catalytic kinase activity (Latour et al., 2003).

F. PROLINE-RICH TYROSINE KINASES

Proline-rich tyrosine kinase (Pyk2 - also designated RAFTK, FAK2, CAK-β or CADTK) is a member of the non-receptor, proline-rich protein tyrosine kinases, of which focal adhesion kinase (FAK) is also a member (Avraham et al., 2000; Yu et al., 1996). Pyk2 is a 123-kDa protein with a central kinase domain flanked by large N-terminal and proline-rich C-terminal domains (Girault et al., 1999). Unlike FAK, which is expressed rather ubiquitously, Pyk2 is most predominantly expressed in the central nervous system and in cells and tissues from hematopoietic lineages. Cellular localization shows that Pyk2 is

diffusely expressed throughout the cytoplasm as opposed to FAK which is localized to focal adhesion complexes in adherent cells (Andreev et al., 1999).

A number of stimuli in various cells types have been shown to mediate an increase in Pyk2 phosphorylation and thus activation; in almost every case activation of Pyk2 is sensitive to chelation of calcium. Upon activation, Pyk2 autophosphorylates on several key tyrosine residues including Tyr^{397} and Tyr^{402}, which promotes the association of SH2 domain containing proteins, such as c-Src or other Src-family kinases (Avraham et al., 1995). This in turn leads to activation of c-Src, which further phosphorylates tyrosine residues in the C-terminus leading to enhanced Pyk2 kinase activity. Phosphorylation of Pyk2 recruits Src-family kinases and has been predominantly shown to increase the activation of the MAPK, Erk (Lev et al., 1995). Pyk2 is also activated in response to cellular stress signals (such as tumor necrosis factor-α, osmotic shock and UV (Tokiwa et al., 1996)) or in response to stimuli that increase intracellular calcium levels, which leads to activation of c-jun N-terminal kinase (JNK) (Yu et al., 1996).

G. EPIDERMAL GROWTH FACTOR RECEPTORS

The EGFR was the first receptor protein identified to possess tyrosine kinase activity (Carpenter et al., 1978). Currently, 4 different EGFR-tyrosine kinases (EGFR-TK) make up this family including, ErbB1 or EGFR, ErbB2 or HER2 (Coussens et al., 1985), ErbB3 or HER3 (Kraus et al., 1989), and ErbB4 or HER4 (Plowman et al., 1993). The EGFR-TK family all share a common structural composition with an extracellular region containing the ligand-binding domain, a single transmembrane-spanning region, and the intracellular cytoplasmic region. Within the cytoplasmic region is the highly conserved tyrosine kinase domain and the C-terminal tail, which possesses many tyrosine residues that upon

autophosphorylation serve as docking site for interacting adaptor proteins (Schlessinger, 2002). Activation of the EGFR-TK family is induced by a number of ligands, including EGF TGFα, betacellulin, heparin binding-EGFR (HB-EGF), epiregulin, and amphiregulin. Upon ligand binding, the receptor can form homo- or heterodimers of the various EGFR-TK family members. Receptor oligomerization results in trans-autophosphorylation of key tyrosine residues within the activation loop of the kinase domain, stimulating kinase activity and leading to autophosphorylation of tyrosine residues in the C-terminal tail region of the receptor. These phosphorylated tyrosine residues then serve as docking sites for proteins containing SH2 or PTB domains. Proteins recruited via these domain interactions are often cytoplasmic kinases activated by EGFR-dependent tyrosine phosphorylation or adaptor proteins that couple EGFR-TK activation to downstream signal transduction pathways, most notably, MAPK or PI_3K (Prenzel et al., 2001a). Signaling through the EGFR-TK family is terminated by internalization of the receptor-ligand complex into endosomes and through changes in endosomal pH, the ligand is dissociated from the receptor in order to terminate signaling (Dikic, 2003). Within these intracellular compartments, components are either degraded or recycled back to the plasma membrane.

Early on the physiological importance of this receptor family pointed to a critical role in mammalian organogenesis. However, the EGFR is essential to many signaling pathways. The ability of several different ligands to induced dimerization of different EGFR-TK family receptors results in the activation of different adaptor and effector cascades, thus increasing greatly the signaling specificity and diversity of this receptor family. This is ultimately reflected transcriptionally, where the EGFR influences many genes important to growth and development, as well as affecting apoptosis, cell migration, adhesion, cell differentiation and

proliferation. Because the EGFR participates in so many different signaling pathways, regulation of its activity must be tightly controlled. Receptor over-expression, mutation of key regulatory residues or disregulation of other critical regulatory proteins, such as PTPases, results in persistent receptor activation and is frequently associated with disease states, such as cancer.

H. EGFR TRANSACTIVATION

Traditionally, the EGFR and other members of the EGFR-TK family are activated in response to specific ligand-binding to the extracellular domain of the receptor; this led to the view that receptor activation was predominantly growth factor-selective. However, it is now well accepted that EGFR activation can occur in response to stimuli other than EGF-ligands, which do no physically interact with the EGFR. This activation in the absence of natural ligands was termed EGFR "transactivation" and has been demonstrated in response to G protein-coupled receptor (GPCR) agonists (Shah et al., 2003), sulfhydryl reagents (Meves et al., 2001), cellular stresses (such as osmotic shock, UV radiation (Rosette and Karin, 1996), hydrogen peroxide treatment (Peus et al., 1999), membrane depolarization (Zwick et al., 1997)) and cytokines (Venkatakrishnan et al., 2000). EGFR transactivation broadened the field of signal transduction in that a given stimulus could mediate the activation of a diverse array of signaling pathways through crosstalk and demonstrated the integral role of this receptor at the center of many signaling networks. The physiological and pathological significance of the EGFR is expanded by the broad range of transactivating-stimuli, and thus mechanisms involved in mediating transactivation are important to understand. Our current knowledge of EGFR transactivation shows that the mechanisms involved vary widely and are dependent on a given cell type and stimulus. Nevertheless, several protein families,

including well known kinases such as Src, Pyk2 and PKCs, as well as the non-kinase MMPs and even calcium signaling, have been shown to mediate EGFR transactivation.

1. Intracellular kinases participating in transactivation

While much work has identified the involvement of specific protein kinases in participating in EGFR transactivation, these kinases often act in combination with each other to coordinate transactivation of the receptor. Src has been shown to interact directly with and phosphorylate the EGFR receptor on two specific tyrosine residues, Tyr^{845} and Tyr^{1101} (Biscardi et al., 1999). Interestingly, these sites do not serve as autophosphorylation sites for the receptor's own tyrosine kinase activity. Several other studies have further implicated the involvement of Pyk2; this kinase was shown to be critically involved in angiotensin II (Ang II), carbachol and gonadotropin-releasing hormone-induced EGFR transactivation. Moreover, these experiments demonstrated the formation of Src/Pyk2 complexes that were dependent upon Pyk2 phosphorylation. Thus, while Src can directly phosphorylate the EGFR, evidence suggests a mechanism that would require association of phosphorylated Pyk2 with Src in order to induce EGFR transactivation. PKC has also been shown to be involved in influencing EGFR transactivation; phosphorylation of EGFR by PKC results from direct interaction of these two proteins (Seedorf et al., 1995). Other works have shown that PKC can mediate activation of both Pyk2 (Soltoff et al., 1998), and Src kinase (Levi et al., 1998) and suggest that all three proteins are important for EGFR transactivation (Shah and Catt, 2002).

2. Matrix-metalloproteinase and EGF ligand shedding

Transactivation of the EGFR was thought to be primarily mediated by intracellular kinase signaling pathways until it was shown that transactivation could occur due to the release of EGF-like ligands from the membrane of cells following activation of MMPs (Prenzel et al., 1999). Characterization of the MMPs has identified the a disintegrin and metalloproteinase (ADAM) subfamily of proteases, membrane localized glycoproteins containing a disintegrin and metalloproteinase domain. Specifically ADAM 10, 12, and 17 have been shown to be involved in mediating release of EGF-like ligands resulting in EGFR transactivation in response to GCPR agonists. EGF-like ligands, such as heparin-binding EGF, transforming growth factor α and amphiregulin, are expressed as membrane bound precursors, which upon activation of ADAM, are converted to their active form through metalloproteinase-dependent cleavage. The shed ligands can then interact with and induced EGFR activation. Interestingly, work has shown that ADAM activity can be increased by treatment of cells with phorbol esters, known stimulants or PKC. Moreover, Src kinase has also been shown to mediate phosphorylation of ADAM 12. Thus intracellular signal transduction pathways may also be involved in the regulation of ADAM-mediated EGFR transaction.

3. Role of Calcium in Transactivation

Elevation of intracellular calcium concentration is involved in the regulation of many cellular signaling pathways and can play a role in EGFR transactivation. Following calcium influx through voltage-sensitive calcium channels, there was an increase in phosphorylation of the EGFR and many of the adaptor proteins required for subsequent Erk signaling (Rosen and Greenberg, 1996). Further, other work in PC-12 cells demonstrated that depolarization-induced calcium influx or stimulation with bradykinin, a GCPR agonist known to mediate

changes in calcium, similarly increased tyrosine phosphorylation of the EGFR (Zwick et al., 1997). Interestingly, many EGFR transactivators are known GCPR agonists, which mediate activation of phospholipase C (PLC) and generate inositol 1,4,5-trisphosphate (IP_3). Specific receptors on the ER are activated in response to IP_3 and cause calcium release from this intracellular store. This suggests the involvement of calcium in EGFR transactivation in response to these various GPCR agonists. Additional evidence has accumulated demonstrating that calcium may function to activate the intracellular kinases previously shown to mediate receptor transactivation. Stimulation of EGFR transactivation by angiotensin II treatment in vascular smooth muscle cells involved calcium and Src kinase activation (Eguchi et al., 1998). Further, carbachol-stimulated transactivation of EGFR involved association of the calcium-regulated kinase Pyk2, an event that could be prevented by calcium chelation (Keely et al., 2000).

The source of calcium involved in EGFR transactivation can be derived from intracellular stores or influx across the plasma membrane from the extracellular space as treatment of cells with ionophore or agonists that mediate ER store release have both been shown to mediate EGFR transactivation. Further, the calcium chelator BAPTA-AM, which can effectively block EGFR transactivation does not distinguish between calcium derived from extracellular or intracellular stores. However, the calcium source or the concentration could be critical to the mechanism of transactivation. ER calcium store depletion activates capacitative calcium entry (CCE), which produces a more substantial and prolonged increase in intracellular calcium concentration. Thus stimuli mediating ER calcium release would also cause a secondary influx of calcium, similar to ionophore treatment. Interestingly, work has shown that inhibition of capacitative calcium influx by the store-operated calcium channel (SOCC)

inhibitor SK&F-96365 blocked EGFR transactivation induced by endothelin-1 (ET-1) (Kawanabe et al., 2002). Furthermore, the magnitude of EGFR transactivation was lowered to near basal levels by the removal extracellular calcium. Under these conditions, the release of calcium from ER stores due to ET-1 treatment would not be blocked and suggests that calcium derived from extracellular sources is required or part of the mechanism involved in transactivation of the EGFR.

I. CAPACITATIVE CALCIUM ENTRY

An increase in cytosolic calcium concentrations regulates many cellular processes including exocytosis, contraction, gene regulation, cell proliferation and apoptosis. A key process for intracellular calcium mobilization in non-excitable cells has been termed capacitative calcium entry (CCE) (Figure 1.5) (Putney, 1986). This process involves depletion or release from internal stores, most commonly the ER, followed by influx of calcium across the plasma membrane from the extracellular space. This selective movement of calcium across the plasma membrane has been described as a highly specific calcium-release-activated Ca^{2+} current (I_{crac})(Pauley et al., 2002). Until recently, the molecular mechanisms involved in both the signal transduction from the ER to the plasma membrane and the calcium-specific channels responsible for I_{crac} were unknown; however both stromal interacting molecule (STIM) and Orai proteins have been identified to play required roles in the mechanism of CCE.

1. STIM Proteins

The role of STIM proteins in CCE has only recently been identified, but the protein had been studied for many years. STIM1 was originally identified in a screen of cell surface

expressed proteins on stromal cells and was shown to be a single-span cell surface plasma membrane protein that interacted with and altered B-cell survival and proliferation (Oritani and Kincade, 1996). STIM1 was mapped to human chromosome 11p15.5, a telomeric region known to be associated with many tumor types (Parker et al., 1996). From this the original function was described as a tumor suppressor gene (Sabbioni et al., 1997). Other works further implicated this gene in many tumor types, such as bladder, breast, lung, ovarian, and testicular cancers (Hu et al., 1997). Additionally, STIM1 was shown to be serine phosphorylated; however, the kinases mediating this event are not known and the role phosphorylation plays in STIM1 function has not been determined (Manji et al., 2000).

Recently, nearly 20 years following its cloning, two independent research groups have identified a required role of STIM1 proteins in CCE. The role of STIM1 in CCE was determined by RNA interference (RNAi) screens in thapsigargin-stimulated calcium entry in both insect and mammalian cell lines (Liou et al., 2005; Roos et al., 2005). Further, works have shown that a second gene product exists, stromal interacting molecule 2 (STIM2), a protein that possesses a very similar structure to that of STIM1. Both proteins have a similar domain structural pattern and differ mostly in the C-terminal, praline-rich "variable" region (Pauley et al., 2002). While STIM proteins were originally thought to be localized primarily at the plasma membrane, increasing evidence shows a predominant ER localization (Pauley et al., 2002). The N-terminal portion of the protein contains both a sterile alpha motif (SAM) and EF-hand domain and is thought to extend into the lumen of the ER. The EF-hand domain binds calcium in the lumen of the ER and functions as a calcium sensor; decreases in ER calcium concentration results in a dramatic redistribution of STIM1 protein to punctate regions near the plasma membrane(Pauley et al., 2002).

2. ORAIs

Recently, modified linkage analysis studies identified a mutation in the protein Orai1 (also described as CRACM1 (CRAC modulators-1)) as the cause of a rare immunodeficiency disease in which T-cell calcium entry is completely blocked (Feske et al., 2006). Interestingly, Orai1 knockdown by RNAi, as well as the over-expression of the R91W mutation completely eliminated I_{crac} and blocked CCE (Feske et al., 2006; Vig et al., 2006b; Zhang et al., 2006). Orai1 is a four-transmembrane spanning protein, with both the N- and C-termini extending into the cytoplasm (Prakriya et al., 2006). Initially, it was not known whether Orai1 was another protein similar to STIM1, involved in coupling ER calcium release to SOCCs or if the protein could function as a calcium channel itself. However, mounting evidence has demonstrated that Orai1 serve as the I_{crac} channel for CCE (Vig et al., 2006a). Two other Orai proteins have been identified, Orai2 and Orai3, suggesting a possibility of heteromeric combinations that could result in distinct regulations or coupling mechanisms with STIM proteins (Feske et al., 2006).

TABLE 1.1. *Activation of MAPKs by various PPARα and γ ligands in different cell model systems*

LIGAND	CELL SYSTEM	MAPK	REFERENCE
PPARα			
docosahexaenoic acid	rat VSMC	p38	(Diep et al., 2000)
eretinoic acid	rat adipocytes	Erk, p38	(Teruel et al., 2003)
linoleic acid	rat VSMC	Erk	(Rao et al., 1995)
WY 14,643	ML457	Erk	(Rokos and Ledwith, 1997)
	1º mouse hepatocytes	Erk	(Mounho and Thrall, 1999)
	1º rat hepatocytes	Erk, p38	(Pauley et al., 2002)
clofibrate	ML457	Erk	(Rokos and Ledwith, 1997)
nafenopin	1º rat hepatocytes	Erk, p38	(Cosulich et al., 2000)
PPARγ			
15d-PGJ(2)	hman mesangial cells	Erk, no effect on p38, JNK	(Wilmer et al., 2001)
	1º rat astrocytes	Erk, p38, JNK	(Lennon et al., 2002)
	PC-12	p38	(Jung et al., 2003)
	rat VSMC	Erk	(Takeda et al., 2001)
	C_2C1_2	Erk	(Huang et al., 2002)
Ciglitazone	human mesangial cells	No effect	(Wilmer et al., 2001)
	1º rat astrocytes	Erk, p38, JNK	(Lennon et al., 2002)
	GN4	Erk, p38, JNK	(Gardner et al., 2003)
	C_2C1_2	Erk	(Huang et al., 2002)
Troglitazone	HCT-116	Erk	(Kim et al., 2002)
	rat VSMC	Erk	(Takeda et al., 2001)
	HepG2	p38, JNK, no effect on Erk	(Bae and Song, 2003)
	GN4	p38, Erk, no effect on JNK	(Gardner et al., 2005a)
	MCF-7	Erk, p38, JNK	(Yin et al., 2004)
Rosiglitazone	fetal rat adipocytes	Erk, p38	(Teruel et al., 2003)

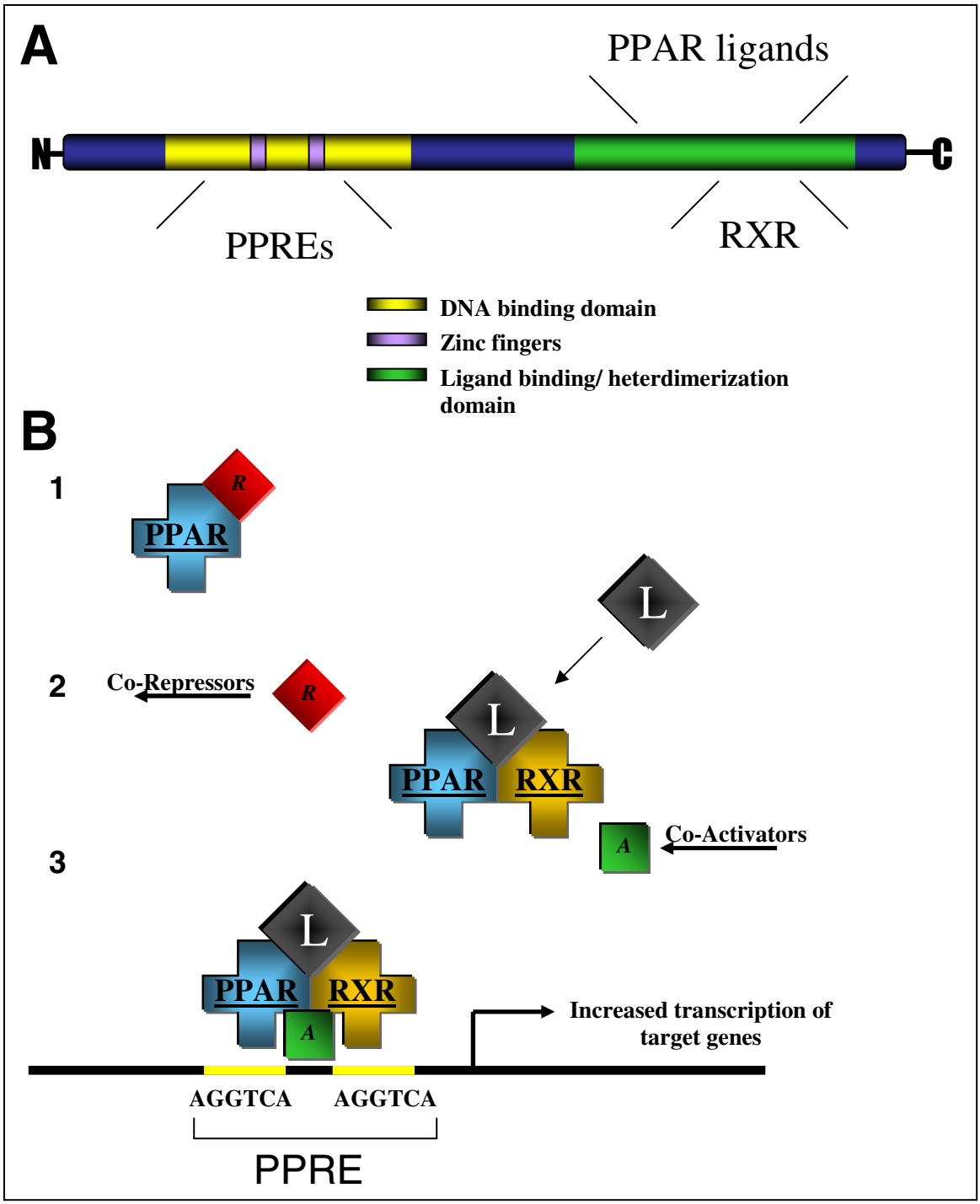

Figure 1.1. Peroxisome Proliferator-Activated Receptors. **A**, Functional domains of PPARs. **B**, Mechanism of ligand-dependent PPAR/RXR heterodimerization and DNA binding. In the absence of ligand (L), nuclear hormone receptors are maintained in the inactive state via interactions with co-repressors (**1**). Ligand binding to PPARs results in a conformational change leading to dissociation of co-repressors (R), heterodimerization of RXR, and recruitment of transcriptional co-activators (A)(**2**). The active transcription complex binds PPREs, two copies of a direct repeat hexanucleotide sequence, in the promoter region of target genes leading to increases in gene transcription (**3**) (Gardner et al., 2005a).

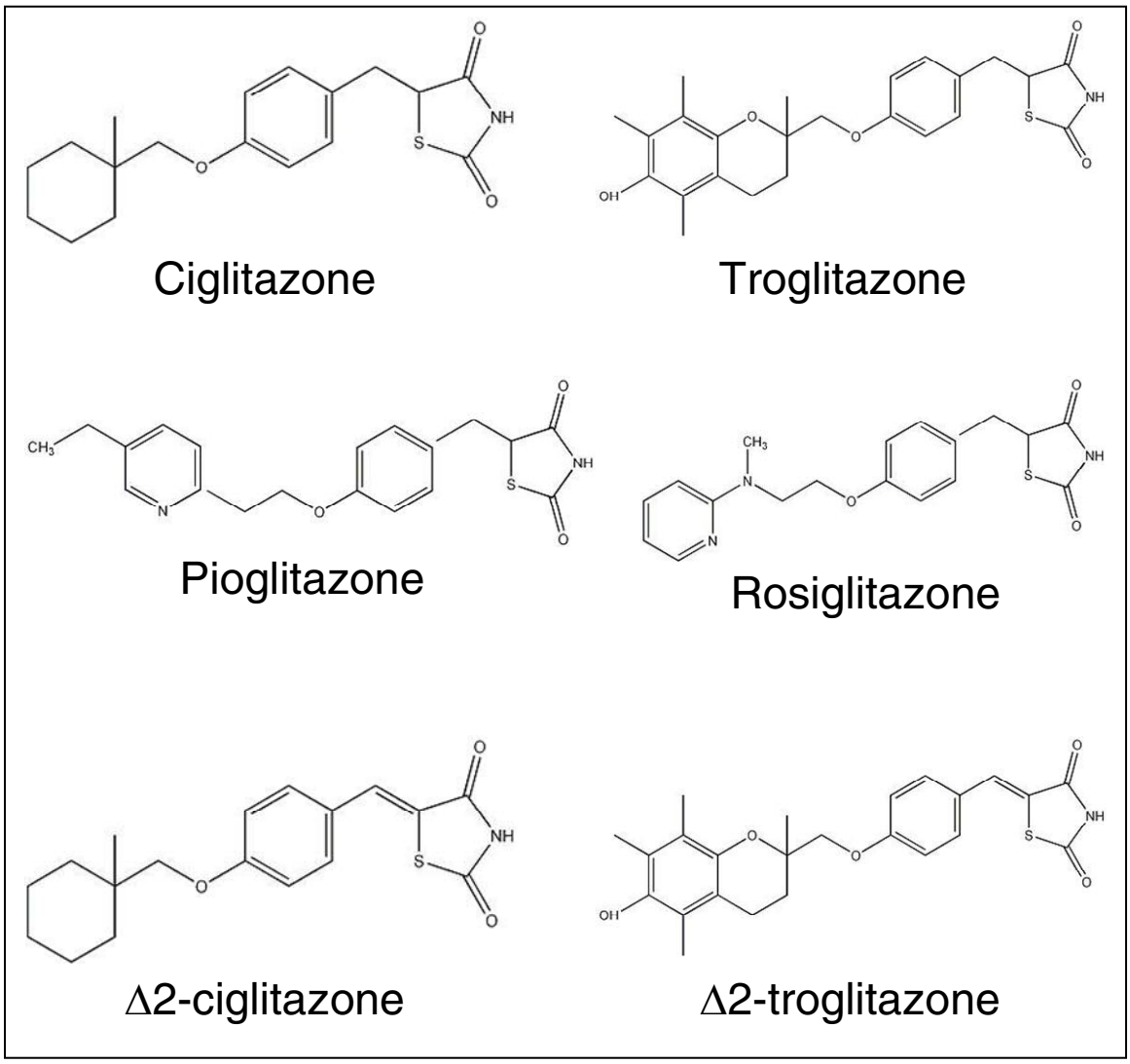

Figure 1.2. Chemical structures of the TZD class of PPARg ligands. Ciglitazone was the first of the TZD family to be synthesized (Sohda et al., 1982). Troglitazone, (Rezulin®; Parke-Davis) was derivative of ciglitazone and was used clinically, but withdrawn after deaths were reported in patients due to liver failure (Kohlroser et al., 2000; Watkins and Whitcomb, 1998). Both pioglitazone, (Actor®; Lilly) and rosiglitazone (Avandia®; GlaxoSmithKline) are currently available therapies against type II diabetes in the U.S. Δ2-ciglitazone and Δ2-troglitazone are TZD derivatives with attenuated or unappreciable activity in PPARγ activation (Huang et al., 2005; Shiau et al., 2005).

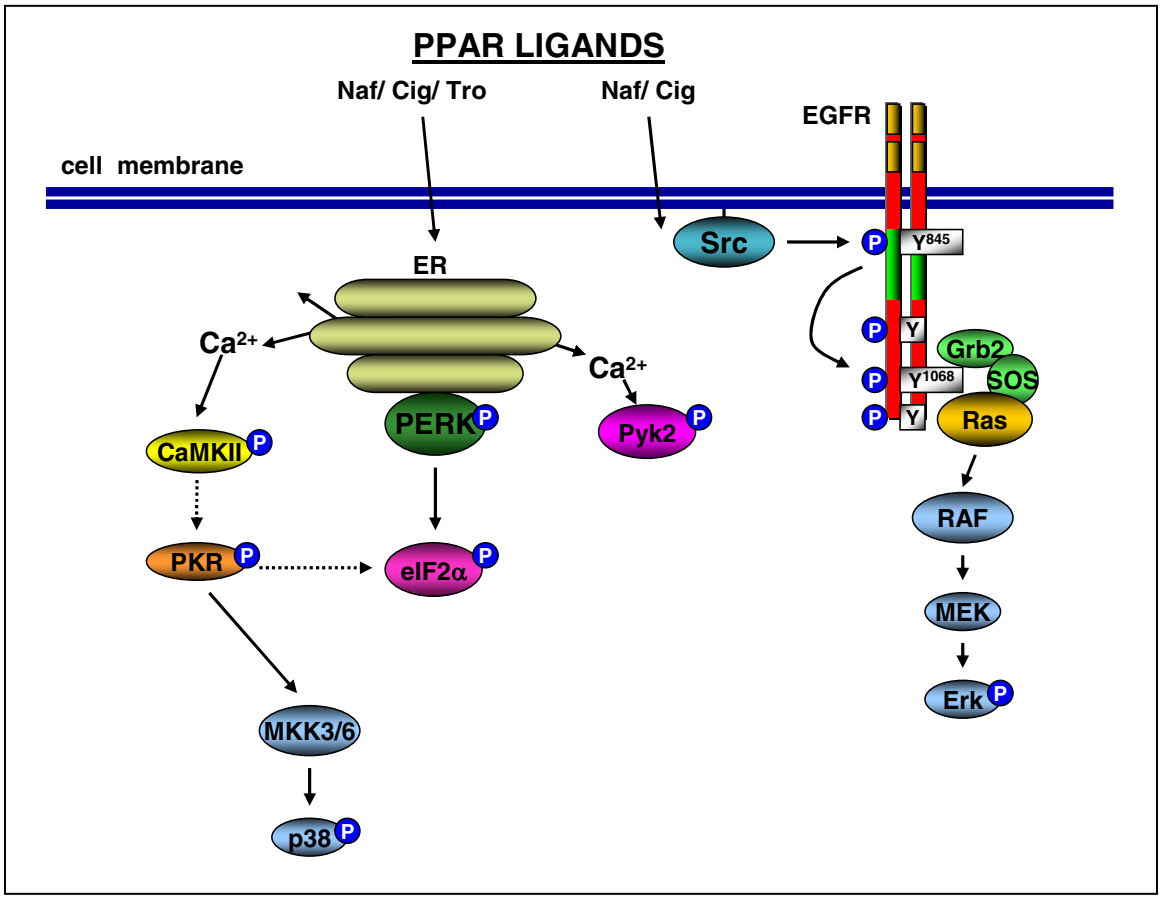

Figure 1.3. Mechanisms of MAPK activation in the rat liver epithelial cells GN4 by PPAR ligands. Nafenopin (naf), ciglitazone (cig) and troglitazone (tro), PPARα and γ ligands, respectively increased the phosphorylation of p38 MAPK. This event was shown to require calcium-dependent CAMK II activation, which in turn mediated phosphorylation of PKR. Activation of p38 was mediated by PKR-dependent MKK3/6 activation. This pathway correlated well with the induction and phosphorylation of ER stress markers PERK and eIF2α. ER calcium release associated with induction of ER stress was thought to mediate activation of Pyk2. A second, independent pathway was shown to be involved in increased Erk phosphorylation following nafenopin and ciglitazone treatment. Erk activation required Src-dependent EGFR transactivation. Following activation of the receptor, the canonical Ras, RAF, MEK cascade led to Erk phosphorylation. Interestingly, in contrast to ciglitazone, troglitazone failed to induce EGFR transactivation and only minimally stimulated Erk phosphorylation. This provided additional evidence that the two pathways were independent of one another and suggested that differences in the PPARγ-independent effects of these compounds could be related to the differential activation of MAPKs.

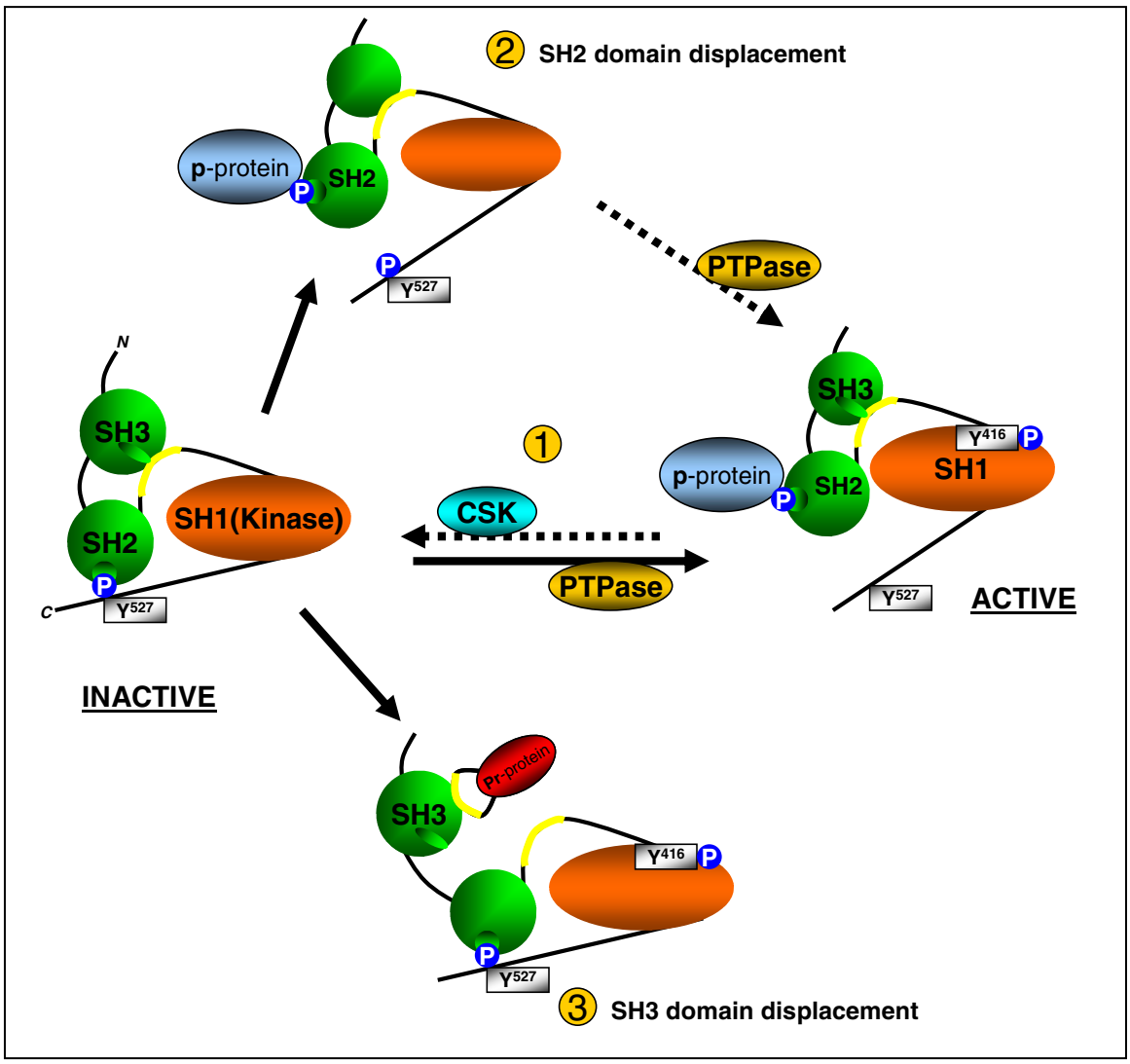

Figure 1.4. General mechanisms of Src family kinases (SFKs) activation. SFKs lobular domains and key tyrosine phosphorylation sites regulate the activity of the kinase domain. Classically, PTPase-mediated tyrosine 527 (Y^{527}) dephosphorylation leads to an intermolecular conformational change allowing for trans-autophosphorylation of tyrosine 416 (Y^{416}) and maximal kinase activity (**1**). CSK phosphorylates SFKs Y^{527} maintaining or returning them to an inactive state. Once activated, the SH2 domain can interact with other phospho.-proteins, which may localize SFKs to other substrates. Alternatively, an increase in tyrosine phosphorylated proteins (**p**-protein) can mediate displacement of the SFKs SH2 domain away from the Y^{527} site in the C-terminal tail (**2**). This exposes the Y^{527} site to PTPases and lead to kinase activation. Lastly, displacement of the SH3 domain can occur through interactions with other proteins containing a specific proline rich target sequence (**Pr**-protein). SH3 domain displacement results in an intermolecular conformational change, which allows for kinase activation and autophosphorylation, but interestingly does involve or require dephosphorylation of Y^{527} site (**3**).

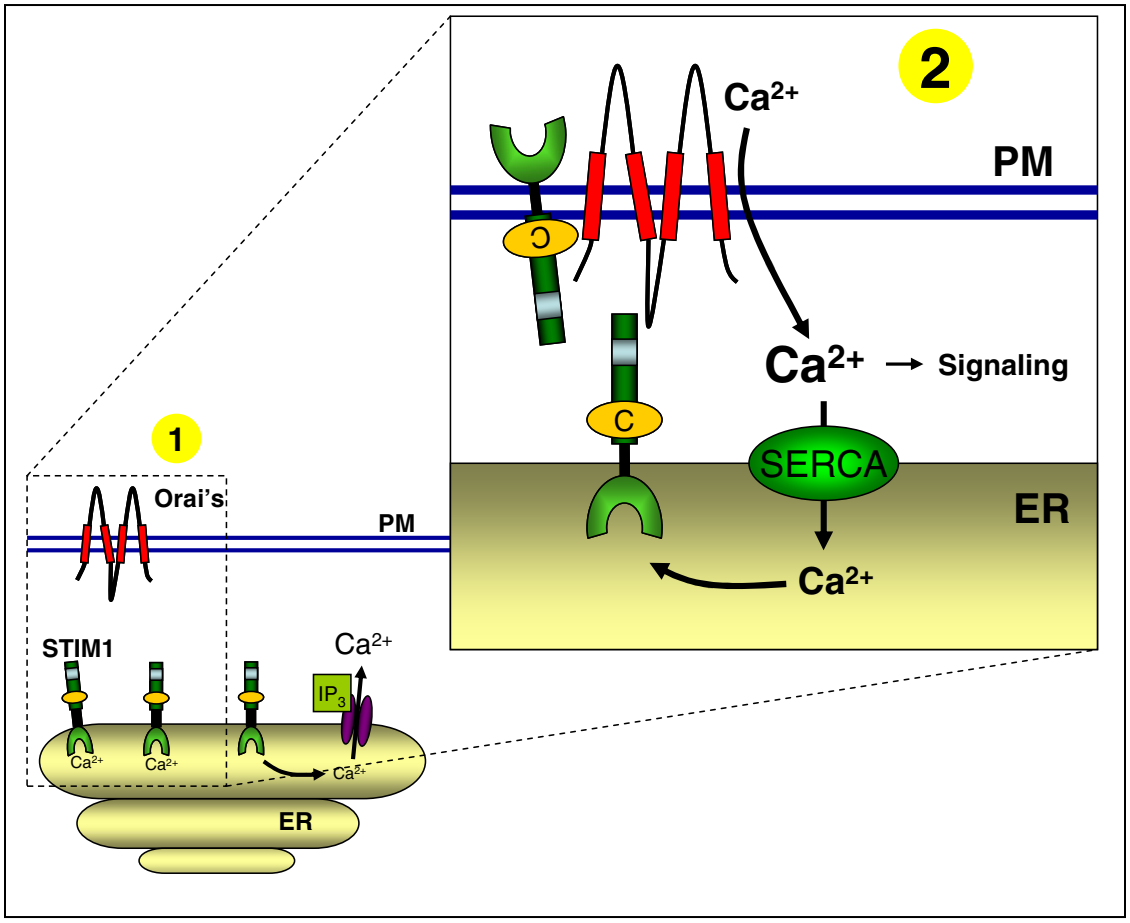

Figure 1.5. Capacitative calcium entry in non-excitable cells. Capacitative calcium entry is initiated through release of endoplasmic reticulum (ER) calcium stores. Typically this is mediated by phospholipase-dependent generation of inositoltriphosphate (IP_3), which activates ER channel proteins to cause calcium release. Additionally, mediators of ER stress or thapsigargin (an inhibitor of the sarco/endoplasmic reticulum calcium ATPases (SERCA)) can also induce ER-store depletion. The ER localized protein STIM1, contains an EF-hand domain that extends into the lumen of the ER and binds calcium; thus STIM1 is thought to act as an ER-calcium sensor. Following ER calcium release, STIM1 proteins assemble into punctate structures close to the plasma membrane (PM). STIM1 then induces extracellular influx by signaling Orai proteins in the PM. Some works suggest that a small population of STIM1 proteins are expressed in the plasma membrane and may interact with ER localized STIM1, thereby mediating activation of Orai-dependent calcium influx. Increases in intracellular calcium concentrations from capacitative calcium entry are important in mediating refilling of intracellular stores and signal transduction.

CHAPTER II

MATERIAL AND METHODS

A. MATERIALS

1. Chemicals

The thiazolidinediones ciglitazone and troglitazone were purchased from Biomol. Δ2-ciglitazone and Δ2-troglitazone (described in (Shiau et al., 2005)) were provided by Dr. Ching-Shih Cheng. TZDs were prepared as stock solutions in DMSO (final concentrations of DMSO following addition to cells did not exceed 0.1%). Human recombinant EGF was purchased from Invitrogen. PD153035, PP2, and PP3 were all purchased from Calbiochem. BAPTA-AM and Fura-2 AM were from Molecular Probes; 2-aminoethyl diphenyl-borinate, thapsigargin, and sodium orthovanadate were purchased from Sigma.

2. Antibodies

Anti-Src monoclonal antibody (B-12) and anti-phosphotyrosine (PY99) pan-monoclonal antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-EGFR (Tyr^{845}) and (Tyr^{1068}), anti-phospho-Src (Tyr416) and (Tyr^{527}) were purchased from Cell Signaling. Anti-EGFR C-terminal polyclonal antibody (#22) and anti-Pyk2 C-terminal polyclonal antibody (#72) were generated as previously described (Li et al., 1998, Yu, 1996 #20).

3. Radioactive Isotopes

[^{32}P]-γ-ATP was purchased from Perkin Elmer Life Sciences.

4. Adenovirus

The recombinant adenoviral vector encoding the C-terminus of Pyk2, termed CRNK (Ad.CRNK), was constructed and amplified as previously described (Li et al., 1999).

Ad.GFP was obtained from the University of North Carolina at Chapel Hill Viral Vector Core.

B. CELL CULTURE

The rat liver epithelial cells designated GN4, were grown in Richter's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/ streptomycin/ amphotericin B as described previously (Earp et al., 1995). Cells at 70-80% confluence were serum-starved 18-24 hours in Richter's minimum essential medium containing 0.1% fetal bovine serum prior to experimental treatment.

C. CELL LYSATE PREPARATION

Following cell stimulation for times indicated, medium was removed and cells were quickly washed twice with ice-cold PBS. The cells were then scraped in ice-cold RIPA buffer (150 mM NaCl, 9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS, pH 7.4) with freshly added 100 μM Na_3VO_4, 250 μM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, and 10 nM microcysteine. Cell lysates were centrifuged at 14,000 rpm for 10 minutes at 4°C to remove cellular debris. Protein concentration of the remaining supernatant was determined using the Coomassie protein assay reagent (Pierce).

D. WESTERN BLOTTING

In a typical experiment, an aliquot of cell lysate (10-30 μg protein) was resuspended in SDS-PAGE sample buffer (0.5 M Tris, pH 6.8, 4.0% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.1% bromphenol blue) and heated for 5 minutes at 95°C in order to denature proteins. The proteins were then resolved by SDS-PAGE on 10% polyacrylamide

gels and transferred to polyvinylidene fluoride (Immobilon-P; Millipore). The membranes were incubated in blocking buffer (TBST + 3.0% gelatin) for 1 hour, followed by addition of the appropriate primary antibody overnight at 4°C. Blots were then washed three times with tris-buffered saline with tween (TBST) followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1-2 hours at room temperature (RT). Immunoblots were developed with enhanced chemiluminescence (ECL) reagent (Amersham Biosciences), according to the manufacture's instructions and visualized by autoradiography (Kodak X-Omay Blue Film). Some immunoblot membranes were stripped in buffer (62.5 mM Tris, pH 6.8, 2.0% SDS, 100 mM β-mercaptoethanol) at 55°C for 30 minutes and then reprobed.

E. IMMUNOPRECIPITATION

After stimulation the cells were rinsed as described above and scraped into ice-cold RIPA buffer without SDS and then cleared by centrifugation. Various amounts of protein (between 150-500 μg depending on protein) were immunoprecipitated by overnight incubation with primary antibody at 4ºC with agitation followed by an additional hour long incubation after the addition of 20 μL of protein A-agarose bead slurry (Santa Cruz Biotechnology). Immune complexes were collected by brief centrifugation and then washed three times in cold lysis buffer and one time in PBS. Following the last wash, the remaining buffer was removed with a Hamilton syringe, the immune complexes resuspended in SDS-PAGE sample buffer and then separated by SDS-PAGE as described above.

F. IN VITRO KINASE ASSAY

Src kinase activity was measured using a standard commercial Src assay kit (Upstate Biotechnology, Inc.). Briefly, Src was immunoprecipitated as described above from 200 μg

of cell lysate by overnight incubation with Anti-Src monoclonal antibody (B-12). Src activity present in immune complexes was assessed by measuring the transfer of the γ-phosphate of [^{32}P]-γ-ATP to a specific Src substrate peptide for 15 minutes at 30°C. Phosphorylated substrate was spotted onto P-81 phosphocellulose paper (Whatman) and quantified with a scintillation counter.

G. INTRACELLULAR CALCIUM MEASUREMENTS

Approximately 1x10^6 GN4 cells were added to 60 mm plastic dishes containing three (No.1) 22-mm round coverslips and allowed to attach overnight in normal growth medium. The medium was removed, cells rinsed with HBSS and Fura-2 acetoxymethyl ester (AM) (Molecular Probes Inc., Eugene, OR) was added in HBSS at a final concentration of 2.5 μM, followed by incubation for 30 minutes at room temperature. The Fura-2-AM-containing buffer was removed and the cells incubated for an additional 30 minutes at room temperature in HBSS to allow for complete de-esterification. The coverslip was then placed in a gravity fed perfusion chamber containing HBSS. Changes in intracellular calcium concentration determined by changes in fluorescence intensity of Fura-2 at excitation wavelengths 340 and 380 nm following addition of stimulus were monitored using a dual-wavelength fluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH); at least four viable cells were monitored in each experiment.

H. RADIOLABELING OF [^{32}P]-PolyGlu:Tyr(4:1)

PolyGlu:Tyr (total of 15 μg) was radiolabeled using approximately 1 μg of recombinant GST-FER kinase in the presence of 200 μCi [^{32}P]-γ-ATP for 30 minutes at 30°C in 300 μl of a buffer consisting of 10 μM $MgCl_2$, 50 μM NaCl and 0.1 μM ATP. The reaction substrate

was precipitated by adding TCA solution to 20% wt/vol and centrifuging at 12,000 rpm for 5 min. The pellet was washed three times in 10% TCA to remove non-specific radioactivity, followed by resuspension in 200 mM TRIS, pH 8.0 at a concentration of 0.3 μg/μL. Incorporation of radiolabel was determined by scintillation counting.

I. IN-GEL PHOSPHATASE ASSAY

The in-gel phosphatase activity assays were carried out using a modification of a method described elsewhere (Burridge and Nelson, 1995). Briefly, protein extracts were prepared as described for Western blots except that sodium vanadate and SDS were omitted and following preparation, the samples were not boiled. Src immunoprecipitates were subjected to SDS-PAGE on 6.0% polyacrylamide gels containing [^{32}P]-PolyGlu:Tyr (approximately 750,000 cpm/7.5 μg PolyGlu:Tyr/gel). The proteins were then renatured by removing the SDS with two 30 minute 20% isopropanol washes followed by extensive washing of the gels with Tris, pH 8.0, buffer containing 0.09% β-mercaptoethanol/0.04% Tween-40. Gels were then incubated in 30% methanol/5% glycerol solution and dried between two sheets of cellophane. Dried gels were exposed to film and clear bands were indicative of PTPase activity.

J. INDENTIFICATION OF PROTEINS BY PEPTIDE MASS FINGERPRINTING

Following separation of proteins by SDS-PAGE, proteins were visualized via coomassie staining. Bands of interest were excised from gels followed by removal of coomassie stain in 10 mM ammonium bicarbonate and 40% acetonitrile (ACN) with vigorous shaking. After destaining, the gel pieces were evaporated to dryness in a speed-vac centrifuge for approximately 15 min, followed by rehydration in sequencing grade

trypsin; peptides were extracted by passive diffusion by incubating overnight at 37ºC in 20 mM ammonium bicarbonate. Peptides were purified using PerfectPure C18 tips (Eppendorf). Briefly, the C18 tips were washed in wetting solution (0.1% trifluoroacetic acid (TFA), 50% ACN) followed wash solution (0.1% TFA). Peptides were bound to C18 tips by aspirating and expelling sample 5-10 times followed by a rinse with the wash solution. Samples were spotted directly onto the MALDI MS target plate using the elution solution (0.1% TFA, 70% ACN, and saturated a-cyano-4-hydroxycinnamic acid) and allowed to air dry. The MALDI-TOF micro MX (Waters) mass spectrometer operating in positive ion mode was used to determine the mass/charge ratio of the peptides in each sample and generate spectra that could then be analyzed by peptide mass fingerprinting. Peptide mass fingerprinting was performed using Mascot (Matrix Science) to identify proteins in samples.

K. PREPARATION OF DETERGENT INSOLUBLE MEMBRANES (LIPID RAFTS)

GN4 cells were grown on 300-cm^2 plates to confluence and then serum-starved overnight. Following treatment, cells were washed on ice two times with ice-cold PBS, followed by rinsing with an isotonic lysis buffer (ISB) containing 10 mM Tris (pH 7.5), 50 mM NaCl, 10 mM EDTA, 20 mM NaF and 10 mM β-glycerol phosphate. Cells were lysed in 2.0 mL ice-cold ISB containing 1.0 mM Na_3VO_4, 0.5% Trito X-100 and protease inhibitor cocktail. Following scrapping and collection, lysates were incubated on ice for 10 minutes. A total of 2.0 mL of lysate was mixed with 2.0 mL of 80% sterile sucrose, and transferred to a thick-walled centrifuge tube. The resulting 40% sucrose/lysate solution was then layered with 4.0 mL of 30% sucrose, followed by 4.0 mL of 5.0% sucrose. Centrifuge tubes were balanced to the nearest 0.01 of a gram followed by 18

hours of centrifugation at 40,000 x g at 4ºC. Equal volume fractions (14) were collected using a Gradient Fractionator (Biocomp, Fredericton, NB, Canada). The detergent insoluble membranes (DIMs - lipid rafts) were routinely found in fractions 5 and 6.

CHAPTER III

Peroxisome proliferator-activated receptor γ-independent mechanisms of activation of the non-receptor tyrosine kinase Src by thiazolidinediones

A. ABSTRACT

c-Src is an important kinase involved in many cellular signal transduction pathways and is required for EGFR transactivation in response to the TZD, ciglitazone. TZDs are synthetic ligands for the PPARγ, but have been also shown to elicit PPARγ-independent effects, most notably activation of MAPKs. Indeed, the activation of the MAPK Erk was mediated by Src-dependent EGFR transactivation by both ciglitazone and Δ2-ciglitazone, a derivative compound that does not activate PPARγ. The mechanisms mediating Src activation in response to these compounds, however, remains unclear. In this study, the TZDs ciglitazone and troglitazone were both shown to increase Src kinase activation and induce changes in Src phosphorylation. Similar effects were observed with Δ2-derivatives of each TZD, indicating that Src activation in response to these TZDs was a PPARγ-independent event. Intracellular calcium chelation reduced Src Tyr^{416} phosphorylation following TZD treatment, but did not affect TZD-induced Src Tyr^{527} dephosphorylation. While TZD treatment did not increase general PTPase activity, inhibition of PTPase activity with pervanadate completely blocked Tyr^{527} dephosphorlytaion and TZD stimulated increases in Src kinase activity. Src was shown to be localized to lipid rafts following TZD treatment, and disruption of lipid raft function with methyl-β-cyclodextrin prevented Try^{527} dephosphorylation; however, no significant PTPase activity was observed in lipid raft fractions. Immunoprecipitation of Src from treated and untreated samples showed the association of a 120-kDa protein that possessed PTPase activity; neither TZD treatment nor inhibition of PTPase activity affected this association. Using MALDI mass spectrometry this associated PTPase was putatively identified as phosphatidylinositol polyphosphate-5 phosphatase.

B. INTRODUCTION

The TZDs are synthetic ligands for the PPARγ, a nuclear hormone receptor that functions as a ligand-activated transcription factor, and are used to promote glycemic homeostasis in patients with type II diabetes. The ability of TZDs to modulate blood glucose levels through improvement of impaired glucose intolerance has largely been attributed to their ability to modulate gene transcription in a PPARγ-dependent manner. However, the exact mechanism of action of TZD remains unclear.

In addition to mediating PPARγ-dependent gene transcription, TZDs have been shown to influence cellular events in a PPARγ-independent manner. This is most readily shown by the ability of these compounds to activate cellular signal transduction pathways at times too rapid to account for changes in gene transcription. The TZDs ciglitazone and troglitazone have been shown to activate specific kinases influenced by the induction of ER stress. Additionally, many works have demonstrated that TZDs modulate the activation of MAPKs, a family of protein kinases important in a diverse array of cellular functions. Investigations into the signal transduction pathways responsible for MAPK activation by TZDs demonstrated the involvement of the kinase Src.

c-Src, the first proto-oncogene discovered, is a member of the Src family kinases (SFKs), a subclass of membrane-associated non-receptor tyrosine kinases involved in a variety of cellular signaling pathways. SFKs are activated in response to cellular signals that promote proliferation, survival, motility, and invasiveness, including activation of cytokine receptors, receptor protein tyrosine kinases, G-protein coupled receptors and integrins (Thomas and Brugge, 1997). Recent evidence showed the involvement of Src kinase in mediating phosphorylation and transactivation of the EGFR following exposure

of cells to the TZD ciglitazone. These events were further shown to be PPARγ-independent as a structural derivative of ciglitazone, which lacked the ability to activate PPARγ, also mediated EGFR transactivation. Interestingly, another TZD troglitazone, did not effectively induce EGFR transactivation, suggesting that these two closely related compounds had potentially differential effects on Src kinase activation. However, the mechanisms involved in the activation of Src in response to TZDs have not been fully elucidated.

The regulation of Src kinase activity is maintained primarily by phosphorylation of tyrosine 527 (Tyr^{527}) in the C-terminal tail region of the protein. Src's own SH2 domain binds this site when it is phosphorylated and induces a protein conformation in which the catalytic cleft of the kinase domain cannot access substrates, thereby keeping Src in a "closed" and inactive state. Phosphorylation of Tyr^{527} is maintained by the closely related enzyme CSK (Okada and Nakagawa, 1988) and conversely is dephosphorylated by a number of PTPases, including the cytoplasmic PTP1B, Shp1 and Shp2 and the transmembrane localized CD45, PTPα, PTPε, and PTPλ (Roskoski, 2005). Upon dephosphorylation of this site, the intramolecular interaction of the SH2 domain is lost, resulting in a conformational change that opens the kinase domain, which leads to subsequent autophosphorylation of Tyr^{416} within the kinase activation loop and induction of maximal kinase activity. Once active, Src can phosphorylate multiple substrates involved in a variety of cellular events. Compounds that could decrease the activity of CSK or increase the activity of a Src-specific PTPase, would be expected to induced an increase in Src kinase activity.

In this study we observed unexpectedly, that both ciglitazone and troglitazone effectively increased Src kinase activity. Changes in Src kinase activity were accompanied by a decrease in Tyr^{527} phosphorylation and an increased in Tyr^{416} autophosphorylation following TZD exposure. The loss of Src Tyr^{527} phosphorylation appears to require the involvement of a PTPase, which may be persistently associated with Src, and movement of Src into lipid rafts, specific plasma membrane structures that localize many signaling proteins. Collectively, these data provide novel evidence as to the mechanisms involved in mediating Src kinase activation in response to TZDs.

C. RESULTS

1. Both ciglitazone and troglitazone increased c-Src phosphorylation and activity independent of PPARγ

Previously we showed that PPAR α and γ agonists increased the phosphorylation of the MAPK Erk in the rat liver epithelial cell line, GN4, an event that required Src-dependent EGFR transactivation (Gardner et al., 2003). Interestingly, ciglitazone but not troglitazone, a related TZD family member, was capable of inducing EGFR transactivation. We therefore investigated the ability of TZDs to activated Src kinase. Additionally, Src activation was examined for Δ2-derivatives of these compounds, which bind but do not activate PPARγ. Autophosphorylation of Src on tyrosine 416 (Tyr^{416}), which is known to correlate well with elevated levels of Src kinase activity, increased after treatment of cells with ciglitazone, troglitazone or their respective Δ2-derivatives (Figure 3.1A and 3.1B). Src Tyr^{416} phosphorylation was maximal at 5 and 10 minutes following ciglitazone treatment; Δ2-ciglitazone was slower and showed maximal

activation around 10 minutes, consistent with previous studies (i.e. EGFR transactivation, Erk phosphorylation) (Gardner et al., 2003). Unexpectedly, both troglitazone and Δ2-troglitazone, compounds that do not affect EGFR transactivation, similarly increased Src Tyr^{416} phosphorylation, with maximal activation occurring at 10 minutes (Figure 3.1B).

We further examined the phosphorylation status of Src Tyr^{527}, the inhibitory phosphorylation site. As expected, Src was highly phosphorylated on Tyr^{527} in unstimulated serum-starved cells; however following treatment of cells with ciglitazone, troglitazone, or their respective Δ2-derivatives, a time-dependent increase in Src Tyr^{527} dephosphorylation was observed. Ciglitazone reduced Src Tyr^{527} phosphorylation after 10 minutes and maximally at 30 minutes when compared to vehicle (DMSO) treated cells; again the response obtained with Δ2-ciglitazone was slightly slower when compared to ciglitazone (Figure 3.1A). Both troglitazone and Δ2-troglitazone decreased Src Tyr^{527} phosphorylation in a comparable time-dependent manner, with maximal loss occurring at 30-minutes (Figure 3.1B).

Because ciglitazone, troglitazone and their Δ2-derivatives affected phosphorylation events involved in regulation of Src activation, we further examined the effects of these compounds on Src kinase activity. Endogenous Src was immunoprecipitated from lysates from treated and untreated cells, and *in vitro* kinase assays performed using a Src-specific substrate peptide. Following treatment of cells for 10 minutes with ciglitazone, troglitazone or their respective Δ2-derivatives, there was a significant increase in Src kinase activity, approximately 3-fold over DMSO vehicle controls (Figure 3.2A). Treatment of cells for 30 minutes, where Src Tyr^{527} loss was maximal, resulted in a 6-fold increase in Src kinase activity over DMSO vehicle controls for ciglitazone and Δ2-

ciglitazone, while troglitazone and Δ2-troglitazone increased Src activity nearly 7-fold over DMSO controls (Figure 3.2A). Additionally, Src was immunoprecipitated from unstimulated serum starved cells, where Src Tyr^{527} phosphorylation was high and activity low. These immune complexes were then incubated in the *in vitro* kinase reaction mixture with or without 50 μM ciglitazone or troglitazone for 10 and 30 minutes. Neither ciglitazone nor troglitazone significantly affected Src kinase activity directly (Figure 3.2B). Collectively, these data show that ciglitazone, troglitazone and their respective Δ2-derivatives modulate key regulatory phosphorylation sites in Src kinase, resulting in an increase in Src kinase activity. Moreover, Src kinase activity was not directly affected by TZD treatment, suggesting that cellular components are required in the mechanism of Src activation. These data further suggest 1) that both ciglitazone and troglitazone affect a common PPARγ-independent mechanism mediating Src activation and 2) that additional mechanisms are required for EGFR transactivation, as both TZDs effectively increase Src activity, but only ciglitazone is capable of mediating transactivation of the EGFR (Gardner et al., 2005b).

2. Calcium chelation prevents Src Tyr^{416} phosphorylation, but does not affect Tyr^{527} dephosphorylation

The ability of both ciglitazone and troglitazone to induce Src activation suggests a common mechanism of activation. Previously we observed that both compounds mediated ER stress, an event often associated with ER calcium release (Gardner et al., 2005b). We therefore examined the role of calcium in modulating Src activation in response to TZD treatment. Serum-starved cells were pretreated for 20 minutes with 50 μM BAPTA-AM, an intracellular calcium chelator. Cells were then treated with 50 μM

ciglitazone, troglitazone, the Δ2-derivatives or DMSO (vehicle control) for 10 and 30 minutes. At 10 minutes ciglitazone, Δ2-ciglitazone and troglitazone effectively increased Src Tyr^{416} phosphorylation; Δ2-troglitazone only mildly increased Src Tyr^{416} phosphorylation in this experiment (Figure 3.3A). Pretreatment of cells with BAPTA-AM effectively lowered TZD-induced Src Tyr^{416} phosphorylation, and in most cases decreased this phosphorylation to below basal levels (Figure 3.3A). Because BAPTA-AM effectively reduced Src Tyr^{416} phosphorylation, we further examined the affects of calcium chelation on the ability of the TZDs to modulate Src Tyr^{527} dephosphorylation. We anticipated that loss of Tyr^{527} following treatment of cells with TZDs would also be prevented by calcium chelation. Interestingly, after treatment of cells for 30 minutes with TZDs, the time point at which maximal Src Tyr^{527} dephosphorylation was previously observed, BAPTA-AM pretreatment had no effect on Src Tyr^{527} dephosphorylation in response to TZD treatment (Figure 3.3B).

3. Inhibition of protein tyrosine phosphatases prevents loss of Tyr^{527} phosphorylation

The status Src Tyr^{527} phosphorlyation is regulated by both CSK and PTPases; CSK maintains phosphorylation of this site while PTPase mediate its dephosphorylation. To determine if a PTPase was involved in the mechanism of activation of Src by TZDs, we pretreated cells with either 50 μM H_2O_2, sodium orthovanadate or vanadyl hydroperoxide [$V^{(4+)}$-OOH] (pervanadate). Pervanadate, a mixture of sodium orthovanadate and hydrogen peroxide, has been shown to be a more potent inhibitor of PTPase activity than sodium orthovanadate and penetrates cells more readily than sodium orthovanadate or H_2O_2 alone in intact cells (Kadota et al., 1987; Trudel et al., 1991). Following treatment

with ciglitazone, troglitazone or their respective Δ2-derivatives, Src Tyr^{527} phosphorylation was decreased as expected (Figure 3.4A and 3.4B). Pretreatment of cells with either H_2O_2 or sodium orthovandate alone did not affect basal Src Tyr^{527} phosphorylation. Further, neither of these compounds affected the ability of any TZD to mediate Src Tyr^{527} dephosphorylation. However, pervanadate pretreatment blocked dephosphorylation of Src Tyr^{527} following TZD treatment (Figure 3.4A and 3.4B) suggesting that PTPase activity was required for Src Tyr^{527} dephosphorylation and subsequent Src activation.

We further examined the effects of PTPase inhibition by pervanadate on Src kinase activity in cells treated with TZDs or their respective Δ2-derivatives to determine if dephosphorylation was necessary for TZD-induced Src kinase activation. Following exposure of cells to TZDs, Src kinase activity was increased nearly 6-fold in each condition (Figure 3.5), similar to previous results. Pretreatment of cells with either H_2O_2 or sodium orthovanadate did not significantly reduce Src kinase activity; however, TZD-induced Src kinase activity was completely inhibited by pervanadate pretreatment to below basal levels (Figure 3.5). These data demonstrated that PTPase-mediated Src Tyr^{527} dephosphorylation was required for Src kinase activation following TZD treatment.

Because TZD-induced Src Tyr^{527} dephosphorylation was prevented by inhibition of PTPase activity, we examined whether ciglitazone or troglitazone were mediating increased PTPase activity. Lysates from 10 and 30 minute ciglitazone or troglitazone treated cells were used in an *in vitro* PTPase activity assay (Molecular Probes). There

was no significant increase in PTPase activity observed following TZD treatment (Figure 3.6).

4. Disruption of Lipid Rafts prevents Src Tyr^{527} dephosphorylation

Src and other Src family kinases have been shown to localize to discrete areas on the plasma membrane called lipid rafts. These sphingomyelin and cholesterol rich domains act like platforms in the plasma membrane and recruit or localize signaling proteins containing glycolsylphosphatidylinositol (GPI) anchors. Because we did not observe a direct increase in PTPase activity following TZD treatment we examined the possibility that TZDs were altering the cellular localization of Src and/or a PTPase important in Src activation to lipid rafts. To isolate lipid rafts, untreated or treated cells were lysed with ice-cold lysis buffer containing 0.5% Triton X-100. Detergent-insoluble fractions were separated using a sucrose density gradient and centrifugation, followed by collection using a gradient fractionator. Fractions from each sample were subjected to SDS-PAGE followed by immunblotting with Src and caveolin-1, a protein commonly localized in raft fractions (Figure 3.7). Caveolin-1, a marker protein for lipid rafts was routinely found in fractions 5 and 6. Following treatment of cells with either ciglitazone or troglitazone, we could not detect any noticeable increase in the total amount of Src protein in raft fractions when compared to un-stimulated or DMSO vehicle controls (Figure 3.7). These data suggested that TZDs do not significantly alter the cellular localization of Src.

We further examined if a PTPase was co-localized in raft fractions. Here non-raft fractions and raft fractions from TZD treated or untreated cells were separated by 6.0% SDS-PAGE impregnated with [^{32}P]-PolyGlu:Tyr(4:1) and IN-GEL PTPase activity assays were performed. Similar to what was observed for total Src protein, little PTPase

activity was observed in raft fractions (Figure 3.8). We did observed significant PTPase activity in non-raft fractions in a protein with a molecular weight of approximately 120 kDa (Figure 3.8, Fractions 10-12). These data suggest that like Src, the association of a PTPase to lipid rafts is rather weak and that TZDs do not significantly alter a change in PTPase localization.

Lastly, we examined if lipid raft function was important in the mechanism of TZD-induced Src activation. Here cells were pretreated with the cholesterol chelator and known lipid raft disrupter, methyl-β-cyclodextrin (MβC) for 30 minutes prior to TZD treatment. TZD-induced Src Tyr^{416} phosphorylation at 10 minutes was relatively unaffected by MβC-induced lipid raft disruption. Interestingly, at 30 minutes, Src Tyr^{416} phosphorylation in TZD-treated samples was reduced to lower than basal levels (this was similarly observed in the initial time course experiments (Figure 3.1A and B)); however in samples pretreated with MβC, Src^{416} phosphorylation at this time point was maintained (Figure 3.9). In contrast, $SrcTyr^{527}$ dephosphorylation, which was lowered and significantly reduced following 10 and 30 minute TZD treatments, respectively, was completely prevented by MβC pretreatment at both time points (Figure 3.9).

5. **A protein tyrosine phosphatase is associated with Src kinase**

Several PTPases have been reported to mediate dephosphorylation of the negative regulatory Tyr^{527} site of Src and other SFK members. These include PTPα, PTP1B, CD-45, and the dual-specific phosphatases Shp1 and Shp2. As TZDs decreased Src Tyr^{527} phosphorylation in a PTPase-dependent manner, but do not appear to increase the activity of PTPase directly or alter its cellular localization, we examined if TZDs affected the association (i.e. localization) of a specific PTPase with Src. Cells were treated with

ciglitazone or troglitazone for 10 and 30 minutes and then lysed in RIPA (No SDS) buffer without the addition of sodium vanadate. Initially, immunoprecipitation from 400 μg of total protein was performed using either total-Src or HA antibodies. Immune-complexes were then separated on 6.0% SDS-PAGE and coomassie stained. Several Src-associated proteins were observed, including a strong protein band with an approximate molecular weight of 120 kDa, in the Src IPs; this same protein as well as others, was not observed in the HA immune-complexes demonstrating that these proteins were only associated with the Src immunoprecipitates (Figure 3.10A). Interestingly, we previously observed an approximate 120 kDa protein in non-raft fractions that possessed PTPase activity (Figure 3.8). To determine if any of these Src-associated proteins possessed PTPase activity, similar IN-GEL PTPase assays were preformed on the Src-immune-complexes from treated and untreated samples. Again PTPase activity was observed in the 120 kDa protein band; however TZD treatment for 10 and 30 minutes did not enhance this PTPase's activity or induce an increase of association. Moreover, when cells were pretreated with pervanadate, the PTPase activity of this protein was completely blocked, but the association with Src was not affected (Figure 3.10B).

To identify the Src-associated PTPase and other Src-associated proteins, trypsin digests were performed on bands excised from gels (See Figure 3.10A – highlighted, numbered bands), and tryptic peptides were analyzed by MALDI-TOF mass spectrometry. A spectrum of the tryptic peptides generated from the protein band in which PTPase activity was observed (Band #2) is shown in Figure 3.11; spectra were obtained for the other Src-associated proteins (data not shown). The top 70 MS peaks (Table 3.1 – PTPase peaks only), by intensity, from each spectra were searched using

MASCOT peptide mass fingerprint (Matrix Science) to identify proteins. The PTPase associated with Src was putatively identified as phosphatidylinositol polyphosphate-5 phosphatase, (Accession: XP_229106) from rat. This protein has a calculated molecular weight of 119,669 Da, which closely matches the observed 120 kDA size of the protein isolated from the gel. Other proteins identified included myosin heavy chain, tubulin and actin (Figure 3.2).

6. **Association of Src with C-terminal Src Kinase**

While PTPase-dependent activity appears to be involved in mediating dephosphorylation of Src Tyr^{527} following TZD treatment, we did not detect any enhancement of PTPase activity nor a difference in the association with Src of a PTPase following TZD treatment. In fact, there appeared to be a PTPase constitutively associated with Src. Therefore, PTPase mediated Tyr^{527} dephosphorylation may be allowed due to a loss of association of CSK following TZD treatment. To determine if Src/CSK association was altered by TZD treatment we immunoprecipitated either Src or CSK from GN4 lysates from treated or untreated samples and immunoblotted for the other protein, respectively. Following immunoprecipitation of CSK from unstimulated samples we did not detect any associate Src protein (Figure 3.12A, lane 2). Similarly, no CSK was associated with immunoprecipitated Src from untreated samples (Figure 3.12A, lane 6). Ciglitazone and troglitazone treatment did not promote an increase in the association between CSK and Src at 10 or 30 min (Figure 3.12B.) These data suggest that the transient association between CSK and Src is relatively weak and can not be maintained following cell lysis.

D. DISCUSSION

TZDs are well known for their anti-hyperglycemic actions and their ability to activate and induce PPARγ-dependent gene transcription. In addition to these PPARγ-mediated effects, TZDs have been shown to rapidly activate MAPKs independent of PPARγ . Previous work from our lab examining pathways contributing to MAPK activation demonstrated a required role for Src kinase in EGFR transactivation in response to the PPARα agonist, nafenopin or the PPARγ agonist, ciglitazone (Gardner et al., 2003). Moreover, nafenopin and ciglitazone treatment was shown to increase phosphorylation of the EGFR at tyrosine 845 (Tyr^{845}), a Src-specific site (Biscardi et al., 1999). These data suggested PPARγ agonists effectively mediated Src kinase activation; however the mechanisms by which these compounds are able to mediate Src activation have not been examined. Here we show that both ciglitazone and troglitazone, two related TZD family members, effectively mediated Src kinase activation. This was also observed with Δ2-derivatives of each parent compound, which are incapable of PPARγ activation and suggest that these effects are indeed, PPARγ-independent. Src kinase activation by these compounds was shown to be sensitive to PTPase inhibition and we further identified a putative PTPase associated with Src. TZDs did not appear to directly affect the association or the activity of this Src-associated PTPase, suggesting that additional mechanisms were important in mediating Src activation.

Previously we have observed that inhibition of Src kinase activity, prevented ciglitazone-induced EGFR transactivation (Gardner et al., 2003). Other works have demonstrated a role of Src kinase in EGFR transactivation in response to physiological PPARγ agonists (Ichiki et al., 2004) and to ciglitazone specifically (Slomiany and

Slomiany, 2004). Interestingly, troglitazone, a related TZD family member was ineffective at mediating EGFR transactivation suggesting that there were differential effects of these compounds on kinase signaling pathways, specifically Src activation. Our data here show that both ciglitazone and troglitazone effectively increased Src kinase activity (Figure 3.2A). Further, neither of these compounds increased Src kinase activity directly suggesting that these compounds were mediating activation by affecting cellular signaling mechanisms (Figure 3.2B). Additionally, Δ2-derivatives of each compound increased Src activity, suggesting that like other signal transduction pathways activated in response to TZDs, Src kinase activation was independent of PPARγ activation (Gardner et al., 2003; Huang et al., 2005).

Previous work from our lab demonstrated that both ciglitazone and troglitazone increased the phosphorylation of PERK, PKR, and eIF2α, events associated with the induction of ER stress (Gardner et al., 2005b). Other work has demonstrated that induction of ER stress can lead to release of calcium from this intracellular store (Kuang et al., 2005) and both ciglitazone and troglitazone mobilize calcium from intracellular stores (Palakurthi et al., 2001). The ability of both TZDs to mediate Src activation pointed to a common mechanism of activation. Interestingly, activation of Src kinase has been shown to be increased by treatment of cells with high extracellular calcium or by thapsigargin, a known modulator of ER calcium release (Zhao et al., 1992). We observed that intracellular calcium chelation with BAPTA-AM lowered ciglitazone and troglitazone-induced Src Tyr^{416} phosphorylation (Figure 3.3A), supporting previous data demonstrating a role of calcium in Src activation (Buitrago et al., 2001). To be consistent with its inhibitory effects on Tyr^{416} phosphorylation, we expected that BAPTA

pretreatment would prevent TZD-induced Src Tyr^{527} dephosphorylation; however this was not the case. In fact BAPTA appear to enhance loss of Tyr^{527} phosphorylation in the presence of TZDs (Figure3.3B). Thus the role calcium plays in Src kinase activation is not completely understood and should be further examined (See Chapter IV).

Classically, the activity of Src kinase is predominantly thought to be regulated by phosphorylation of Tyr^{527} in the C-terminal region of the protein. The viral Src protein, v-Src lacks this C-terminal tail region and phosphorylation site and possesses constitutive kinase activity (Collett and Erikson, 1978; Levinson et al., 1978). Further, mutation of this tyrosine site in Src to phenylalanine produces a protein with greater kinase activity when compared to wild-type Src (Kmiecik and Shalloway, 1987). Consistent with their ability to increase Src kinase activity, ciglitazone, troglitazone and Δ2-derivatives of each compound, mediated Tyr^{527} dephosphorylation (Figure 3.1). The phosphorylated Tyr^{527} is targeted by Src's own SH2 and this intramolecular interaction helps maintain Src in a "closed" and inactive conformation (Brown and Cooper, 1996). Dephosphorylation of this site, as observed with the TZDs, results in a conformational change and an unconstrained form of the kinase domain which autophosphorylates on Tyr^{416} demonstrating kinase activity (Smart et al., 1981). In agreement with the TZDs ability to mediate Src Tyr^{527} dephosphorylation, we further observed a transient increase in Tyr^{416} phosphorylation following TZD treatment (Figure 3.1). Thus our phosphorylation data agree with our results demonstrating an increase in kinase activity in response to TZDs. Interestingly, while dephosphorylation of Tyr^{527} appeared maximal at 30 minutes following TZD treatment, Tyr^{416} phosphorylation was observed to peak at 10 minutes followed by a reduction at 30 minutes. It would be expected that enhanced loss of Tyr^{527}

would result in greater Tyr^{416} phosphorylation; however it has been shown that exogenous substrates decrease Src autophosphorylation (Sun et al., 2002). Thus the decrease in Src Tyr^{416} phosphorylation following the 10 minute peak could be due to enhanced catalytic activity of Src towards other cellular substrates, such as the EGFR.

The phosphorylation of Src's Tyr^{527} site is maintained by the related kinase, CSK (Okada and Nakagawa, 1988). Additionally, the phosphorylation status of this site can be regulated by PTPases. Several have been shown to mediate dephosphorylation of this site, including the cytosolic PTP1B, Shp1 and Shp2, and the transmembrane localized CD45, PTPα, PTPε, and PTPλ (Roskoski, 2005). Troglitazone and pioglitazone were shown to up regulate the activity of Shp2 in smooth muscle cells; however this was primarily due to increase Shp2 protein expression (Wakino et al., 2004). Others though have shown PPARγ-independent activation of PTP1B by troglitazone, a mechanism important in reduction of STAT3 phosphorylation (Akasaki et al., 2006). Here, inhibition of PTPase activity completely prevented TZD-induced Src Tyr^{527} dephosphorylation and increases in Src kinase activity (Figure 3.4 and 3.5). However, there was no effect of TZDs on PTPase activity (Figure 3.6). These data show that a PTPase is involved in mediating Src kinase activation in response to these compounds, but suggest that different mechanism other than increased PTPase activity must account for the changes in Src Tyr^{527} dephosphoylation observed. Moreover, these results suggest that TZDs do not affect CSK activity. CSK possesses similar structural domains as SFKs, but lacks the C-terminal tail negative regulatory phosphorylation site and is therefore thought to be constitutively active in cells (Ogawa et al., 2002). Here, pretreatment of cells with pervanadate completely blocked Src Tyr^{527} dephosphorylation following TZD treatment

(Figure 3.4). If TZDs were reducing Src Tyr^{527} phosphorylation through an inhibitory effect on CSK activity, then pervanadate pretreatment would not have resulted in enhancement of Src Tyr^{527} phosphorylation following TZD treatment.

Lipid rafts are discrete sphingolipid, ceramide and cholesterol enriched domains that exist in the plasma membrane and are thought to serve as "platforms" for plasma membrane bound signaling proteins (Simons and Toomre, 2000). Src and other SFK members by virtue of an N-terminal lipid modification are primarily localized at the plasma membrane and further can be enriched into lipid rafts. Some PTPase's involved in regulating Src kinase activation, such as CD45 and PTPα, are also membrane localized proteins. Therefore intracellular localization could be an important mechanism in Src regulation. However, our data show that there no noticeable change in the amount of Src in lipid rafts following TZD treatment (Figure 3.7); similarly, there was no detectable PTPase activity found in these same fractions (Figure 3.8). Interestingly, when cells were pretreated with MβC, a cholesterol chelator known to disrupt lipid raft function, TZD-mediated Src Tyr^{527} dephosphorylation was prevented (Figure 3.9). Other work has shown that MβC lipid raft disruption induces a loss of lipid raft associated proteins including members of the SFK family (Ilangumaran and Hoessli, 1998). Thus while little PTPase activity was observed in lipid rafts, localization of Src to these specific membrane structures could be important to the mechanism regulating Src activation in response to TZDs.

Because we were unable to observe the association of either Src or a PTPase in lipid rafts, we examined Src protein directly following TZD treatment for associated proteins that possessed PTPase activity. Work has demonstrated that the ER localized PTP1B is

cleaved by the calcium-dependent protease, calpain, which leads to a 2-fold increase in its PTPase activity and a release of its ER localization (Frangioni et al., 1992; Frangioni et al., 1993). The catalytically active PTP1B fragment can redistribute to the plasma membrane and thereby interact with Src. Src immunoprecipitated from cells showed the association of several proteins including a protein with the approximate molecular weight of 120-kDa. This same protein was observed to possess PTPase activity (Figure 3.10). Interestingly, neither treatment of the cells with TZDs or inhibition of its PTPase activity with pervanadate affected its association with Src. This suggested that contrary to the idea that TZDs were affecting the association of PTPase with Src thereby mediating Src's activation, that in GN4 cells, Src is strongly associated with a regulatory PTPase. Using MADLI mass spec, this PTPase was putatively identified a phosphatidylinositol polyphosphate-5 phosphatase (Figure 3.11 and Table 3.1).

There are eight defined mammalian 5-phosphatase enzymes originating from separate genes that have been cloned and characterized (Majerus et al., 1991). These enzymes are characterized as dual-specificity PTPases in that they hydrolyze phosphate groups from proteins and lipids; specifically multiple phosphorylated inositol and phosphatidylinositol and phospho-tyrosine proteins. Based on their substrate specificity for either IP_3, Ins 1,3,4,5-P_4 (IP_4) and the lipids PtdIns-4,5-P_2 and PtdIns-3,4,5-P_3, 5-phosphatase are categorized into 4 general groups (Jefferson and Majerus, 1996). Group III enzymes hydrolyze substrates with a 3-position phosphate group and include the enzymes Shp1 and Shp2. Interestingly, in addition to mediating dephosphorylation of lipids substrates, Shp1 and 2 have been shown to be involved in Src kinase regulation through protein dephosphorylation. Here we have putatively identified a PTPase, which is found

associated with Src kinase that is very similar to Shp2, a PTPase known to regulate Src activation (Figure 3.11). Shp1 has been shown to directly catalyze the dephosphorylation of Src (Somani et al., 1997). However, Shp2 is thought to target dephosphorylation of Cbp, a lipid raft localized tyrosine-phosphorylated adaptor protein that recruits CSK to the plasma membrane. Dephosphorylation of Cbp abolishes the CSK binding interaction and reduces CSK-mediated Src Tyr^{527} phosphorylation, leaving the Tyr^{527} site exposed to dephosphorylation by Shp2 or another PTPase (Figure 3.13). TZDs did not appear to affect the localization or the activity of the Src-associated phosphatidylinositol polyphosphate-5 phosphatase, therefore TZD treatment could be influencing the association of regulatory proteins maintaining Src in an inactive state. Initially we examined the association of Src and CSK to determine if TZD treatment affected this interaction. However, we did not observe an interaction between these two proteins in either basal conditions or after TZD treatment (Figure 3.12). Additional experiments should be performed to determine the phosphorylation status of Cbp or its interaction with CSK before and after TZD treatment to better define this mechanism.

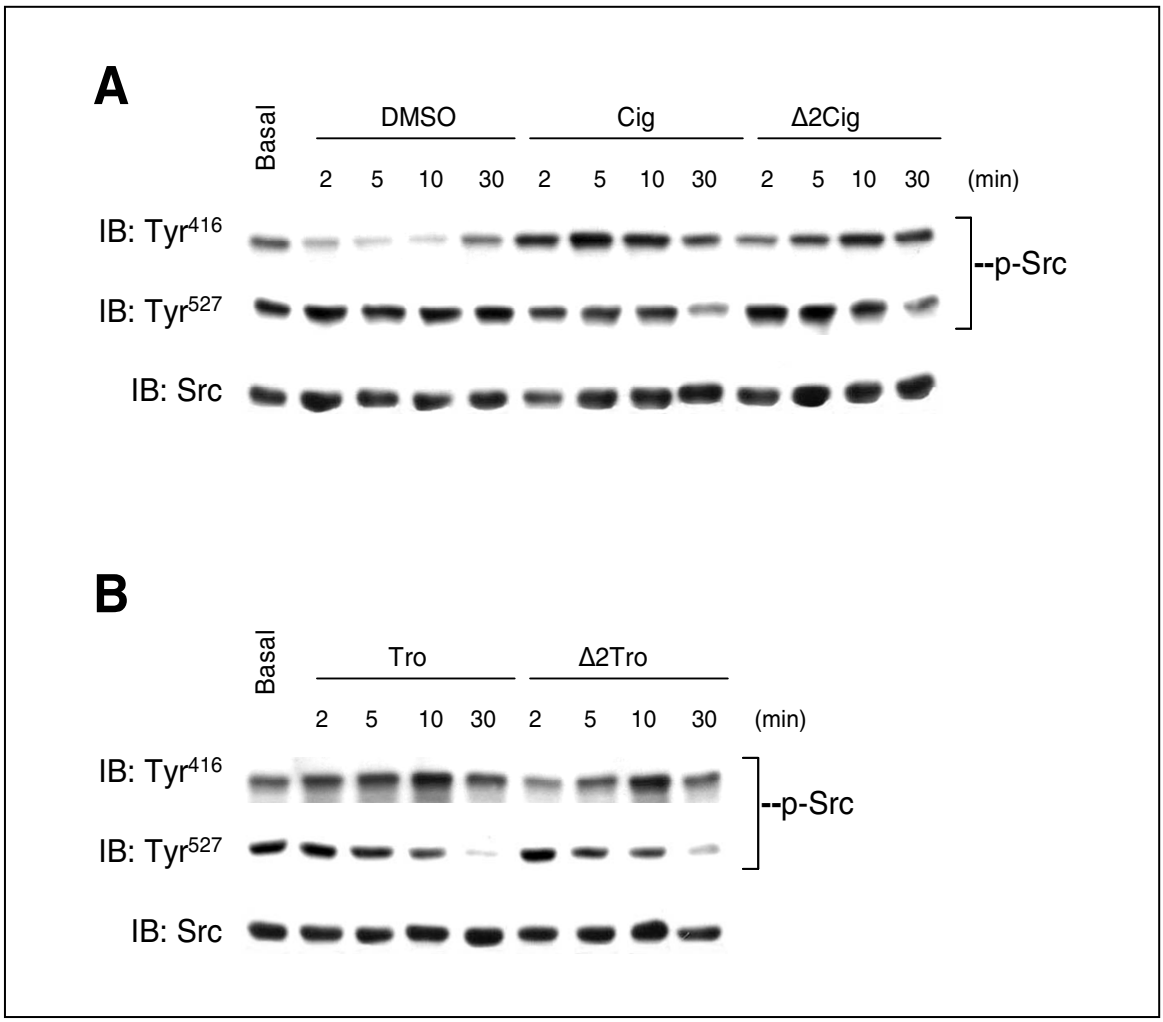

Figure 3.1. Ciglitazone, troglitazone, and their respective Δ2-derivatives alter Src kinase phosphorylation. Rat liver epithelial cells (GN4) were grown to confluence and serum-deprived overnight in media containing 0.1% fetal bovine serum. Cells were stimulated with (**A**) 50 μM ciglitazone (Cig), Δ2-ciglitazone (Δ2Cig), or 0.1% DMSO (vehicle control), and (**B**) 50 μM troglitazone (Tro) or Δ2-troglitazone (Δ2Tro) for the times indicated in minutes (min). Cell lysates were prepared and subjected to 10% SDS-PAGE. Src tyrosine phosphorylation was determined by immunoblotting (IB) using either an anti-phospho-Src (Tyr^{416}) or (Tyr^{527}) antibodies. The blots were stripped and reprobed using antibodies directed against total Src to determine equal protein loading.

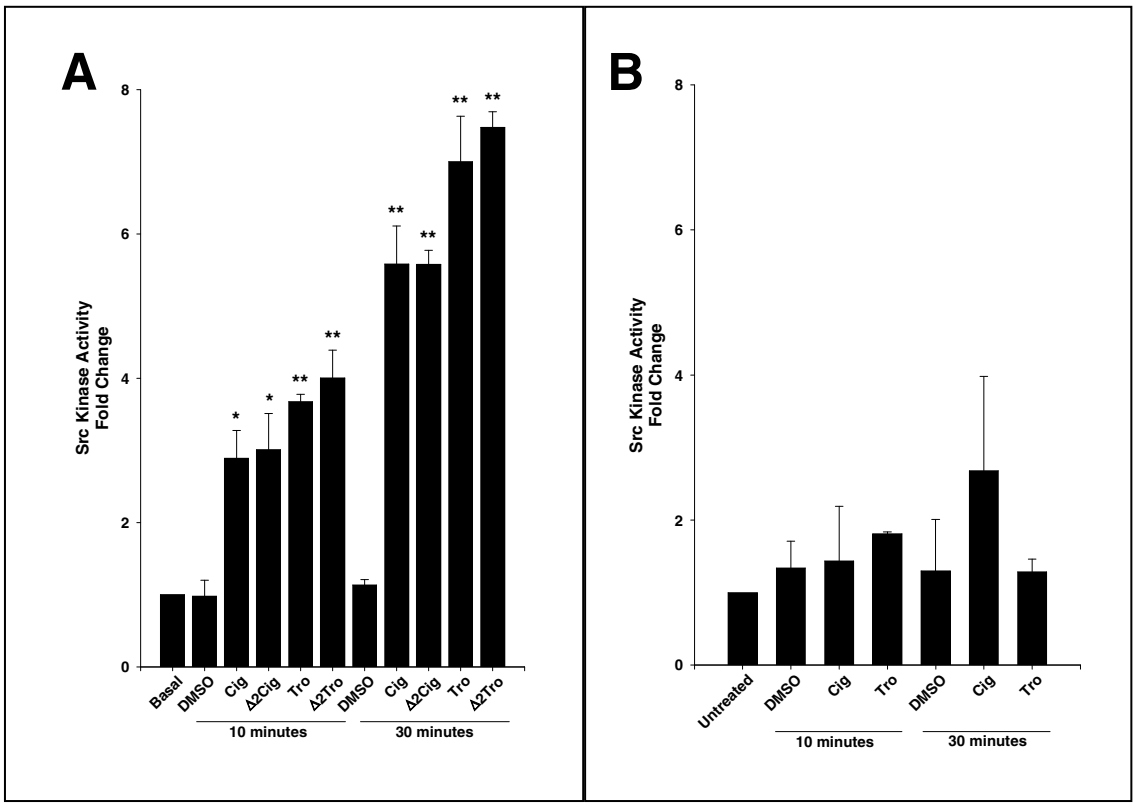

Figure 3.2. TZDs increase Src kinase activity. Src kinase activity was measured as described in MATERIALS AND METHODS. **A**, cells were incubated with 50 μM ciglitazone, troglitazone, or their respective Δ2-derivatives for the time indicated. Src kinase activity was determined by immunoprecipitating endogenous Src from 200 μg of total protein from treated lysates. **B**, Src was immunoprecipitated from 200 μg of total protein from unstimulated serum starve GN4 cells. Immuncomplexes were incubated in the presence of DMSO, 50 μM ciglitazone or troglitazone for the times indicated. Results are mean ± SEM (n=3). * $p< 0.05$, ** $p< 0.001$ for comparison with respective DMSO vehicle control by ANOVA with post hoc comparisons using Tukey's multiple comparisons test.

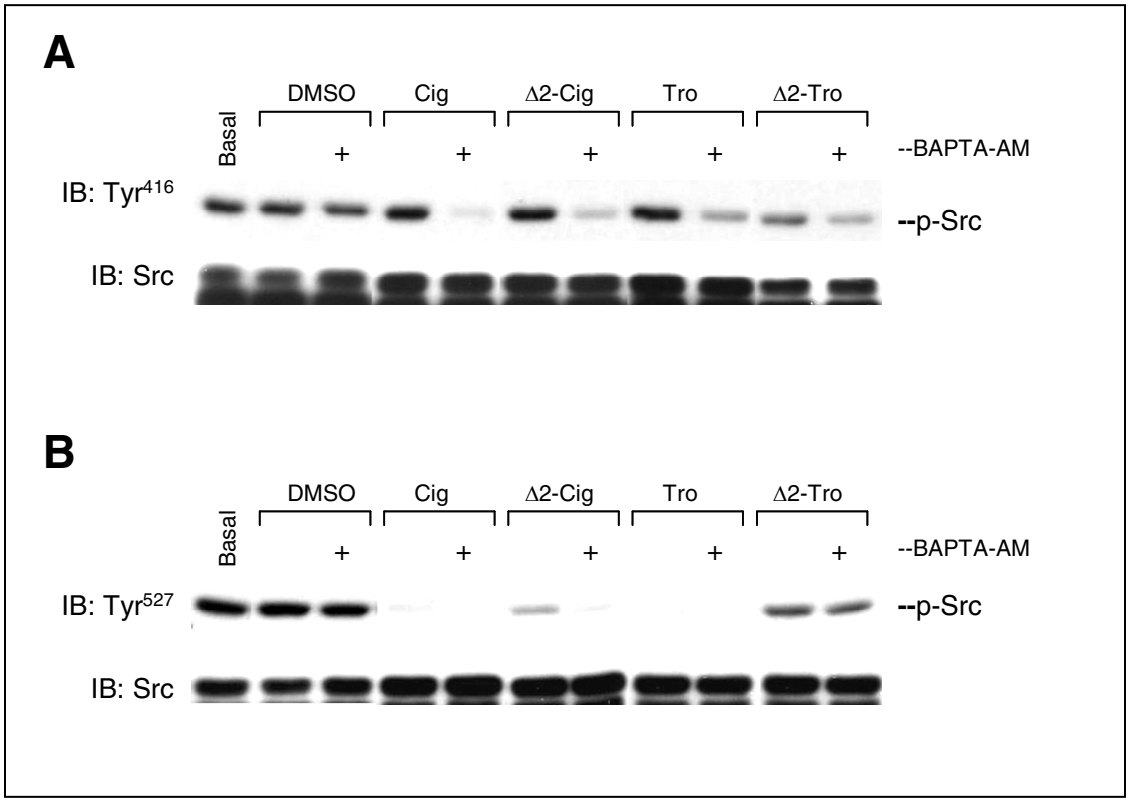

Figure 3.3. BAPTA-AM lowers Src Tyr^{416} phosphorylation, but does not affect Src Tyr^{527} dephosphorylation by TZDs. Serum-starved GN4 cells were pretreated 20 minutes with 50 μM BAPTA-AM followed by treatment with 50 μM ciglitazone, troglitazone or their Δ2-derivatives for 10 minutes and 30 minutes. Lysates were prepared and subjected to SDS-PAGE. **A**, lysates from 10 minute treated samples were immunoblotted (IB) for Src Tyr^{416} phosphorylation. **B**, lysates from 30 minute treated samples were IB for Src Tyr^{527}. Blots were stripped and reprobed for total protein to determine equal loading.

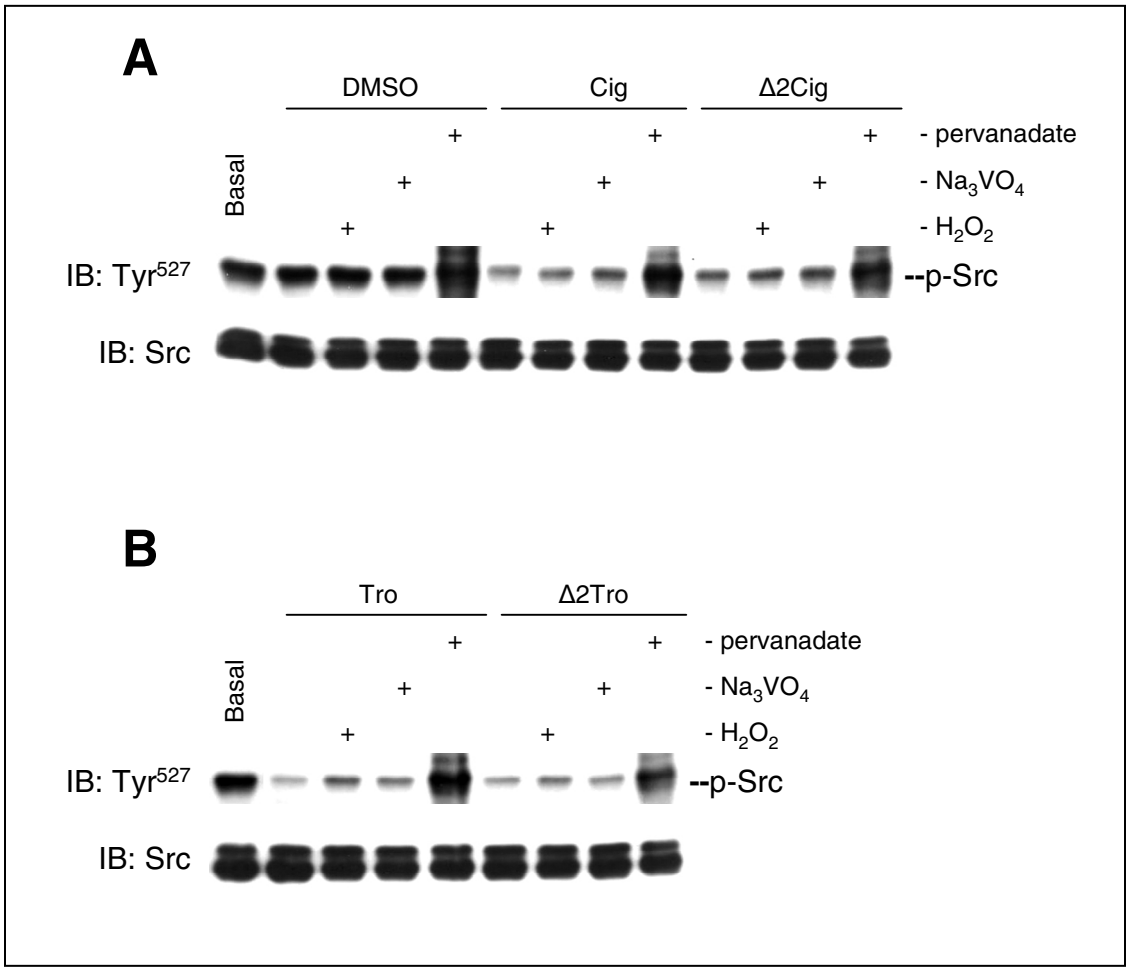

Figure 3.4. Inhibition of PTPase activity prevents loss of Src Tyr^{527} phosphorylation. Cells were grown to confluence, serum-deprived overnight and then pretreated for 5 min with either 50 μM H_2O_2, sodium orthovanadate (Na_3VO_4) or pervanadate followed immediately by a 30 min treatment with (**A**) 50 μM ciglitazone (Cig), Δ2-ciglitazone (Δ2Cig), or (**B**) troglitazone (Tro), Δ2-troglitazone (Δ2Tro) or 0.1% DMSO (vehicle control). Cell lysates were prepared and subjected to 10% SDS-PAGE. Src tyrosine phosphorylation was determined by immunoblotting (IB) using anti-phospho-Src Tyr^{527} antibody. The blots were stripped and reprobed using antibodies directed against total Src.

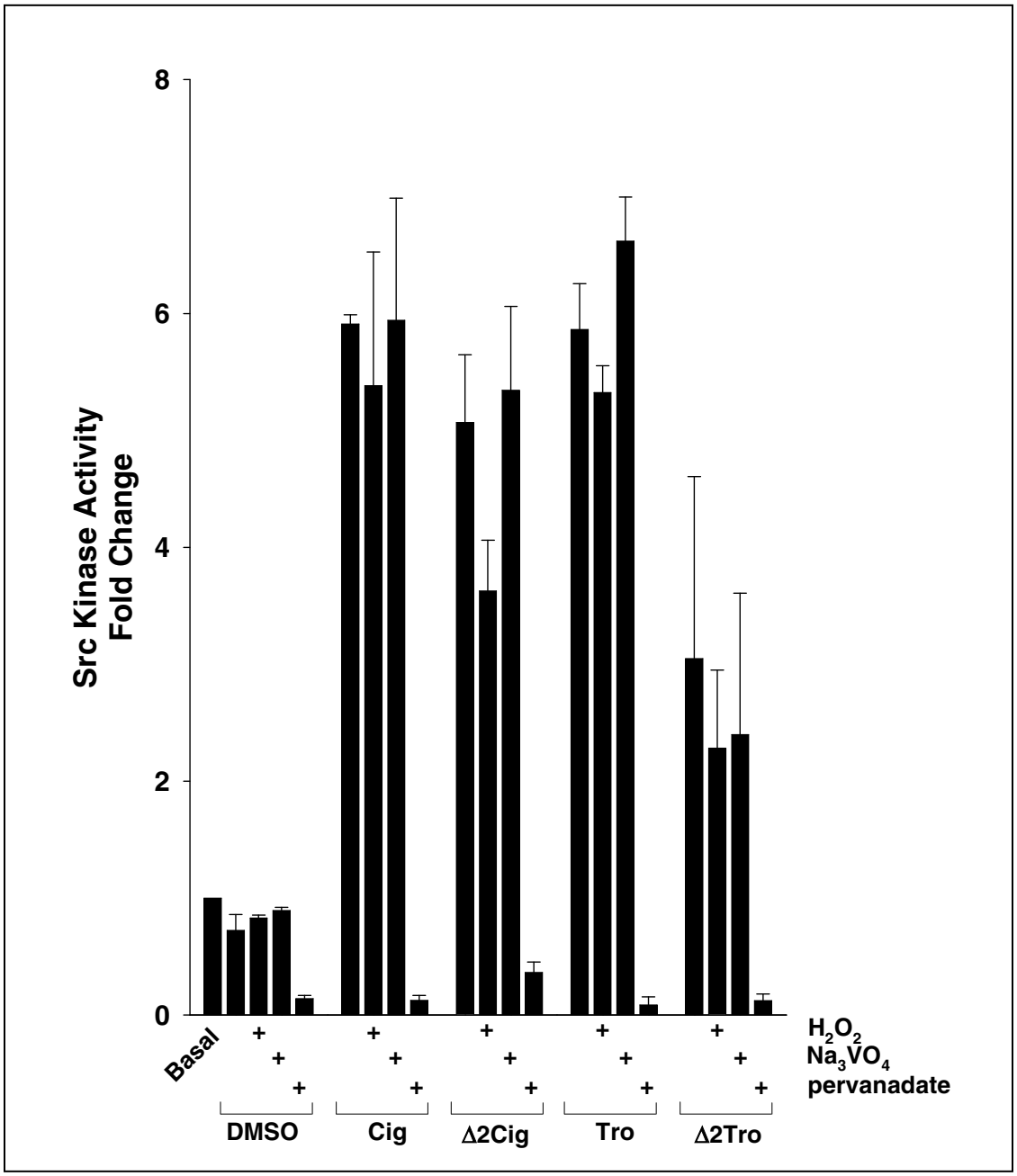

Figure 3.5. Pervanadate blocks TZD-induced Src kinase activity. Cells were grown to confluence, serum-deprived overnight and then pretreated for 5 min with either 50 μM H_2O_2, sodium orthovanadate (Na_3VO_4) or pervanadate followed immediately by 30 min with 50 μM ciglitazone (Cig), Δ2-ciglitazone (Δ2Cig), troglitazone (Tro), Δ2-troglitazone (Δ2Tro) or 0.1% DMSO. Endogenous Src was immunoprecipitated from prepared lysates and kinase activity in immuncomplexes was determined as described in MATERIALS AND METHODS. Results are mean ± SEM (n=3).

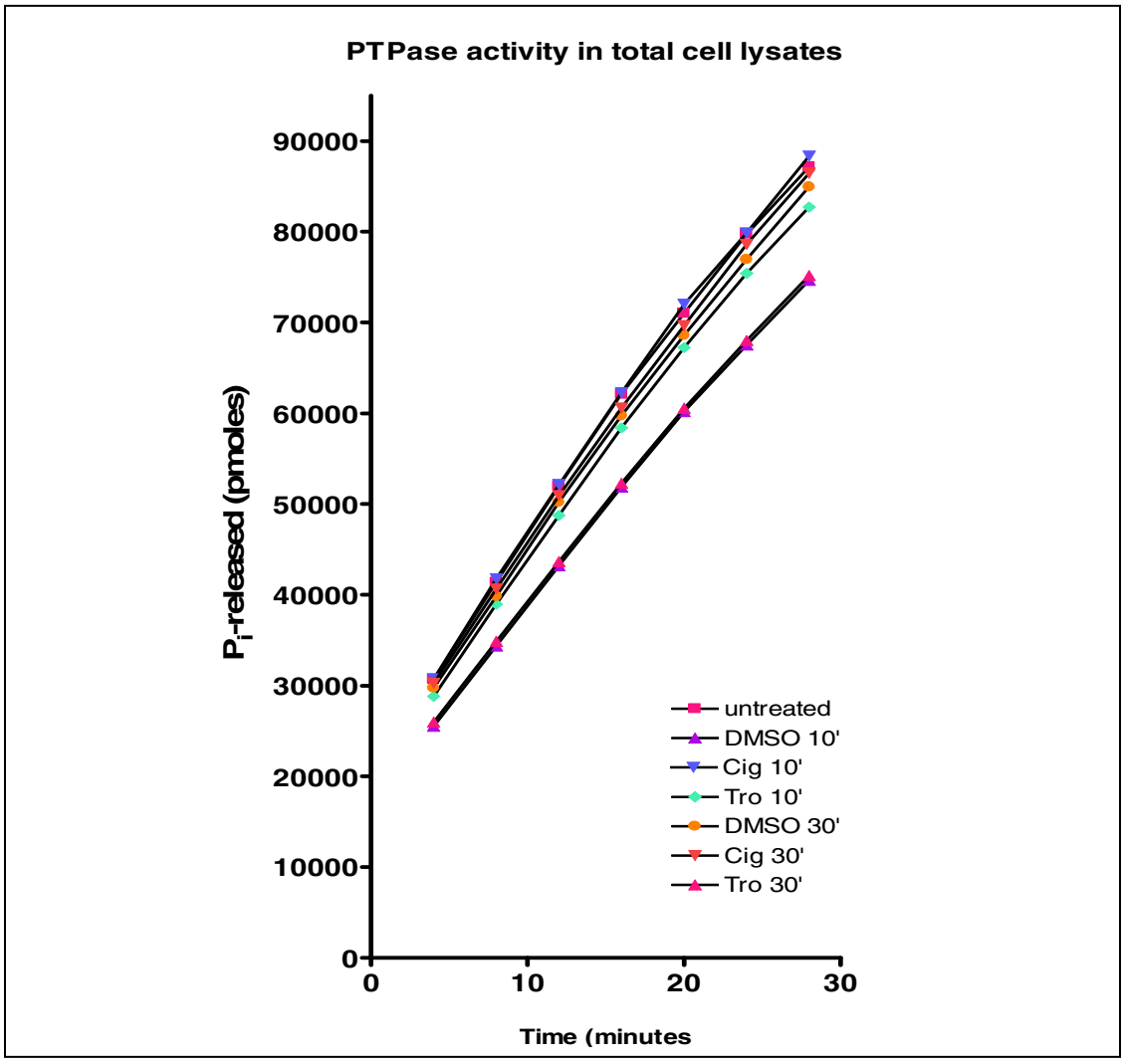

Figure 3.6. TZDs do not increase general PTPase activity. GN4 cells were serum-starved and then treated with 50 μM ciglitazone or troglitazone for 10 and 30 minutes, respectively. Cell lysates were prepared as previously described except that vanadate was left out of the lysis buffer. Approximately 50 μg total protein were incubated

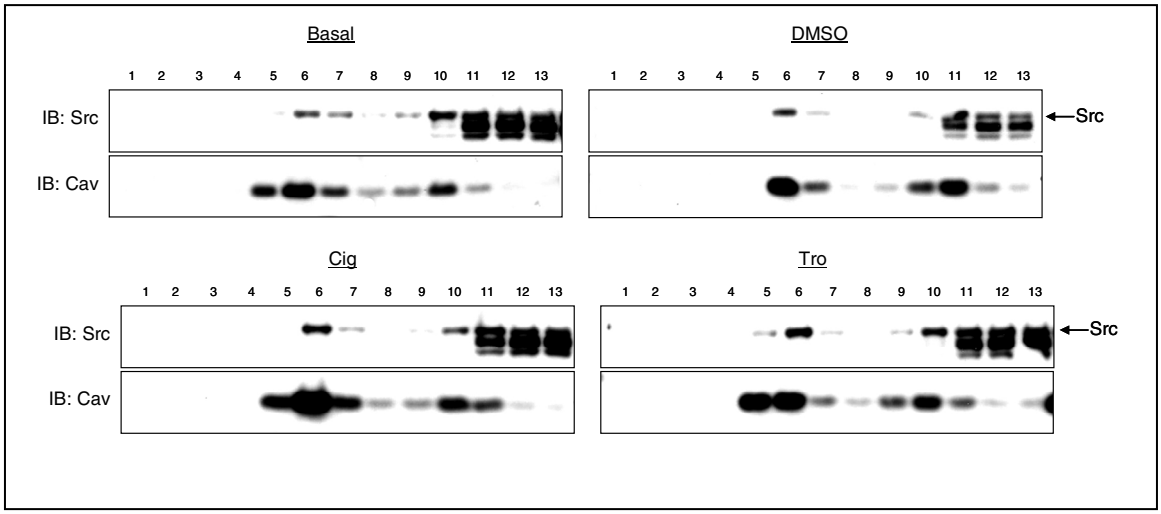

Figure 3.7. Effect of TZD on relocalization of Src in lipid rafts. GN4 cells were grown to confluence in 300-cm^2 flasks and then serum-starved overnight. Each flask was treated with 50 μM ciglitazone or troglitazone for 30 min; DMSO was used as a vehicle control. Cell lysates for detergent insoluble fractions were prepared as describe in MATERIALS AND METHODS. Lysates from each sample were diluted into an 80% sucrose solution 1:1 and then layered with 30% and 5% sucrose in centrifuge tubes. Samples were centrifuged for 18 hr at 40,000 x g at 4ºC. Equal volume fractions were collected sequentially from top to bottom, 1 representing the top or first fractions collected, using a gradient fractionator and then subjected to SDS-PAGE. Immunoblots (IB) were probed for total Src protein and caveolin-1 (Cav).

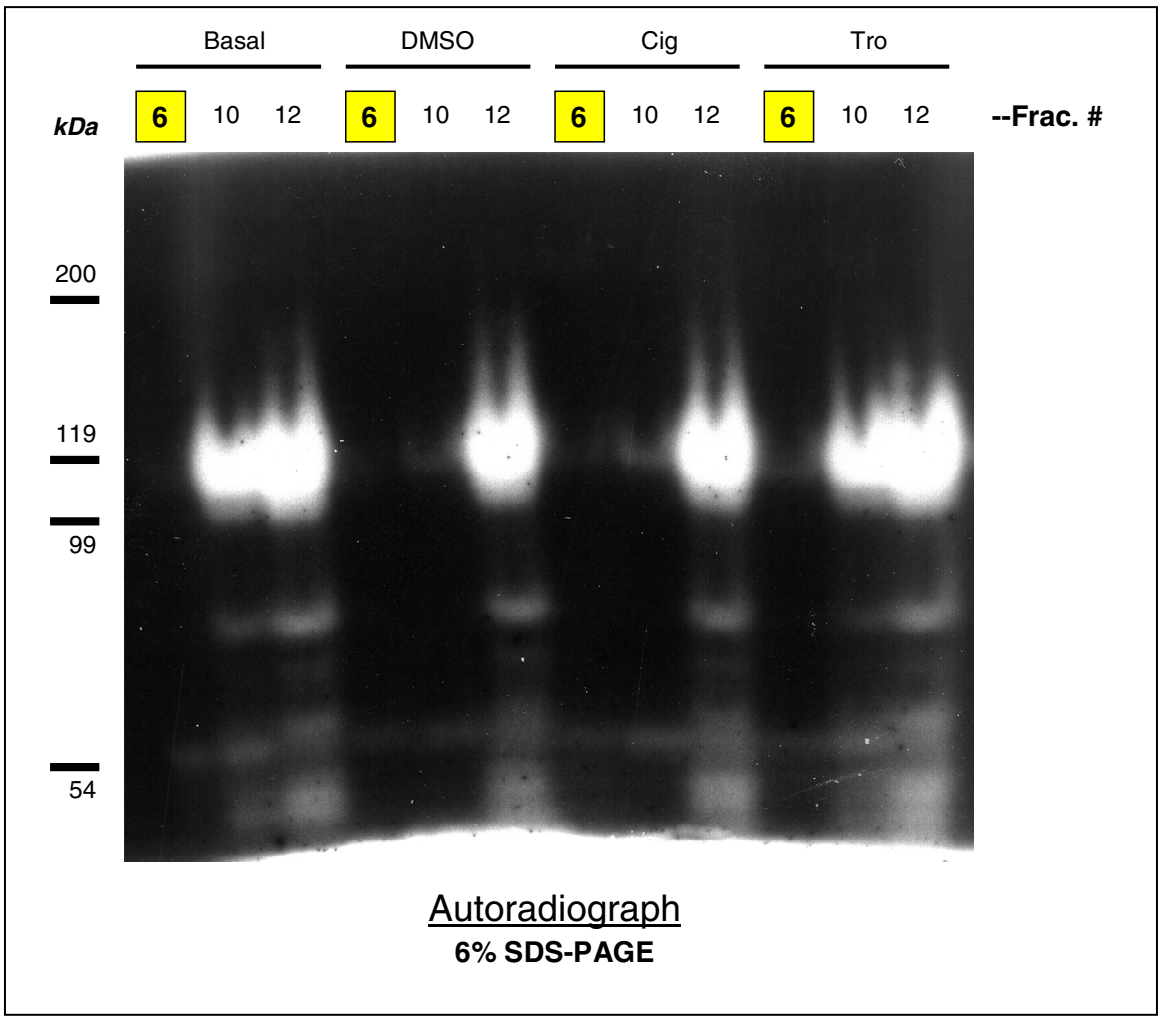

Figure 3.8. Localization of PTPase activity in raft and non-raft fractions from TZD treated cells. Raft (6) and non-raft fractions (10 and 12) were separated by 6.0% SDS-PAGE impregnated with [^{32}P]-PolyGlu:Tyr(4:1). IN-GEL phosphatase activity assays were carried out as described in MATERIALS AND METHODS.

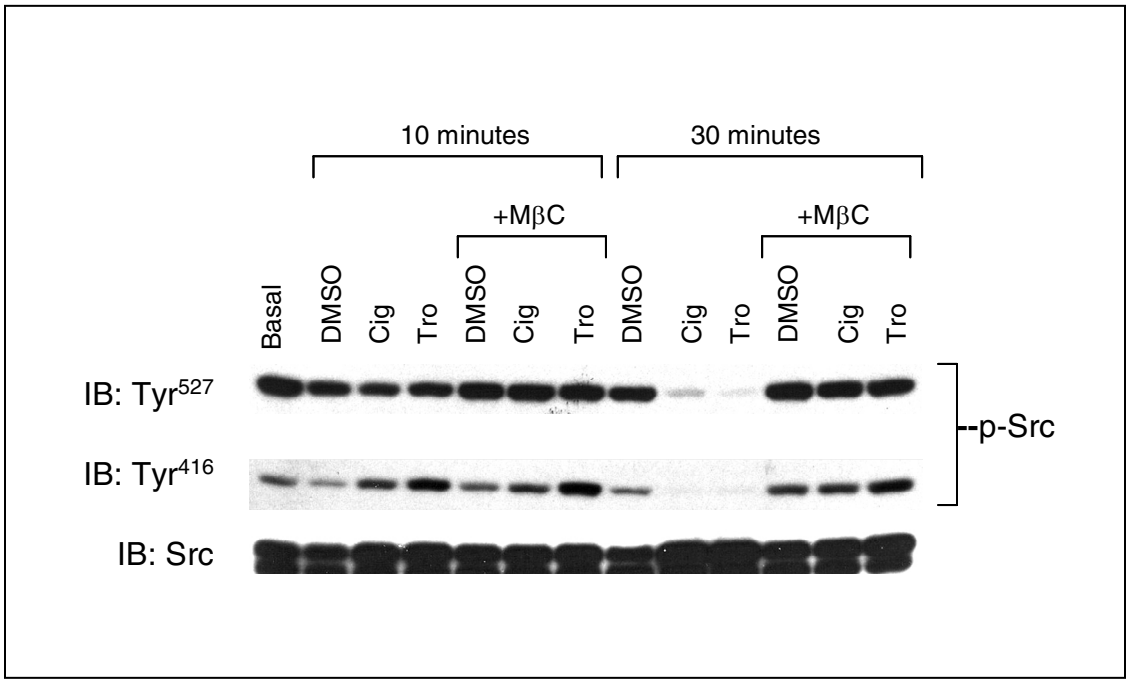

Figure 3.9. Disruption of lipid rafts with methyl-β-cyclodextrin prevents TZD-induced changes in Src Tyr^{527} dephosphorylation. GN4 cells were serum-starved overnight followed by pretreatment in 1.0% methyl-β-cyclodextrin (MβC) for 30 min. The cells were then washed 2X with 0.1% FBS containing media to remove any excess MβC, followed by stimulation with 50 μM ciglitazone or troglitazone for 10 and 30 min; DMSO was used as a vehicle control. Lysates were prepared and subjected to SDS-PAGE. Src phosphorylation was determined by immunoblot (IB) using either an anti-phospho-Src Tyr^{416} or Tyr^{527} antibodies. Blots were stripped and reprobed with total Src to determine equal protein loading.

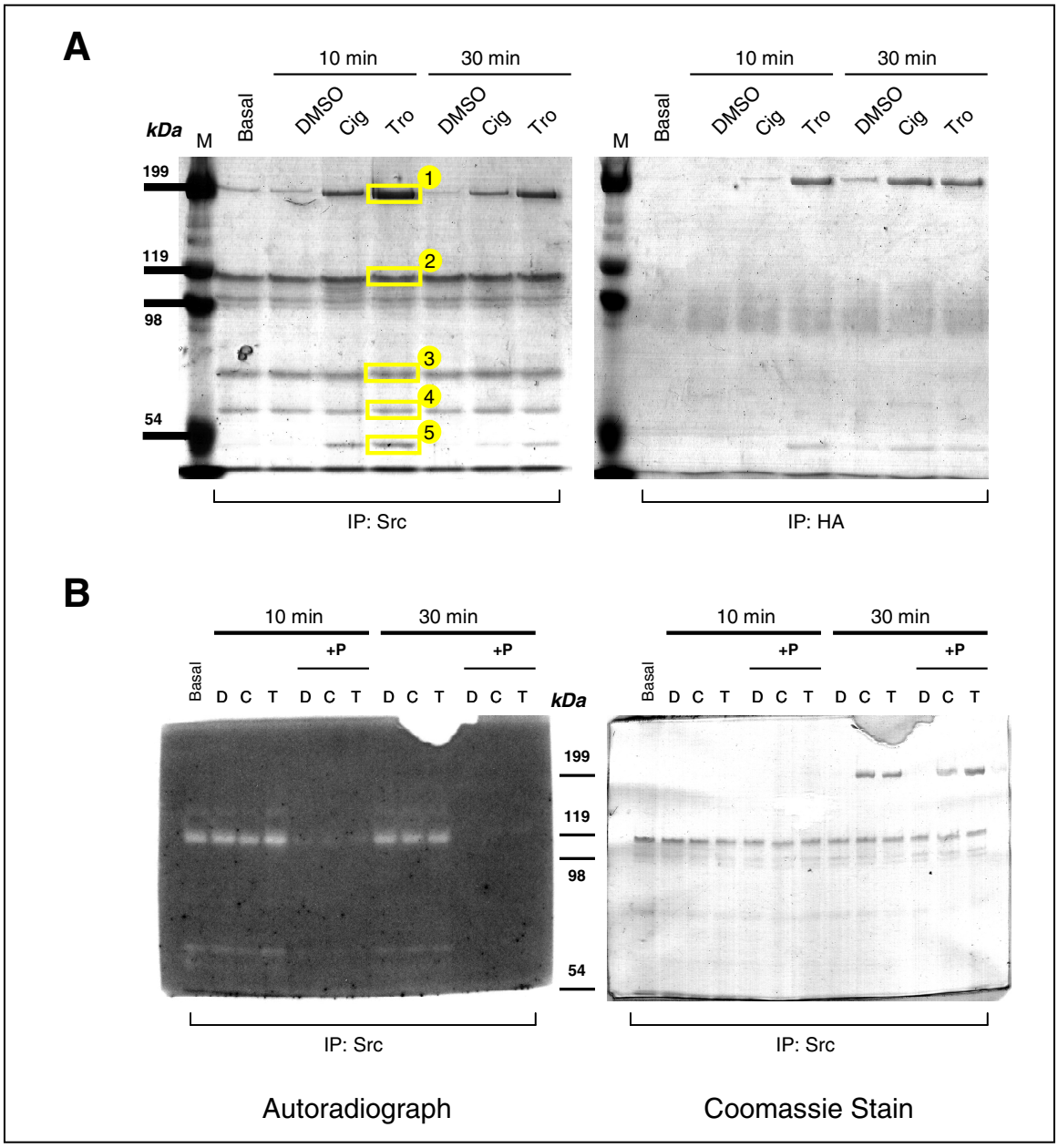

Figure 3.10. Src associated proteins from TZD treated cells. Confluent, 24 hr serum-starved cells were treated with 50 μM ciglitazone (Cig or C) or troglitazone (Tro or T) for 10 and 30 min; DMSO (D) served as a vehicle control. Some samples were treated for 5 min with pervanadate (+P) prior to TZD exposure. Cell lysates were prepared. **A**, 400 μg total protein were immunoprecipitated (IP) with either Src or HA antibodies. Immunecomplexes were subjected to 6% SDS-PAGE followed by coomassie staining to visualize proteins. **B**, Src immunecomplexes were separated by 6.0% SDS-PAGE impregnated with [^{32}P]-PolyGlu:Tyr(4:1) and IN-GEL PTPase activity assays were preformed as described in MATERIALS AND METHODS. Gels were further coomassie stained to visualize proteins.

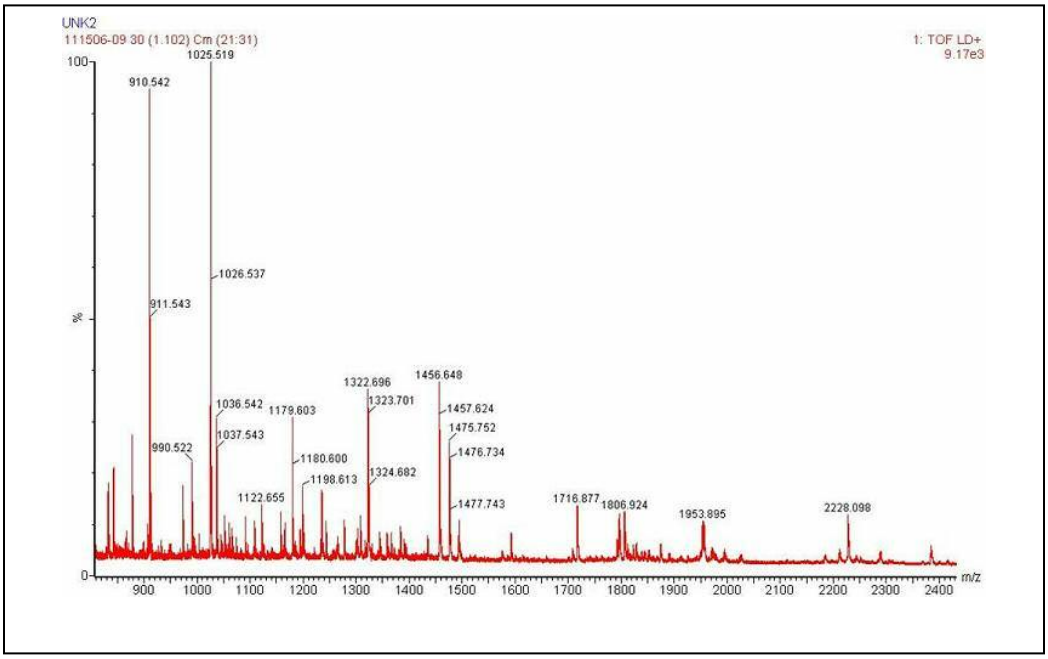

Figure 3.11. Peptide Mass Fingerprint of Src-associated PTPase (Band #2). Numbered bands were excised from gels followed by removal of coomassie stain in 10 mM ammonium bicarbonate and 40% acetonitrile (ACN). Gel pieces were evaporated to dryness in a speed-vac centrifuge followed by rehydration in sequencing grade trypsin. Trypsin digests were carried out by an overnight incubation at 24ºC and peptides were extracted by passive diffusion in 20 mM ammonium bicarbonate. Peptides were purified using PerfectPure C18 tips (Eppendorf) and spotted onto MALDI target plate. Spectra were generated using MALDI-TOF micro MX (Waters).

TOP 70 PEAKS (by intensity) from MS SPECTRA

1	1025.56		36	1090.5831	
2	910.5747		37	1165.6204	*keratin*
3	1456.693	r.ERHFEIPDEER.c	38	1052.5453	k.MGNKGGVAMR.f
4	1322.737		39	831.5295	r.EKEALNK.e
5	1475.781	*keratin*	40	2211.2068	*trypsin*
6	1179.633	*keratin*	41	1434.8009	*keratin*
7	2227.12	r.GIRWQSAAPQSLEAALMEPR.i	42	1344.7211	
8	1716.956	*keratin*	43	1107.5919	
9	1805.994		44	1365.7015	k.MNTQNPPTGIHR.e
10	1036.549	k.MGNKGGVAMR.f	45	1994.0549	
11	1796.026		46	1193.6472	*keratin*
12	1952.969		47	1060.6169	*keratin*
13	1198.651		48	1390.6967	k.FDQLNIQRTQK.k
14	1234.696	*keratin*	49	1968.9686	
15	877.551	k.VFEDIVR.i	50	1852.0239	
16	990.5622		51	2510.1479	
17	1493.77	k.KMNTQNPPTGIHR.e	52	1265.6937	
18	1308.685		53	2025.9895	
19	2384.058		54	1949.9774	r.MSFSVPNQTLPQVNIMK.h
20	973.5897	*keratin*	55	1232.6219	
21	1122.668		56	2184.1099	
22	1383.703	*keratin*	57	1316.5767	
23	842.6	trypsin	58	1889.9742	
24	1277.725		59	2243.1094	
25	1157.624	r.EPCVLTLARR.n	60	1300.6096	
26	1791.872		61	1707.8855	k.LIDLEEDSFLEKEK.s
27	1243.574		62	1066.5662	*keratin*
28	1873.993		63	1925.115	
29	1591.798		64	1003.6125	
30	2288.156		65	1045.6046	
31	1972.064	(-).MCPFVLLQYDTRQTLK.s	66	807.4896	
32	1302.711		67	1263.6853	
33	1828.004		68	1574.7889	r.EPPPPSSNRMLPR.e
34	1821.989		69	1867.9636	
35	1357.733	r.CLDSAHDPRICK.q			

TABLE 3.1. MASCOT SEARCH RESULTS. MS peaks were searched using MASCOT peptide mass fingerprint (Matrix Science). A putative candidate PTPase protein was identified as **phosphatidylinositol polyphosphate 5-phosphatase** (Accession: XP_229106) from rat. Calculated molecular weight = 119,669 Da with a pI value of 8.77. The yellow highlighted m/z peak values correspond to the shown peptide sequences found in the identified protein.

Sample	Protein ID
1	Myosin heavy chain 10
2	**Phosphatidylinositol polyphosphate-5 phosphatase**
3	IgG (heavy chain)
4	tubulin
5	actin

Table 3.2. Src associated proteins identified by mass Spec. Bands of interest were excised from gels and destained in 10 mM ammonium bicarbonate and 40% acetonitrile (ACN) with vigorous shaking. Gel pieces were evaporated to dryness followed by rehydration in sequencing grade trypsin; peptides were extracted by passive diffusion in 20 mM ammonium bicarbonate. Peptides were purified using PerfectPure C18 tips (Eppendorf). Peptides bound to C18 tips were eluted and spotted directly onto the MALDI MS target plate using the elution solution (0.1% TFA, 70% ACN, and saturated a-cyano-4-hydroxycinnamic acid) and allowed to air dry. The MALDI-TOF micro MX (Waters) mass spectrometer operating in positive ion mode was used to determine the mass/charge ratio of the peptides in each sample and generate spectra that could then be analyzed by peptide mass fingerprinting. Peptide mass fingerprinting was performed using Mascot (Matrix Science) to identify proteins in samples.

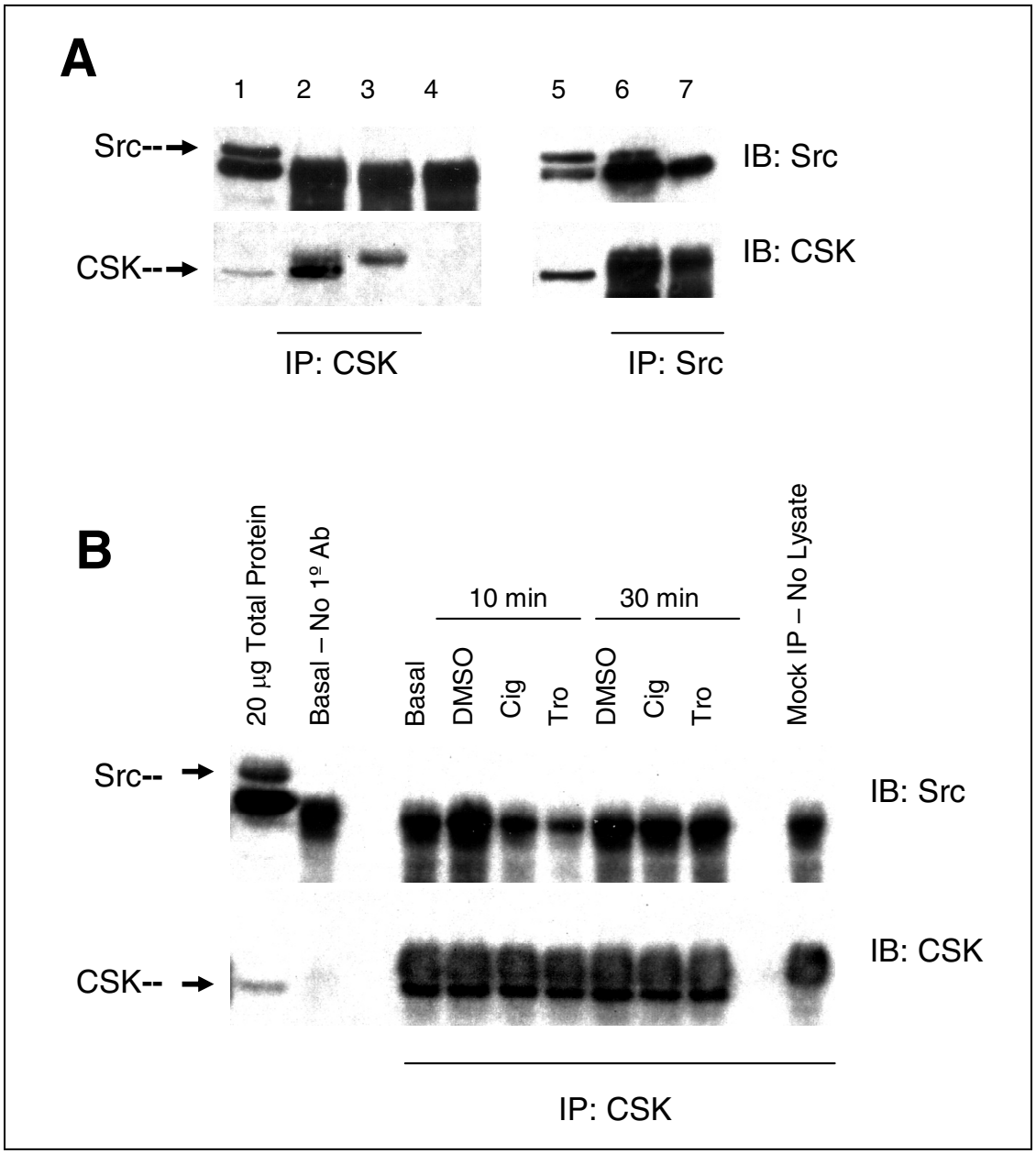

Figure 3.12. Src and C-terminal Src Kinase association. Lysates were prepared from unstimulated (basal), DMSO, ciglitazone (50 μM) or troglitazone (50 μM) treated serum-starved GN4 cells for 10 and 30 min. **A**, CSK or Src was immunoprecipitated (IP) from unstimulated cells; lanes 1 and 5 are 20 μg of total cell lyate, 2 and 6 are immunoprecipitates from 500 μg total protein with CSK or Src, respectively, lane 3 and 7 are no lysate controls, and lane 4 contains no CSK primary anitbody. **B**, CSK was immunoprecipitated from treated or untreated samples and then immunoblotted (IB) with either anti-Src or anti-CSK. Total cell lysates were run to show effectiveness of IB.

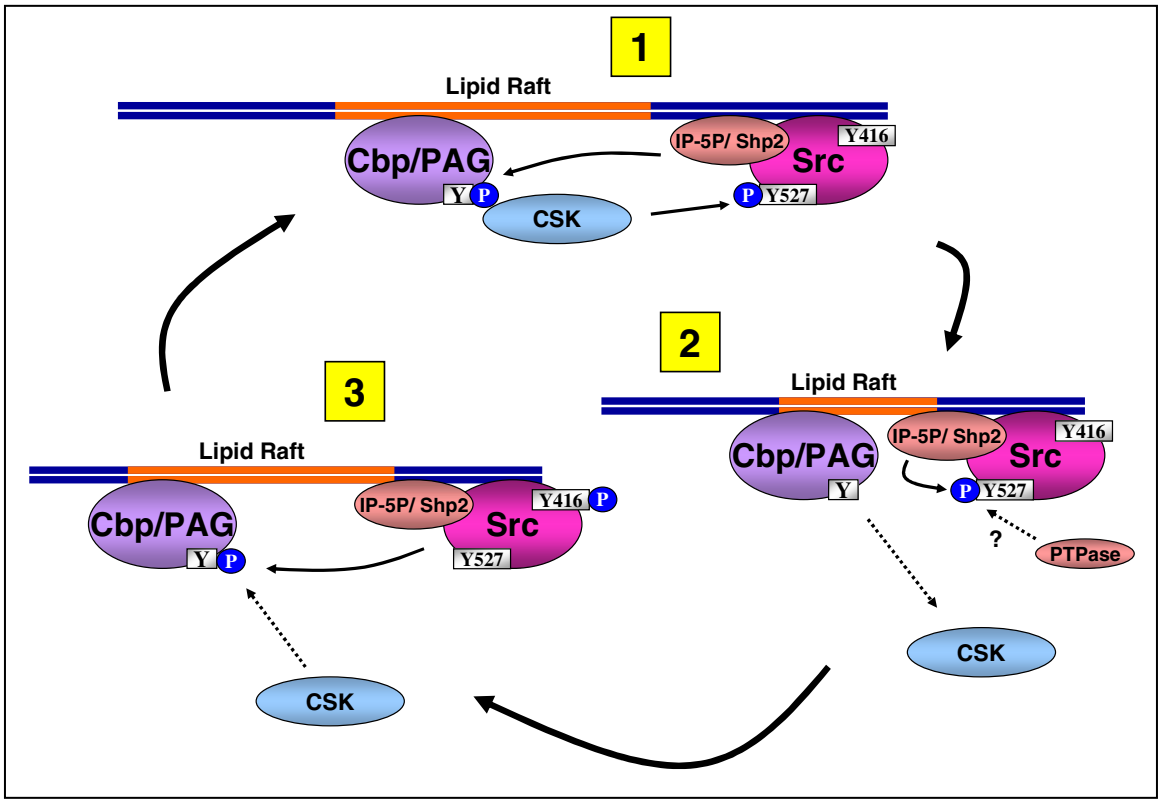

Figure 3.13. Model of TZD mediated Src activation. We have observed the association of a putative Src-PTPase, phosphatidylinositol polyphosphate-5 phosphatase, which is related to another known Src-PTPase, Shp2. TZDs mediated the activation of Src kinase, by influencing known phosphorylation sites important in increasing Src kinase activity. Dephosphorylation of Tyr^{527}, the negative regulatory site was prevented by PTPase inhibition. However, TZDs neither increased PTPase activity nor association of the PTPase with Src. Thus, it appears an additional mechanism may be involved. A proposed model would be related to Shp2 mediated Src kinase activation. Here CSK maintains Src Tyr^{527} due to a phospho-tyrosine mediated association with Cbp, an adapter protein traditionally localized in lipid rafts. The Src associated PTPase (IP-5P/ Shp2) has been shown to mediate Cbp dephosphorylation (**1**). This induces loss of CSK association from Cbp and renders the Src Tyr^{527} phosphorylation site susceptible to either IP-5P/Shp2 or an unknown PTPase-mediated dephosphorylation and subsequent Src kinase activation (**2**). Interestingly, Cbp serves as a Src substrate; thus as Src activity increases it becomes susceptible to Src-mediated tyrosine phosphorylation (**3**). This phosphorylation redirects CSK to Cbp thereby promoting CSK mediated Src Tyr^{527} phosphorylation and inhibition of kinase activity (**1**).

CHAPTER IV

Capacitative calcium entry contributes to the differential transactivation of the epidermal growth factor receptor in response to Thiazolidinediones

A. ABSTRACT

Src-dependent epidermal growth factor receptor (EGFR) transactivation has been previously shown to be an important mechanism regulating the activation of Erk in response to the thiazolidinedione (TZD) ciglitazone. Interestingly, another TZD family member, troglitazone, effectively mediated Src kinase activation, but failed to induce either Erk activation or EGFR transactivation. This suggested the involvement of additional mechanisms regulating EGFR transactivation. Here we show that neither inhibition of EGFR kinase activity nor Pyk2 activation affected the ability of either TZD to mediate Src kinase activation. However, inhibition of Pyk2 phosphorylation lowered ciglitazone-induced EGFR transactivation. Inhibition of Src kinase activity prevented Pyk2 activation by either compound suggesting that the differential mechanism responsible for EGFR transactivation was downstream of these kinases. Intracellular calcium chelation reduced ciglitazone-induced EGFR transactivation, suggesting a role for calcium. Interestingly, both TZDs induced a rapid and transient increase in cytosolic calcium concentrations indicative of intracellular store release. However, only ciglitazone produced a secondary, more sustained calcium influx in the presence of extracellular calcium. Removal of extracellular calcium or inhibition of capacitative calcium entry (CCE) by 2-APB prevented ciglitazone-induced EGFR transactivation and Erk activation, but did not affect upstream kinase signaling pathways. These results demonstrate that upstream kinases (i.e. Src and Pyk2) are required, but not sufficient elements involved in EGFR transactivation. Moreover, these data suggest that extracellular calcium influx through CCE may be an unrecognized novel component involved in EGFR transactivation and a mechanism that explains the differential effects observed with different TZD compounds.

B. INTRODUCTION

Recent work from our lab and others has shown that TZDs, ligands of the nuclear transcription factor PPARγ (Lehmann et al., 1995) and used to reverse hyperglycemia in type II diabetes, mediate the activation of MAPKs, important intracellular signal proteins playing significant roles in coordinating a variety of cellular processes such as cell growth and differentiation, and under some conditions cellular apoptosis (reviewed in (Gardner et al., 2005a)). Specifically, the TZDs ciglitazone and troglitazone affected two distinct kinase signaling cascades culminating in the activation of either Erk or p38 MAPKs. These effects were maintained by structural derivatives lacking the ability to activate PPARγ, demonstrating a PPARγ-independent mechanism (Gardner et al., 2005b). Interestingly, while both ciglitazone and troglitazone were effective activators of the p38 pathway, only ciglitazone was shown to mediate Erk activation, an event requiring Src kinase-mediated EGFR transactivation (Gardner et al., 2003); however, both ciglitazone and troglitazone effectively activate Src kinase. The underlying mechanism, which must be subsequent to Src kinase activation, involved in the differential effects of ciglitazone and troglitazone on EGFR transactivation and Erk activation, has not been examined.

The receptor tyrosine kinase EGFR family consists of four members, ErbB1 (EGFR), ErbB2, ErbB3 and ErbB4 (Ushiro and Cohen, 1980). The prototypical member of this family, the EGFR, is a single membrane spanning glycoprotein of 1186 amino acids and is expressed in many different cell types (Ullrich et al., 1984; Wells, 1999). EGFR ligands, including EGF, TGFα, betacellulin, HB-EGF, epiregulin, and amphiregulin, induce receptor homo- or heterodimerization, which leads to activation of its intrinsic tyrosine kinase activity. This results in the autophosphorylation of multiple tyrosine residues in the C-

terminal tail region of the protein, sites that serve as binding sites for cytosolic adaptor proteins that lead to activation of downstream kinase signaling cascades (Ullrich and Schlessinger, 1990). These signaling cascades have been shown to affect cell migration and differentiation, adhesion, proliferation and apoptosis (Carpenter et al., 1978; Prenzel et al., 2001b). However, receptor activation can also occur in the absence of physiological ligands via a mechanism termed EGFR 'transactivation'. Proteolytic cleavage of EGF-like ligands by MMPs, nonreceptor tyrosine kinases, stress factors, cell adhesion, G protein-coupled receptors and cytokine receptor have all been associated with EGFR transactivation (Wetzker and Bohmer, 2003). The EGFR has emerged as a critical transducer in many different intracellular signal transduction pathways and therefore the mechanism(s) regulating transactivation are important to understand.

Cytosolic calcium is a well known second messenger and regulates many processes including exocytosis, contraction, cellular metabolism and signaling pathways controlling gene expression, cell proliferation and apoptosis. Cells increase cytosolic calcium by either releasing compartmentalized calcium from intracellular stores, such as the ER or mitochondria, or by regulated influx from the extracellular space. Intracellular store release is transient and many processes require a sustained increase. A key cellular process for sustained intracellular calcium mobilization in non-excitable cells is CCE (Putney, 1986). This process involves transient depletion of internal ER calcium stores followed by a sustained and highly specific calcium influx or calcium-release-activated calcium current, I_{crac}, across the plasma membrane (Hoth and Penner, 1992). The secondary influx of calcium helps replenish ER calcium stores and can serve as an activator of many kinases important in signal transduction.

In this study we examined mechanisms contributing to the differential transactivation of the EGFR in response to TZDs ciglitazone and troglitazone. While both ciglitazone and troglitazone mediate the activation of upstream kinases necessary in EGFR transactivation, we demonstrate here that ciglitazone, but not troglitazone induces a large secondary calcium influx from extracellular sources. Both removal of extracellular calcium and the CCE inhibitor 2-APB prevented ciglitazone induced EGFR transactivation and Erk activation. Interestingly, activation of Src or Pyk2 was not affected by extracellular calcium removal, but their activation in response to both TZDs could be prevented by intracellular calcium chelation. Collectively these data suggest both ciglitazone and troglitazone modulate the required signals upstream of EGFR transactivation, but that only ciglitazone is capable of inducing EGFR transactivation through modulation of extracellular calcium influx via CCE.

C. RESULTS

1. EGFR kinase activity is not involved in Src activation by TZDs

Previous data show that ciglitazone and troglitazone activate Src kinase (Chapter III). Src has been shown to directly bind the EGFR leading to transactivation of the receptor through phosphorylation of two tyrosine residues, distinct from known EGFR tyrosine autophosphorylation sites (Biscardi et al., 1999). Conversely, other studies suggest that Src, through interactions of its SH2 domain, can interact with autophosphorylated EGFR; EGF-stimulated Jak/STAT activation was shown to require Src kinase activity and others showed EGF-stimulation of the EGFR was capable of Src activation through the GTPase Ral (Goi et al., 2000; Olayioye et al., 1999). As the EGFR can serve as an upstream activator of Src, we examined the role of EGFR in Src activation following TZD treatment. GN4 cells were pretreated with the EGFR kinase inhibitor PD153035 and Src

Tyr^{416} phosphorylation under these conditions was examined. Similar to previous data, both ciglitazone and Δ2-ciglitazone treatment increased EGFR phosphorylation and this was blocked by PD153035 (data not shown) (Gardner et al., 2003). However, PD153035 pretreatment did not affect the ability of ciglitazone or troglitazone to increase Src Tyr^{416} phosphorylation (Figure 4.1A). PD153035 was affective at blocking EGF-stimulated phosphorylation of the EGFR (Figure 4.1B). These data show that EGFR kinase activity was not required for TZD-induced Src activation, and support our previous work indicating EGFR as a downstream target of Src kinase (Gardner et al., 2003).

2. Pyk2 phosphorylation is mediated by Src activation

The non-receptor, calcium-dependent, praline-rich tyrosine kinase, Pyk2, has been implicated in ligand-independent EGFR transactivation (Shah et al., 2003) and has been shown to play a role in the activation of Src (Dikic et al., 1996). We therefore examined the role of Pyk2 in mediating both Src activation and EGFR phosphorylation. To prevent Pyk2 activation, cells were infected with either adenovirus containing a C-terminal inhibitory form of Pyk2 (Ad.CRNK) or green fluorescent protein (Ad.GFP) to serve as a negative control. As an alternative potential splice variant of Pyk2 (Schaller and Sasaki, 1997), Ad.CRNK expression negatively regulates endogenous Pyk2 autophosphorylation (Li et al., 1999). Addition of 8 x 10^6 plaque forming units/mL (the concentration used in these studies) effectively blocked Ang II-dependent Pyk2 phosphorylation to basal levels (data not shown). As expected, Ad.CRNK over-expression lowered both ciglitazone and troglitazone induced Pyk2 phosphorylation (Figure 4.2A). In contrast to evidence supporting a role for Pyk2 in mediating Src activation, Ad.CRNK over-expression did not alter the increase in Src Tyr^{416} phosphorylation following treatment of cells with

ciglitazone or troglitazone (Figure 4.2B). We further examined EGFR tyrosine phosphorylation under these conditions. As expected, troglitazone did not affect EGFR Tyr^{845} or Tyr^{1068} phosphorylation; however, ciglitazone increased EGFR phosphorylation on both of these tyrosine residues and this was blocked to near basal levels by Ad.CRNK (Figure 4.2B). Neither Ad.GFP nor Ad.CRNK affected EGF-stimulated EGFR phosphorylation (data not shown).

Because inhibition of Pyk2 had little effect of TZD-stimulated Src activation, we alternatively examined whether Src was required for Pyk2 activation in response to TZD treatment. When cells were pretreated with the Src kinase inhibitor PP2, ciglitazone and troglitazone-induced increases in Src Tyr^{416} phosphorylation were reduced to basal levels, but were unaffected by the inactive analogue, PP3 (Figure 4.3A). Under these conditions, PP2 similarly lowered the increase in Pyk2 phosphorylation following incubation of cells with either ciglitazone or troglitazone (Figure 4.3B). Collectively these data show that Pyk2 tyrosine phosphorylation is mediated by Src activation and additionally, suggest a potential role of Pyk2, in addition to the requirement of Src, in EGFR phosphorylation and transactivation.

3. Influence of ciglitazone and troglitazone on intracellular calcium mobilization

A rapid increase in Src kinase activity was observed in keratinocytes following treatment with either a high concentration of extracellular calcium or ionophore (Zhao et al., 1992); Pyk2 kinase is regulated by stimuli that increase intracellular calcium (Li et al., 1999). Further, Src Tyr^{416} phosphorylation and Pyk2 activation were prevented by intracellular calcium chelation. Therefore, modulation of intracellular calcium could affect the ability of TZDs to activate signaling events. We investigated the ability of

ciglitazone and troglitazone to mobilize calcium when cells were incubated in HBSS containing no calcium. GN4 cells were loaded with Fura-2 AM and then challenged with 50 μM ciglitazone or troglitazone, first in the absence of extracellular calcium. Under these conditions both ciglitazone and troglitazone increased intracellular calcium concentrations to a peak of approximately 150 nM (Figure 4.4); this influx of calcium was both rapid and transient and suggested that both ciglitazone and troglitazone mediated intracellular store depletion. We next examined the effects of ciglitazone and troglitazone on calcium influx in HBSS media containing 1.0 mM calcium. Interestingly, both TZDs produced the same initial rise in intracellular calcium concentrations observed in the absence of extracellular calcium, but only ciglitazone produced a secondary and sustained influx of calcium in the presence of extracellular calcium (Figure 4.5, black line). By contrast, troglitazone failed to produce this secondary response (Figure 4.5, red line). These data suggest that the increase in intracellular calcium was due to release from intracellular stores, such as the endoplasmic reticulum. These data demonstrate that ciglitazone, but not troglitazone mediated a large secondary increase in intracellular calcium concentration, indicative of CCE.

4. Effect of intracellular calcium chelation on TZD-induced EGFR transactivation

The ability of the TZDs to mobilize calcium from different sources suggested this may be an important factor contributing to the differential transactivation of the EGFR. We first examined the effect of intracellular calcium chelation by BAPTA-AM on ciglitazone-induced EGFR phosphorylation. Serum-starved cells were incubated for 20 minutes with 50 μM BAPTA-AM followed by stimulation with DMSO, ciglitazone or troglitazone for 10 minutes, the time at which maximal ciglitazone-induced EGFR

transactivation was observed. Under these conditions where BAPTA-AM pretreatment reduced ciglitazone and troglitazone-induced Src Tyr^{416} phosphorylation (Figure 3.3), as well as Pyk2 activation (Gardner et al., 2003), EGFR Tyr^{845} and Tyr^{1068} phosphorylation induced by ciglitazone was similarly reduced (Figure 4.6). Consistent with previous data, troglitazone did not increase EGFR tyrosine phosphorylation at either site. There was no effect of calcium chelation on EGF-stimulated EGFR phosphorylation.

5. Removal of extracellular calcium prevents EGFR transactivation and Erk activation

Intracellular calcium chelation reduced ciglitazone-stimulated EGFR transactivation, however BAPTA-AM does not distinguish between calcium derived from intracellular or extracellular sources. To determine if this secondary influx of calcium was involved in the ability of ciglitazone to induce EGFR transactivation and subsequent Erk activation, cells were stimulated with ciglitazone in the presence and absence of extracellular calcium and EGFR and Erk phosphorylations were examined. In the absence of extracellular calcium the ability of ciglitazone and Δ2-ciglitazone to cause EGFR phosphorylation was blocked, whereas the effect of EGF stimulated EGFR phosphorylation was not affected (Figure 4.7A). Further, activation of Erk was similarly prevented when cells were stimulated with either ciglitazone or Δ2-ciglitazone in the absence of extracellular calcium (Figure 4.7A). In contrast, the ability of either ciglitazone or troglitazone to induced Src Tyr^{416} phosphorylation was not affected by removal of extracellular calcium (Figure 4.7B) and moreover, induction of p38 phosphorylation, an event previously shown to be dependent upon ER-stress derived calcium-dependent CAMK II activation, was also not affected (Figure 4.7B).

Collectively, these data show that extracellular calcium influx is needed for ciglitazone-induced transactivation of the EGFR and subsequent Erk activation, but is not a required element mediating Src kinase or p38 activation, events thought to be linked to ER calcium release.

6. Inhibition of capacitative calcium entry prevent ciglitazone-induced EGFR transactivation

Because extracellular calcium was observed to be required for EGFR transactivation following ciglitazone treatment, the possible role of CCE in this process was further investigated. Cells were pretreated with 2-aminoethyl diphenyl-borinate (2-APB), a known inhibitor of CCE (Braun et al., 2003) followed by ciglitazone, troglitazone or EGF treatment. As expected, ciglitazone but not troglitazone induced EGFR Tyr^{845} and Tyr^{1068} phosphorylation. 2-APB effectively prevented ciglitazone-induced EGFR transactivation but had little effect on EGFR phosphorylation following EGF treatment (Figure 4.8A). Consistent with the reduction of EGFR phosphorylation, 2-APB similarly reduced ciglitazone induced Erk phosphorylation (Figure 4.8B). In contrast, 2-APB did not inhibit ciglitazone or troglitazone-induced Src Tyr^{416} phosphorylation (Figure 4.8C). These data suggest a role for CCE in EGFR transactivation and subsequent Erk activation in response to ciglitazone and provide a potential mechanism for the differential effects on MAPK activation observed with these compounds. Moreover, these data demonstrate that extracellular calcium is not required for TZD-induced Src activation.

7. Inhibition of matrix metalloproteinases does not affect ciglitazone-induced EGFR transactivation

Calcium influx has been shown to increase the expression of MMPs (Kohn et al., 1994) and further, kinases such as Src have been shown to mediate MMP activation leading to EGFR transactivation (Shah and Catt, 2002). We examined whether MMP activation played a role in ciglitazone-induced EGFR transactivation and subsequent Erk phosphorylation. Cells were pretreated with the MMP inhibitor GM6001 or the inactive analogue, GM6001(-) for 60 min followed by treatment of cells with either ciglitazone (50 μM) or EGF (50ng/mL) for 10 min. GM6001 was previously shown to reduce EGFR phosphorylation in response lysophosphatidic acid (LPA) in GN4 cells (data not shown), a compound that mediates MMP-dependent diffusible ligand shedding. Here, we do not observe any inhibition of either ciglitazone or EGF-induced EGFR phosphorylation by GM6001 (Figure 4.9). Similarly, Erk phosphorylation induced by both ciglitazone and EGF was not affected. These data demonstrate that diffusible ligand shedding by MMP activation was not involved in ciglitazone-induced EGFR transactivation.

D. DISCUSSION

The ability of PPAR ligands to elicit PPAR-independent effects is well documented. The PPARγ ligands, TZDs have been shown to mediate such effects as cell growth inhibition or induction of cell death independent of their PPARγ activating abilities. Moreover, work from our lab and others demonstrate that TZDs activate members of the MAPKs in different cell models (Gardner et al., 2005a; Lennon et al., 2002; Mounho and Thrall, 1999; Rokos and Ledwith, 1997). As MAPK signaling affects a diverse array of transcription factors, ultimately leading to enhanced gene expression, our lab has sought

to define the molecular mechanisms involved in MAPK activation by TZDs. Recent work demonstrated that ciglitazone, but not troglitazone rapidly activated Erk, an effect dependent upon Src-mediated EGFR transactivation (Gardner et al., 2003). Previously we found that both ciglitazone and troglitazone effectively activated Src kinase independent of PPARγ activation (Chapter III – Figure 3.1). Thus, we concluded that an undefined mechanism specifically activated by ciglitazone, in addition to Src kinase, was necessary for EGFR transactivation and subsequent Erk activation. In this study we demonstrate that calcium influx from extracellular sources through CCE is a critical factor involved in the transactivation of the EGFR by ciglitazone. Although both TZDs effectively mediated release of calcium from intracellular stores and activation of upstream kinases (Src and Pyk2), only ciglitazone induced a large secondary calcium influx. Removal of extracellular calcium or pharmacological inhibition of CCE prevented ciglitazone-induced EGFR transactivation and Erk activation.

EGF stimulated EGFR activation has been shown to recruit Src into receptor complexes leading to its activation (Olayioye et al., 1999). Further, other work in human epidermoid carcinoma cells demonstrated that the Shc adaptor protein was a novel mediator of EGF stimulated Src activation through the EGFR; activation was associated with Src autophosphorylation, but dephosphorylation in the c-terminal tail region was not observed (Sato et al., 2002). In contrast to these studies, our data show that inhibition of EGFR kinase activity had little effect on the ability of TZDs to mediate Src Tyr^{416} autophosphorylation, indicating that EGFR kinase activity was not involved in Src activation (Figure 4.1). This supports other works demonstrating a role of Src kinase in EGFR transactivation in response to various stimuli and our own data suggesting that

ciglitazone mediates EGFR transaction by first activating Src kinase (Gardner et al., 2003). Further, the fact that troglitazone failed to mediate EGFR transactivation, but is still capable of activating Src, suggests that EGFR activation is not involved in Src kinase activation in response to these compounds.

Pyk2 (also designated CAKβ/RAFTK/CadTK) is a member of the focal adhesion kinase family, of which FAK is also a member (Avraham et al., 2000). Pyk2 is highly expressed in GN4 cells and can be activated by stimuli that increase intracellular calcium (Graves et al., 1997) or in response to stress signals (Yu et al., 1996). Both TZDs used in this study have been shown to increase Pyk2 phosphorylation (Gardner et al., 2003). Pyk2 phosphorylation has been shown to recruit and activate members of the Src-family kinases, thereby resulting in activation of downstream MAPKs (Dikic et al., 1996). However, our data demonstrated that Src is the upstream kinase necessary for mediating the activation of Pyk2 following TZD exposure. Following inhibition of Src kinase with PP2, TZDs-induced Pyk2 phosphorylation was also inhibited (Figure 4.3B). However, the ability of either TZD to activate Src kinase was unaffected when Pyk2 activation was prevented by expression of the dominant negative Ad.CRNK (Figure 4.2B). These data suggest that Src mediates the activation of Pyk2 following TZD treatment and are consistent with other reports demonstrating the requirement of Src kinase in the activation of Pyk2 (Bobe et al., 2003).

In this study, ciglitazone and troglitazone lead to a rapid transient increase in intracellular calcium concentrations when cells were stimulated in the absence of any extracellular calcium, suggesting intracellular store depletion (Figure 4.4). These data reinforce other studies showing that both ciglitazone and troglitazone led to a rapid and

transient increase in intracellular calcium in embryonic stem cells; furthermore addition of thapsigargin, a specific inhibitor of the SER-Ca^{2+} ATPase, known to cause ER calcium store depletion, did not induce more Ca^{2+} release, indicating that TZDs were affecting ER calcium stores (Palakurthi et al., 2001). Previous work from our laboratory also suggested that TZDs affect ER calcium in that ciglitazone and troglitazone rapidly activated classical markers of ER stress, including PERK, PKR, and eIF-2α (Gardner et al., 2005b), events known to correlate with ER calcium depletion (Kuang et al., 2005).

CCE is a regulated mechanism of calcium entry in non-excitable cells (Putney, 1986). Depletion of intracellular calcium stores through the actions of IP_3, SERCA inhibition by thapsigargin, or other ER calcium releasing signals, activates a pathway leading to the opening of plasma membrane calcium channels allowing for influx of calcium from extracellular sources. When GN4 cells were exposed to ciglitazone in the presence of extracellular calcium, we observed a large secondary influx of calcium following an initial rise in intracellular calcium (Figure 4.5). While troglitazone produced the initial rise in calcium, no secondary increase in calcium was observed (Figure 4.5). In agreement with our data, ciglitazone has been shown to inhibit cell proliferation in leiomyoma cells through activation of capacitative calcium entry (Kim et al., 2005). Furthermore, other work has demonstrated that troglitazone actually prevented capacitative calcium entry in aortic endothelial cells (Kawasaki et al., 1999). Therefore, the differential effects of these compounds on CCE could contribute to the difference observed in EGFR transactivation.

A variety of intracellular signaling pathways are regulated by changes in intracellular calcium including both Pyk2 and Src activation (Berridge et al., 2000). Previously we

demonstrated that Pyk2 activation following TZD treatment was sensitive to intracellular calcium chelation (Gardner et al., 2003) and similarly, Src kinase Tyr^{416} phosphorylation was lowered (Chapter III – Figure 3.3). This suggests that calcium release from intracellular stores is important to the activation of Src kinase, and in our model, subsequent Pyk2 activation (Figure 4.10). Interestingly, other work in keratinocytes demonstrated that exposure of cells to either a high concentration of extracellular calcium or ionophore rapidly increased Src kinase activity (Zhao et al., 1992). However, our data suggest that extracellular calcium does not play a role as Src kinase activation was unaffected in the presence or absence of extracellular calcium following stimulation of cells with ciglitazone or troglitazone (Figure 4.7B). Therefore we suggest that ER calcium release is important to the activation of upstream kinases required for EGFR transactivation and other related signaling cascades related to TZD-induced p38 activation (Figure 4.7B).

Other works have highlighted the importance of calcium in the mechanism of EGFR transactivation. Our data support these observations showing that BAPTA-AM reduced EGFR transactivation in response to ciglitazone treatment (Figure 4.6). However, activation of other kinases thought to be required for EGFR transactivation was also affected by calcium chelation in this study. Troglitazone activated upstream kinase signals necessary for EGFR transactivation and depleted ER calcium stores, but failed to transactivate the EGFR. Interestingly, our data demonstrated that troglitazone failed to produce a secondary calcium influx characteristic of CCE, suggesting that the secondary influx of calcium by CCE was important to the mechanism of EGFR transactivation. When cells were treated with ciglitazone, which produced a secondary calcium influx

(Figure 4.5), in the absence of any extracellular calcium, EGFR transactivation was prevented (Figure 4.7A). This agrees with previous studies that demonstrated treatment of cells with a calcium ionophore could induce EGFR phosphorylation (Eguchi et al., 1998; Rosen and Greenberg, 1996). Further, bradykinin-induced EGFR transactivation was shown to be prevented by extracellular calcium chelation using EGTA (Zwick et al., 1997).

However, we further hypothesized that in addition to upstream kinase signaling events, the regulated entry of calcium from extracellular sources through CCE is an additional requirement in the mechanism of EGFR transactivation. When CCE was prevented by 2-APB, ciglitazone failed to induce EGFR transactivation even though upstream kinases (i.e. Src) were still activated; EGF stimulated EGFR phosphorylation was not affected by 2-APB (Figure 4.8A and B). Therefore, these data support a required role of CCE in EGFR transactivation and provide an explanation for the difference observed following treatment of cells with TZDs. Since many other stimuli have been shown to induce EGFR transactivation, the requirement of CCE should be further investigated.

These data highlight the importance of different sources of calcium in mediating specific cellular signaling events in the cell and demonstrate a novel role of CCE-derived calcium in the mechanism of EGFR transactivation. Furthermore, the ability ciglitazone, but not troglitazone, to mediate EGFR transactivation is best explained by the exclusive ability of ciglitazone to effectively mediate CCE. The role of extracellular calcium from CCE in the mechanism of EGFR transactivation is unclear and should be explored further.

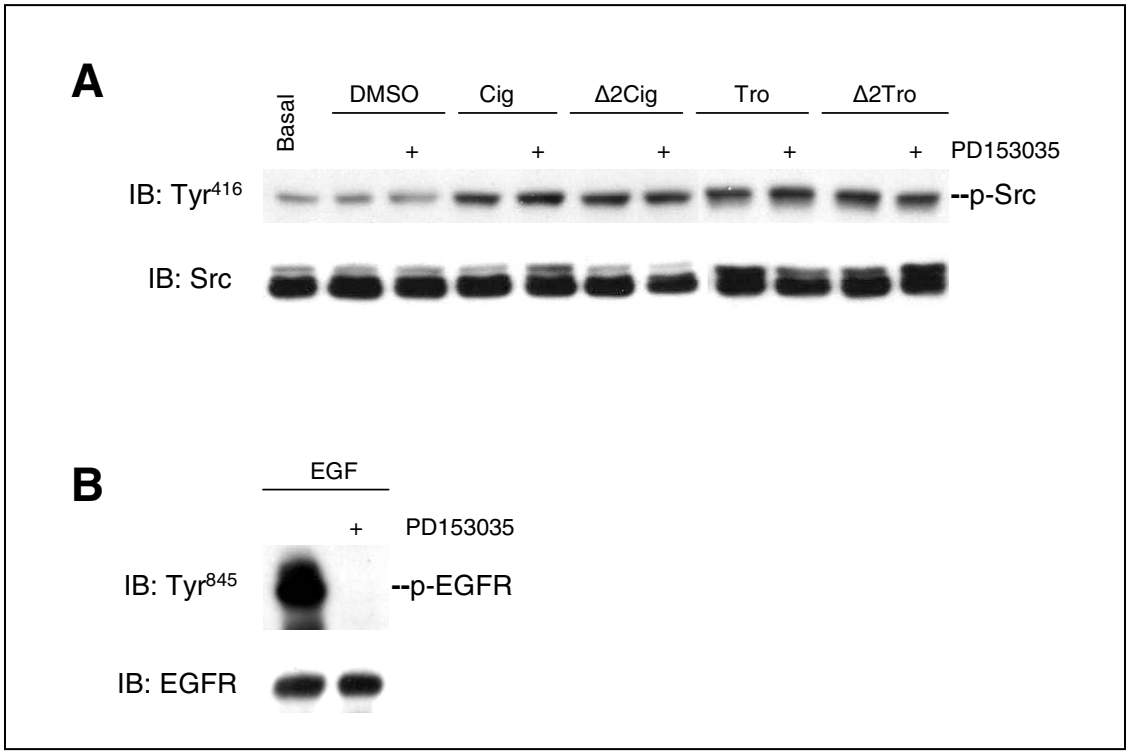

Figure 4.1. EGFR kinase activity is not required for Src phosphorylation in GN4 cells treated with TZDs. Confluent GN4 cells were pre-treated with or without 10 μM PD153035 for 30 min. **A**, Cells were treated in the presence or absence of the EGFR kinase inhibitor with 50 μM ciglitazone (Cig), Δ2-ciglitazone (Δ2Cig), troglitazone (Tro) or Δ2-troglitazone (Δ2Tro) or 0.1% DMSO (vehicle control) for 10 min. Cell lysates were prepared and subjected to SDS-PAGE. Src tyrosine phosphorylation was determined by immunoblotting (IB) using anti-phospho-Src (Tyr^{416}) antibody. **B**, Cells were treated with or without 100 ng/mL EGF for 5 min. EGFR phosphorylation was determined by immunoblotting (IB) using anti-phospho-EGFR (Tyr^{845}) antibody. The blots were stripped and reprobed using antibodies directed against total Src or EGFR, respectively, to determine equal protein loading.

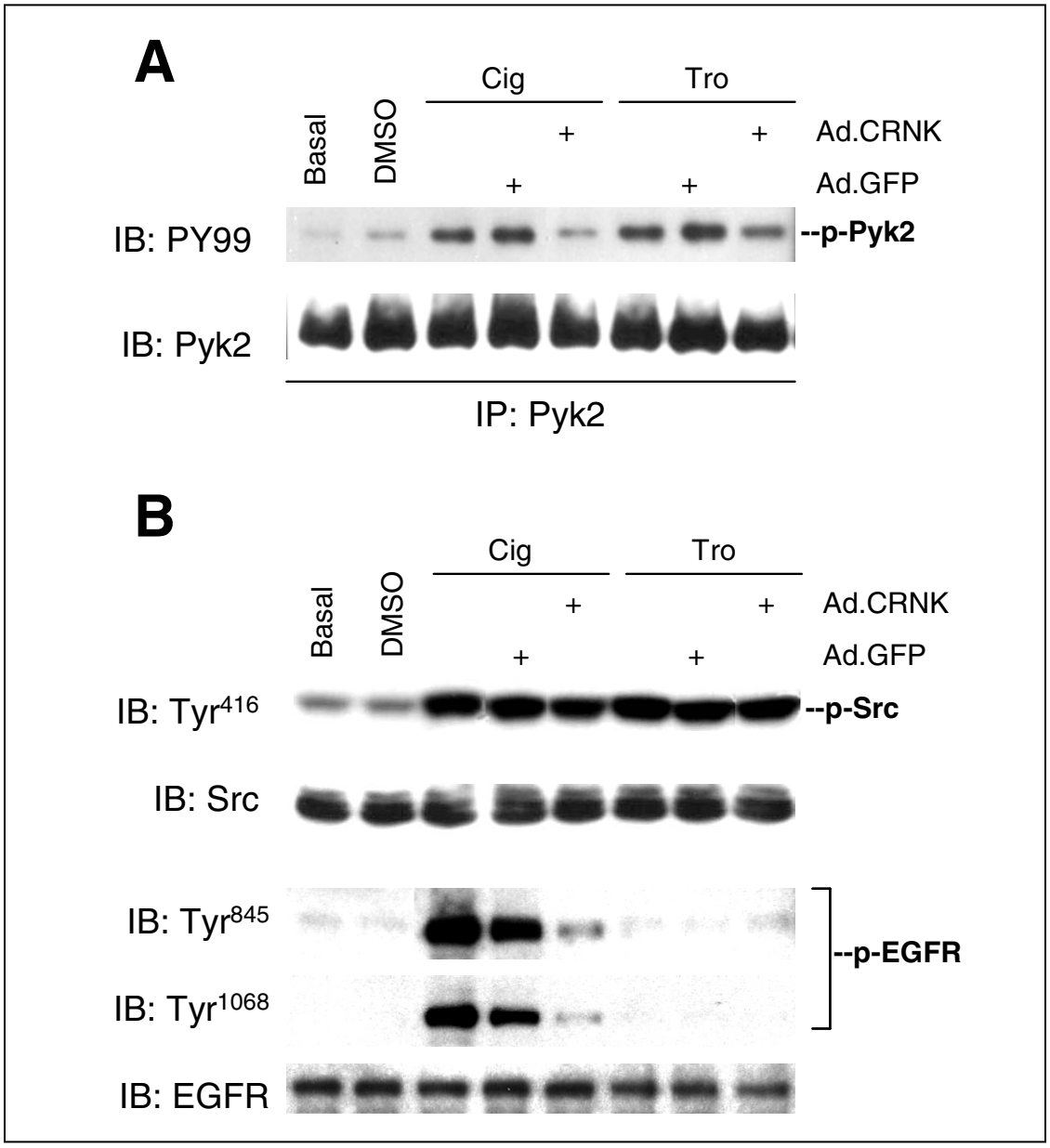

Figure 4.2. Pyk2 inhibition lowers EGFR transactivation, but does not prevent Src kinase activation. Following infection of near confluent GN4 cells with 8 x 10^6 plaque-forming units/ mL Ad.GFP or Ad.CRNK, cells were serum-deprived for 18 hr 0.1% FBS containing medium, and then treated with 50 μM ciglitazone, troglitazone or 0.1% DMSO for 10 min. **A**, Pyk2 was immunoprecipitated (IP) from lysates and immune complexes were subjected to SDS-PAGE. The effect of Ad.CRNK on Pyk2 phosphorylation was determined by immunoblotting (IB) with a pan anti-phosphotyrosine (PY99) antibody; total Pyk2 was determined using an anti-Pyk2 antibody. **B**, total lysates were subjected to SDS-PAGE and then immunoblotted to detect changes in Src Tyr^{416} or EGFR Tyr^{845} or Tyr^{1068} phosphorylation. To determine equal loading, blots were stripped and reprobed with Pyk2, Src or EGFR antibodies, respectively.

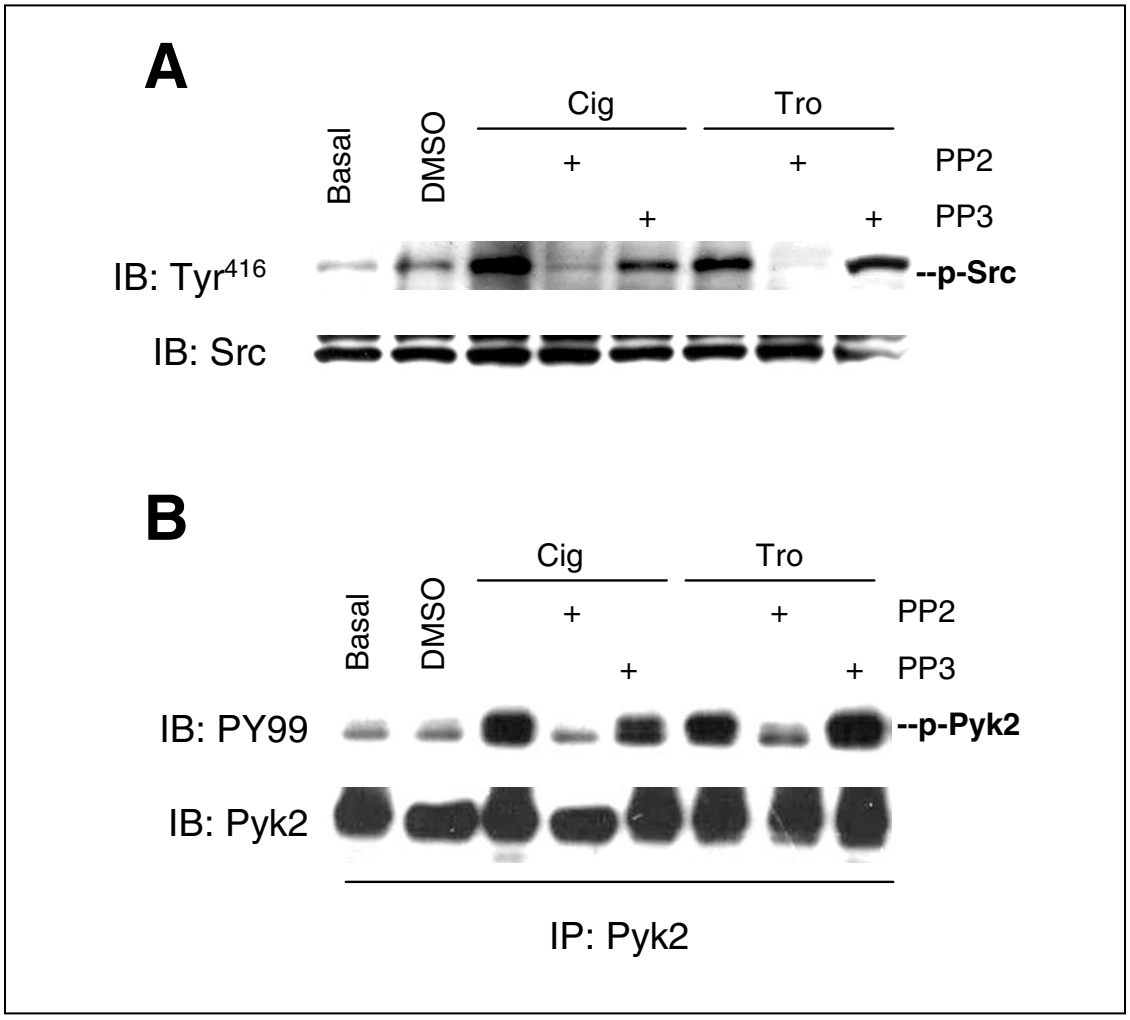

Figure 4.3. Inhibition of Src kinase activity prevents Pyk2 activation. GN4 cells grown to confluence were pretreated with 10 μM PP2 or PP3 for 30 min followed by 50 μM ciglitazone (Cig) or troglitazone (Tro) or 0.1% DMSO for 10 min. Cell lysates were prepared as previously described. **A**, Cell lysates were subjected to SDS-PAGE and immunoblotted (IB) with Src (Tyr^{416}) antibodies. Blots were stripped and reprobed for total Src. **B**, Pyk2 was immunoprecipitated (IP) from lysates and immune complexes were subjected to SDS-PAGE. Phosphorylation of Pyk2 was determined by immunoblot (IB) with a pan anti-phosphotyrosine (PY99) antibody; total Pyk2 was determined using an anti-Pyk2 antibody.

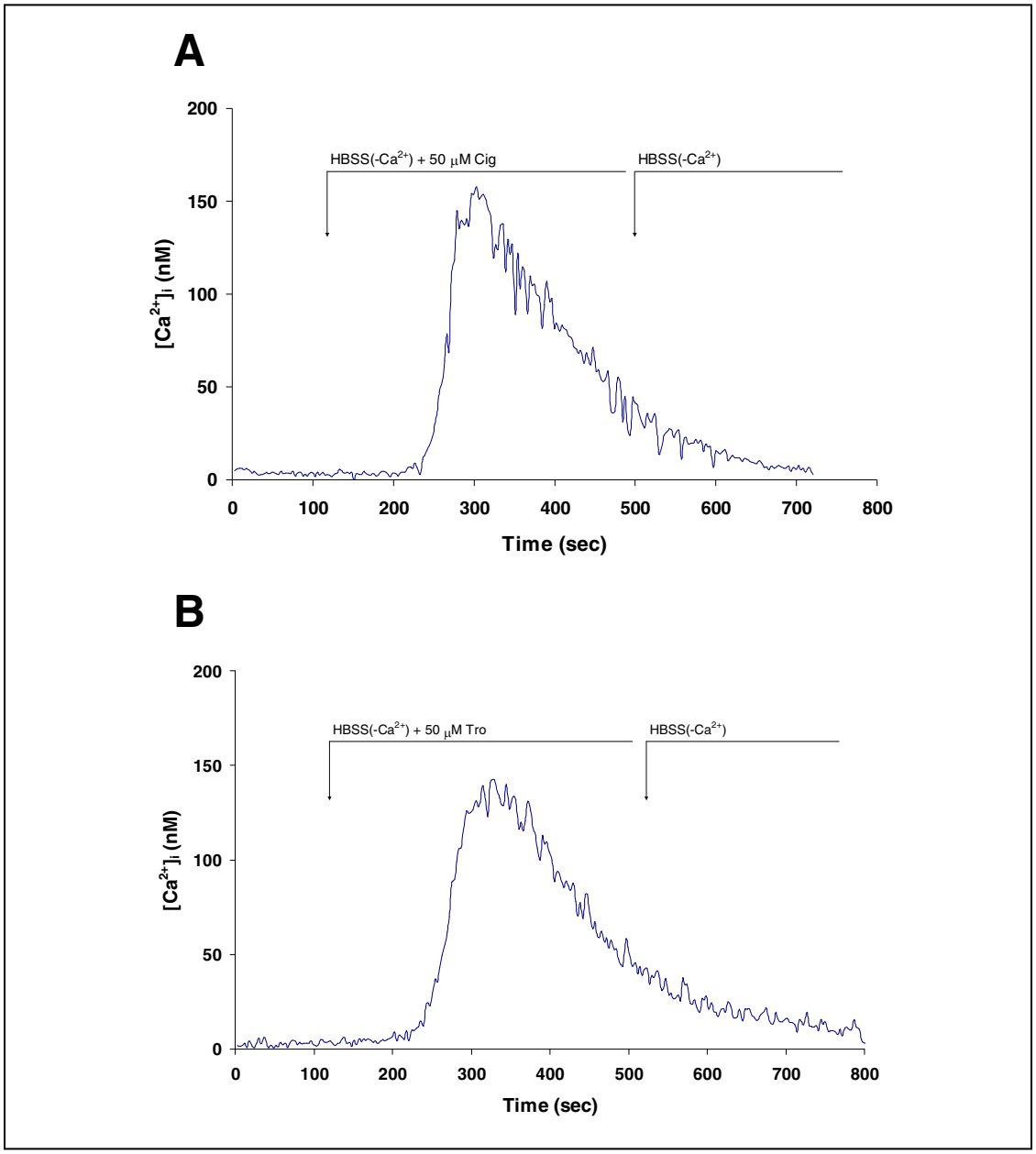

Figure 4.4. Ciglitazone and troglitazone increase intracellular calcium concentrations by depletion of intracellular stores. GN4 cells were loaded with Fura-2 AM and changes in intracellular calcium concentrations were monitored as described in MATERIALS AND METHODS. Following initial perfusion of cells with calcium free HBSS, the perfusion medium was changed to calcium free HBSS containing either 50 μM ciglitazone (**A**) or troglitazone (**B**) (first arrow). The medium was then switched back to normal calcium free HBSS as indicated by the second arrow. Shown is a representative experiment from 3 separate trials.

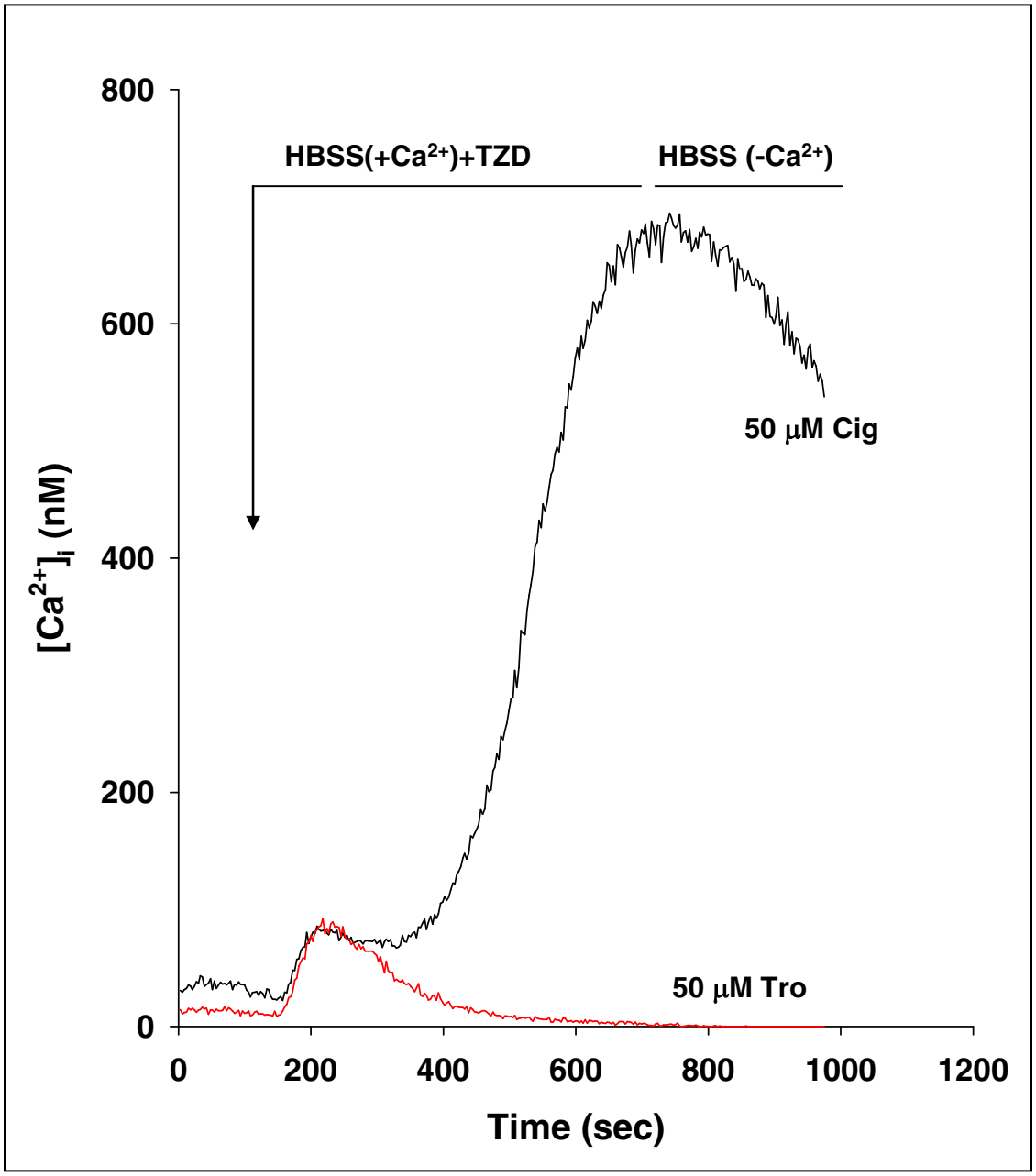

Figure 4.5. The effect of ciglitazone and troglitazone on changes in intracellular calcium concentrations in the presence of extracellular calcium. Fura-2 AM loaded GN4 cells were challenged with 50 μM ciglitazone or troglitazone in HBSS containing 1.0 mM calcium as indicated by the arrow and changes in intracellular calcium concentrations were measured as described in MATERIALS AND METHODS. The perfusion medium was switch to calcium free HBSS as indicated by the second arrow. Shown is a representative experiment from 3 separate trials.

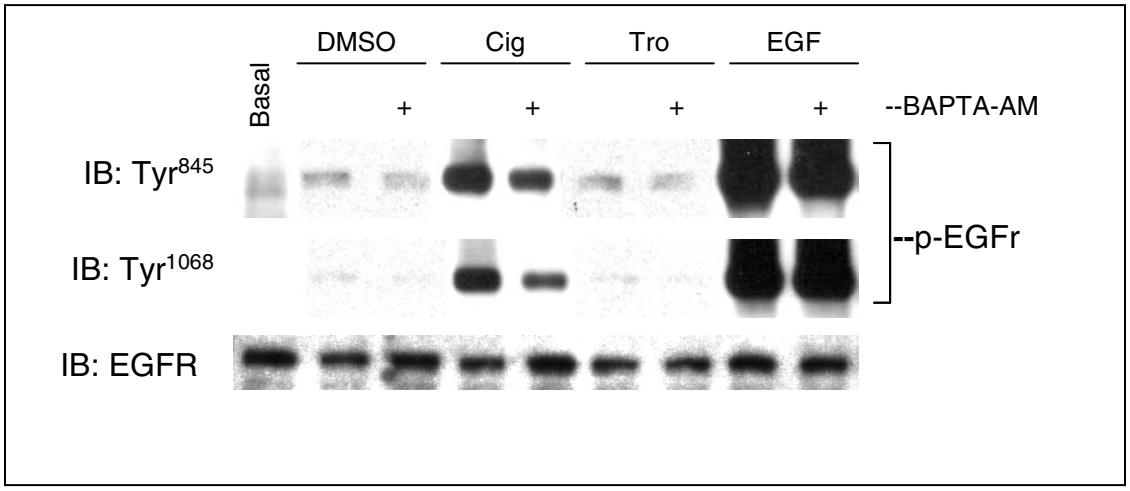

Figure 4.6. BAPTA-AM lowers ciglitazone-induced EGFR Tyr^{845} and Tyr^{1068} phosphorylation. Serum-starved GN4 cells were pretreated 20 min with 50 μM BAPTA-AM followed by treatment with 50 μM ciglitazone or troglitazone for 10 min. Additionally cells were treated with 50 ng/mL EGF for 10 min. Lysates were prepared and subjected to SDS-PAGE. Samples were immunoblotted (IB) for EGFR Tyr^{845} or Tyr^{1068}. Blots were stripped and reprobed for total EGFR protein to determine equal loading.

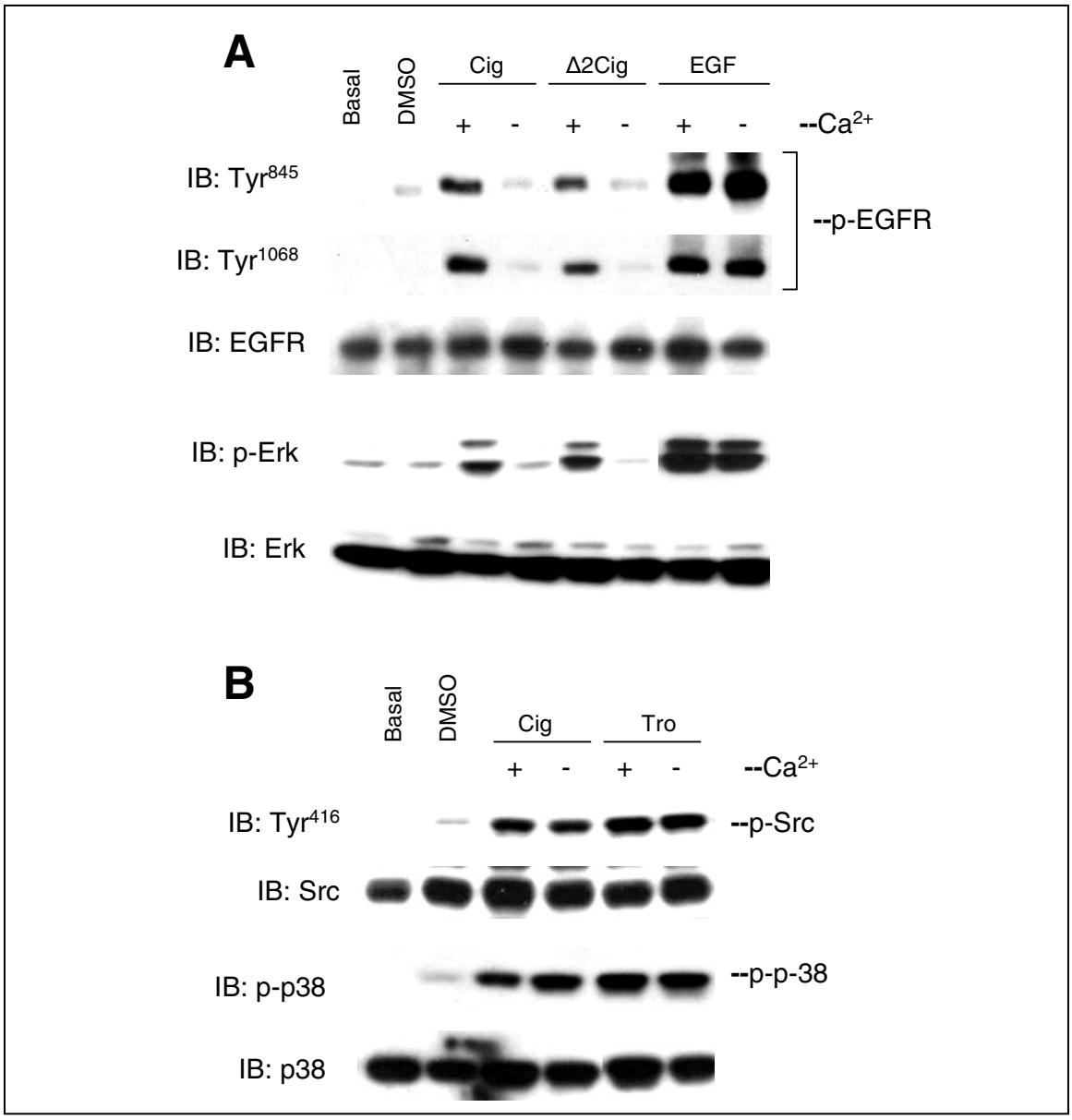

Figure 4.7. Removal of extracellular calcium prevents EGFR transactivation and Erk activation, but does not affect upstream signals. Cells were grown to near confluence and then serum-deprived overnight. The medium was removed and cells were washed with HBSS and then incubated for 30 min in HBSS containing (+) or lacking (-) 1 mM Ca^{2+}. Cells were then exposed to 50 μM ciglitazone (Cig), Δ2-ciglitazone (Δ2Cig), troglitazone (Tro) or Δ2-troglitazone (D2Tro) or 0.1% DMSO (vehicle control) for 10 min. Some cells were also treated with 100 ng/mL EGF for 5 min. Cell lysates were prepared and subjected to 10% SDS-PAGE. **A**, EGFR and Erk phosphorylation were determined by immunoblotting (IB) using anti-phospho-EGFR (Tyr^{845}) and (Tyr^{1068}) or anti-phospho-Erk antibodies. Blots were stripped and reprobed using antibodies against total EGFR and Erk. **B and C**, Src tyrosine phosphorylation was determined by IB using anti-phospho-Src (Tyr^{416}) antibody and p-38 phosphorylation was determined using an anti-phospho-p38 (p-p38) antibody. The blots were stripped and reprobed using total antibodies to its respective protein to determine equal protein loading.

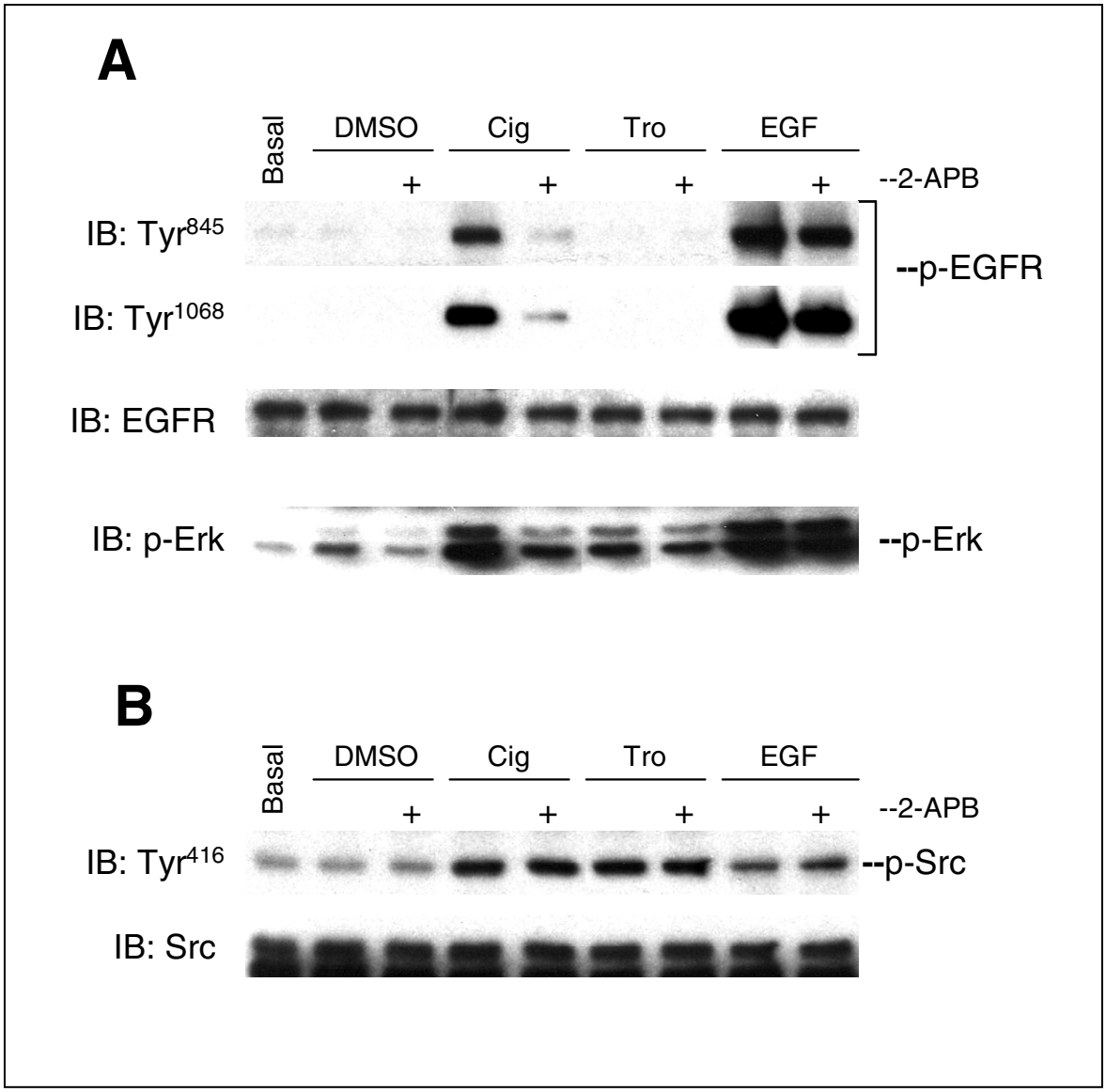

Figure 4.8. 2-APB blocks EGFR transactivation by ciglitazone. GN4 cells were grown to confluence and serum-starved overnight. Some cells were pretreated with 30 μM 2-APB for 15 min followed by 50 μM ciglitazone (Cig) or 50 ng/mL EGF for 10 min. **A**, cell lysates were prepared and EGFR was immunoprecipitated from lysates, immune complexes were subjected to SDS-PAGE, and then immunoblotted (IB) with a pan anti-phosphotyrosine (PY99) antibody. **B**, Src and Erk phosphorylation were determined by immunoblotting (IB) using anti-phospho-Src (Tyr^{416}) or anti-phospho-Erk antibodies, respectively. Blots were stripped and reprobed for total protein.

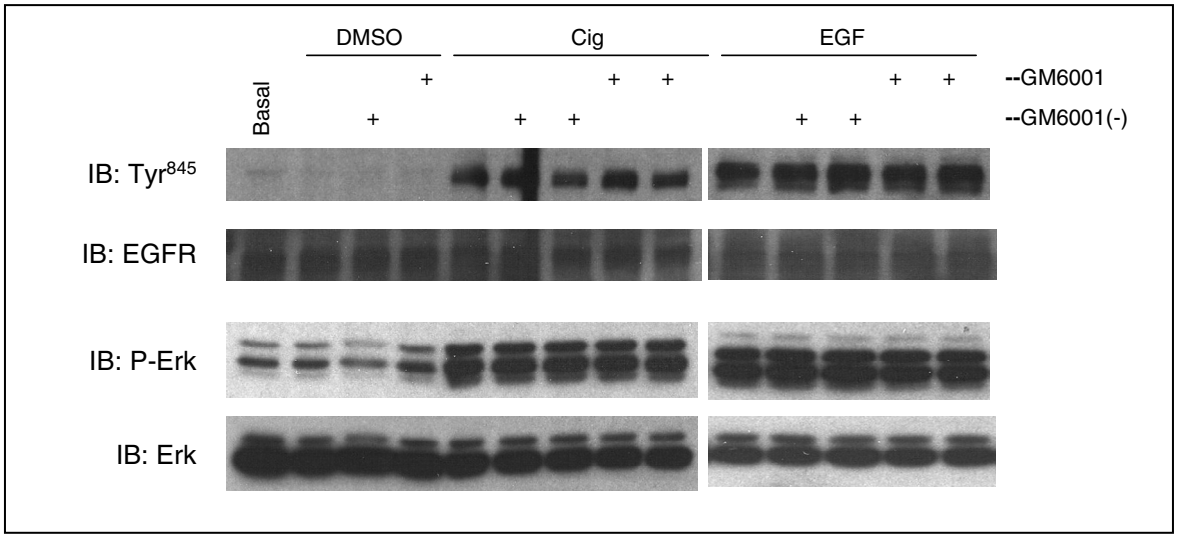

Figure 4.9. Inhibition of MMP activity does not block EGFR phosphorylation by ciglitazone. Serum-starved GN4 cells were pretreated 60 min with 10 μM GM6001 or the inactive analogue GM6001(-), followed by ciglitazone (50 mM) or EGF (50 ng/mL) for 10 min. Lysates were prepared and subjected to SDS-PAGE. Samples were immunoblotted (IB) for phospho-EGFR Tyr^{845} or phospho-Erk. Blots were stripped and reprobed for total EGFR and Erk protein to determine equal loading.

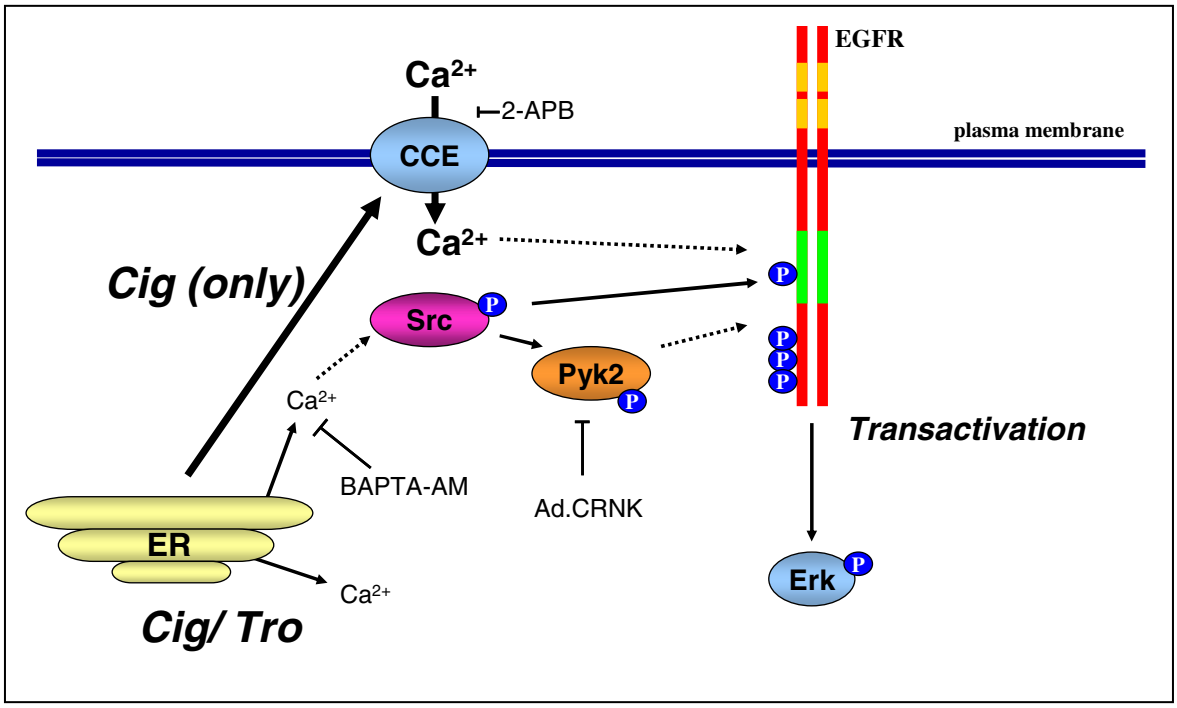

Figure 4.10. Model of differential EGFR transactivation by TZDs. Both ciglitazone and troglitazone mediated a transient increase in intracellular calcium concentration, presumably through ER store release, and increased Src-dependent Pyk2 activation. Intracellular calcium chelation lowered both TZD-induced Src activation and ciglitazone stimulated EGFR transactivation. The mechanism involved in calcium mediated Src activation is not known. Only ciglitazone induced a second influx of calcium; removal of extracellular calcium or inhibition of CCE with 2-APB prevented ciglitazone-induced EGFR transactivation. Moreover, inhibition of Pyk2 also prevented ciglitazone induced EGFR transactivation. While Src has been shown to directly mediate phosphorylation of the EGFR, the mechanisms by which CCE-derived calcium and Pyk2 are involved in mediating EGFR transactivation remain unclear.

CHAPTER V

Concluding Remarks

The primary goal of this research was to examine the mechanisms involved in the differential transactivation of EGFR following TZD treatment. Previously we observed that Src kinase was required for EGFR transactivation, an event mediated by the TZD ciglitazone, but unaffected by a related TZD, troglitazone. Our initial data presented here demonstrated that both TZDs were capable of influencing Src kinase activation. We therefore examined mechanisms regulating Src kinase activation, as well as signals that followed Src activation to explain the differential effects in response to these compounds. We have shown that while both compounds activated kinases important to EGFR transactivation and mediated intracellular calcium store depletion, only ciglitazone induced a secondary and substantial calcium influx. Inhibition of this secondary influx or capacitative calcium entry prevented ciglitazone-induced EGFR transactivation (Figure 5.1).

Although earlier studies suggested that TZDs and other PPARα ligands could mediate Src activation, data shown here clearly demonstrated the ability of the PPARγ ligands, ciglitazone and troglitazone to effectively mediate an increase in Src kinase activity in a PPARγ-independent manner. Importantly, Src signals to a wide variety of downstream effectors, including the regulatory subunit of phosphatidylinositol 3-kinase, p85, phospholipase Cγ, integrin signal proteins and focal adhesion kinases (Brown and Cooper, 1996). Previous work from our lab demonstrated the importance of Src kinase in mediating phosphorylation and transactivation of the EGFR in response to ciglitazone and this corresponds well with other data demonstrating a role for Src in EGFR phosphorylation (Biscardi et al., 1999; Gardner et al., 2003). As Src is an important kinase involved in cellular differentiation, motility, proliferation and survival, other

downstream effectors activated by Src in response to TZDs could be important to understanding the PPARγ-independent cellular effects observed with these compounds. Previous work demonstrated that both ciglitazone and troglitazone activated the non-receptor, proline-rich tyrosine kinase, Pyk2, an event thought to be tied to the ER and calcium release (Gardner et al., 2003). Data here suggests that in addition to the EGFR, Src acts as an upstream mediator of Pyk2 activation. Pyk2 has also been demonstrated to be involved in EGFR transactivation (Shah and Catt, 2002), giving cause for the re-examination of Pyk2 and its involvement in TZD-induced EGFR transactivation. Further experiments should be performed to determined additional downstream targets of TZD-induced Src activation

Many studies have demonstrated that PPARγ ligands, like the TZDs negatively affect cell growth in a variety of cancer cell lines. On the other hand, activation of Src is involved in mechanisms promoting cell survival and proliferation. Thus the significance of Src activation in relation to the physiological effects of TZD appears to be contradictory. However, some studies suggest that Src activation could be relevant and important to the actions of these compounds. For example, in carcinogenic studies the incidence of hemangiosarcomas was increased in female mice treated with TZDs, albeit at the highest doses administered. Moreover, there was an increase in the incidence of hepatocellular carcinomas observed in female mice at the highest dose, 800 mg/kg, in the same study (Herman et al., 2002). Further, other works demonstrated that both troglitazone and rosiglitazone promoted the development of colon tumors in mice (Lefebvre et al., 1998); however it was suggested that PPARγ was involved. Subsequently, a study showed that a diverse panel of PPARγ ligands could promote colon

tumor formation (Pino et al., 2004). As Src is a potent oncogene involved in many cancers, it is tempting to speculate that the ability of TZDs to promote tumor formation could be tied to Src activation or pathways activated in response to Src. Studies could be employed to examine the levels of Src activity in tumors from TZD-treated animals.

While TZDs promote the amelioration of hyperglycemia, they also promote adipocyte differentiation and proliferation (Yki-Jarvinen, 2004). Patients administered TZDs often gain weight due to expansion of subcutaneous fat stores and fluid retention. TZD-induced adipocyte proliferation is thought to be principally linked to high expression of PPARγ in this tissue (Fajas et al., 1997). However, recent work has pointed to a role of Src in adipogenesis. Hormonal induced differentiation of fibroblasts to adipoctyes was sensitive to Src kinase inhibition and did not occur in Src, Yes, Fyn (SYF) knockout cells, demonstrating a clear role for SFKs in adipogenesis (Sun et al., 2005). As TZDs effectively mediated Src activation in rat liver epithelial cells, it would be interesting to examine Src activation in fibroblasts and adipocytes. Moreover, this suggests that PPARγ-independent activation of Src could be linked to an important physiological effect of the TZDs.

Our previous data suggested that generation of ROS was involved in mediating activation of Src kinase in response to TZDs (Gardner et al., 2003). However, we now show data that PTPase activity is necessary for TZD-induced Src kinase activation. Generally, ROS production would lead to an inhibition of PTPase activity. Potentially, this alone could mediate Src activation as inhibition of PTPase activity would lead to an increase in tyrosine phosphorylated proteins, which could then serve as ligands for Src's SH2 domain, leading to SH2 domain displacement and kinase activation. However,

under similar conditions, where PTPase activity was prevented, TZD-induced Src kinase activation was completely blocked. This suggests that ROS generation and SH2 domain displacement by tyrosine-phosphorylated proteins are not mechanisms involved in Src activation in response to TZDs.

Src kinase activation occurs through dephosphorylation of Tyr^{527} in the C-terminal tail region of the protein. This can be mediated by a decrease in the kinase that moderates phosphorylation of this site, CSK or though PTPase-dependent dephosphorylation. Our data demonstrated that TZDs did not directly increase PTPase activity nor did they increase the association of a PTPase with Src. However, clearly a PTPase was involved. Rather we found that the putative phosphatidylinositol polyphosphate-5 phosphatase, a Shp2 related PTPase was associated with Src. As a model system, Shp2 does not directly target dephosphorylation of Src protein, but rather dephosphorylates tyrosine residues on Cbp, an adaptor protein found in lipid rafts on the plasma membrane. CSK is brought to the plasma membrance by virtue of its SH2 targeting tyrosine phosphorylated Cbp; this interaction is thought to keep CSK in close proximity to Src, allowing for C-terminal tail phosphorylation. Dephosphorylation of Cbp causes a disassociation of this transient CSK/Src interaction and allows for and promotes Src dephosphorylation. Whether Src's associated Shp2 mediates this or another PTPase is responsible is not well understood. Once active, Src phosphorylates Cbp, inducing reassociation of CSK and inhibition of Src activity. We did not observe an appreciable increase in Src in lipid raft fractions following TZD treatment, suggesting that these discrete plasma membrane structures may not be involved, even though Cbp is know to localize to these cellular locations. Further, TZDs could mediate Src activation

by initially disrupting the CSK/Src interaction. We examined this possibility; however, our results suggested that the two proteins did not maintain a strong enough interaction to be observed by immunoprecipitation of one protein or the other. It would be of further interest to examine the phosphorylation status of Cbp, before and after TZD treatment. Alternatively, as CSK targets Cbp, this interaction may be a better one to study.

Previously, work demonstrated that TZD increase intracellular calcium concentration through intracellular store depletion, presumably the ER (Palakurthi et al., 2001). Recent data also show that TZDs mediate the activation of markers of ER stress and that CAMK II-mediated p38 activation was linked to the effects of these compounds on ER calcium (Gardner et al., 2005b). Our work here supports these observations demonstrating that in the absence of any extracellular calcium both ciglitazone and troglitazone effectively modulated an increased intracellular calcium levels. Moreover, the increase in cellular calcium observed with each TZD were transient, which is highly indicative of ER store release. Interestingly, intracellular calcium chelation with BAPTA-AM effectively lowered TZD-induced Src Tyr^{416} phosphorylation, Pyk2 activation and ciglitazone-induced EGFR phosphorylation. A role for calcium in these events has been previously demonstrated supporting our observations (Keely et al., 1998; Shah and Catt, 2002; Zwick et al., 1999b). Thus TZD-induced ER stress and calcium store depletion previously considered to be involved only in TZD-induced p38 activation, may be a common link mediating activation of several signaling cascades contributing ultimately to actvation of several MAPKs.

CCE is the retrograde process whereby ER calcium store depletion signals the opening of plasma membrane calcium channels, mediating a sustained influx of

extracellular calcium (Putney, 1986). This process has been extensively study using the SERCA inhibitor, thapsigargin (Putney et al., 2001). Data shown here suggest that ciglitazone but not troglitazone effectively induces CCE. When cells were stimulated with either TZD in the presence of extracellular calcium, only ciglitazone produced a secondary and substantial increase in intracellular calcium concentration. Under these conditions, both ciglitazone and troglitazone cause a transient intial rise in intracellular calcium concentration. These observations support other work demonstrating that ciglitazone is capable of inducing capacitative calcium entry (Kim et al., 2005). Conversely, and in agreement with our data, troglitazone has been demonstrated to inhibit thapsigargin-induced CCE (Kawasaki et al., 1999). Further studies could be done to examine if troglitazone inhibits specific, recently identified molecular mechanisms regulating CCE.

Others have suggested a role of calcium in EGFR transactivation and moreover, a recent study demonstrated that inhibition of calcium channels involved in CCE prevented EGFR transactivation (Kawanabe et al., 2002). Further, stimuli that induce EGFR transactivation, such as GPCR agonists, are well known for their influence on intracellular calcium concentrations and therefore could promote CCE. Indeed, GCPR activation stimulates PLC enzymes, which in turn generate IP_3, a secondary-messenger that affects specific ER localized receptors to mediate ER calcium release, the event that initiates CCE. Our data here point to a novel role of CCE in EGFR transactivation. Only ciglitazone produced an initial transient increase in calcium followed by a second more sustain influx, when extracellular calcium was provided. Further, ciglitazone failed to mediated EGFR transactivation and Erk activation in the absence of extracellular

calcium. And lastly, a CCE inhibitor effectively lowered ciglitazone induced EGFR transactivation. Collectively, these data point to a role of CCE in EGFR transactivation. The exact role calcium is playing, however is not known and should be investigated. Further there are conflicting data as to whether calcium influx is sufficient to mediate EGFR transactivation. Others have shown that EGFR transactivation could occur simply by treating cells with an ionophore. Moreover, others have shown that thapsigargin, a well-known activator of CCE could mediate EGFR transactivation. However, in the cells used in our experiments neither ionophore nor thapsigargin effectively mediated EGFR transactivation alone (Figure 5.2). This suggests that in response to ciligtazone treatment, calcium and intracellular kinases are required elements, but not sufficient in mediating EGFR transactivation. Furthermore, as other stimuli mediated EGFR transactivation, and this event is significant to their physiological mechanisms of action, the role calcium from CCE should be explored in EGFR transactivation in response to these agents.

STIM 1 and 2 have recently been shown to be ER-localized calcium sensors that signal or redistribute to the plasma membrane and activate CCE. Further work has identified Orai proteins as the possible calcium channels involved in CCE. RNAi knockdown of either STIM1 or Orai proteins has been shown to block thapsigargin induced CCE. To definitively demonstrate the involvement of CCE in EGFR transactivation by ciglitazone or in response to other stimuli, experiments examining EGFR transactivation should be performed under conditions where STIM1 protein expression has been blocked.

The ability of ciglitazone and troglitazone to activate signaling pathways is a potential mechanism by which, in part, these compounds moderate PPARγ-independent cellular

effects. We have now seen that the overall stress these compounds place on cells influences the activation of specific cellular pathways leading to MAPK activation. The differential activation of Erk preceded by EGFR transactivation by ciglitazone and not troglitazone is best explained by differences in calcium mobilization and could provide an explanation to the discrepancy in toxicities observed with these compounds. While Erk activation is primarily thought to play a central role in cell proliferation, hyperactivation of Erk can elicit cell cycle arrest by inducing the accumulation of cyclin-dependent kinase inhibitors. We have observed that ciglitazone is a more potent inhibitor of cell viability (data not shown), and this could be due to ciglitazone's unique ability to mediate the EGFR/ Erk pathway.

In addition, PPARγ-independent signaling cascades and subsequent MAPK actvation may extend to the genomic effects of TZDs in that recent work has demonstrated that MAPK mediated phosphorylation of PPARs. Specifically, MAPK phosphorylation of the PPARγ led to decreased transcriptional activity and adipogenesis (Hu et al., 1996). This adds an additional level of regulation of transcriptional activity induced by these compounds. As specific kinase signaling networks extend to the ultimate activation of specific MAPKs, an understanding of these upstream pathways and their regulation is important and could provide better knowledge of how these compounds are working.

Lastly, hepatocytes specifically, are sensitive to disruptions in calcium homeostasis and others have observed activation of similar signaling cascades in response to calcium. Here both ciglitazone and troglitazone influenced calcium, but only in a transient manner. It is therefore possible that the secondary induction of CCE induced by ciglitazone overwhelms the cell to a point at which it is unable to recover leading to cytotoxicity; in

other cell types not sensitive to fluctuations in calcium homeostasis, this secondary calcium influx would serve a different function. A specific understanding of the calcium mobilization process (i.e. – whether CCE is induced) specifically in liver cells may be of importance to drug development and could explain why some drug candidates fail liver toxicity tests.

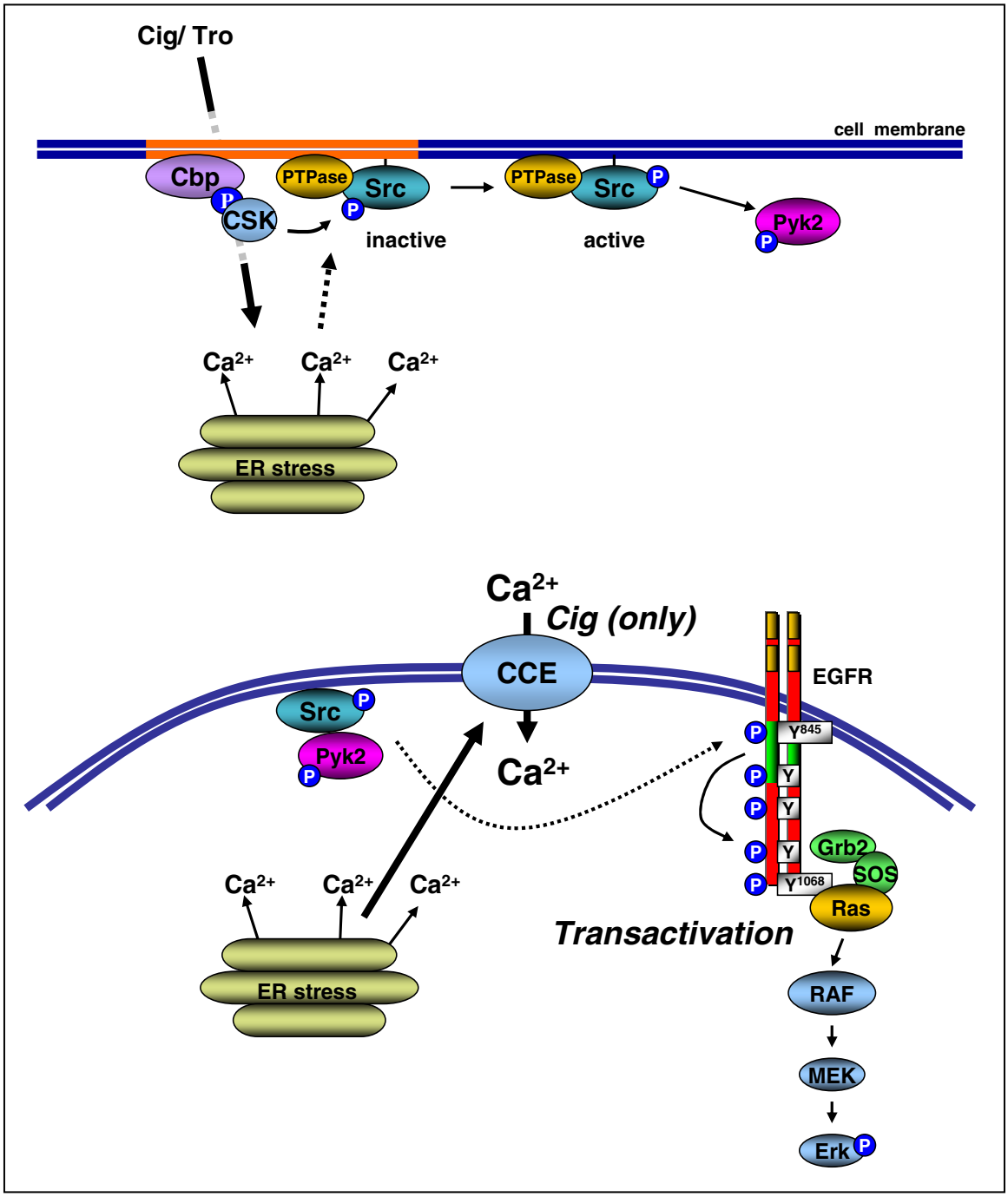

Figure 5.1. Model of differential EGFR transactivation and MAPK activation.

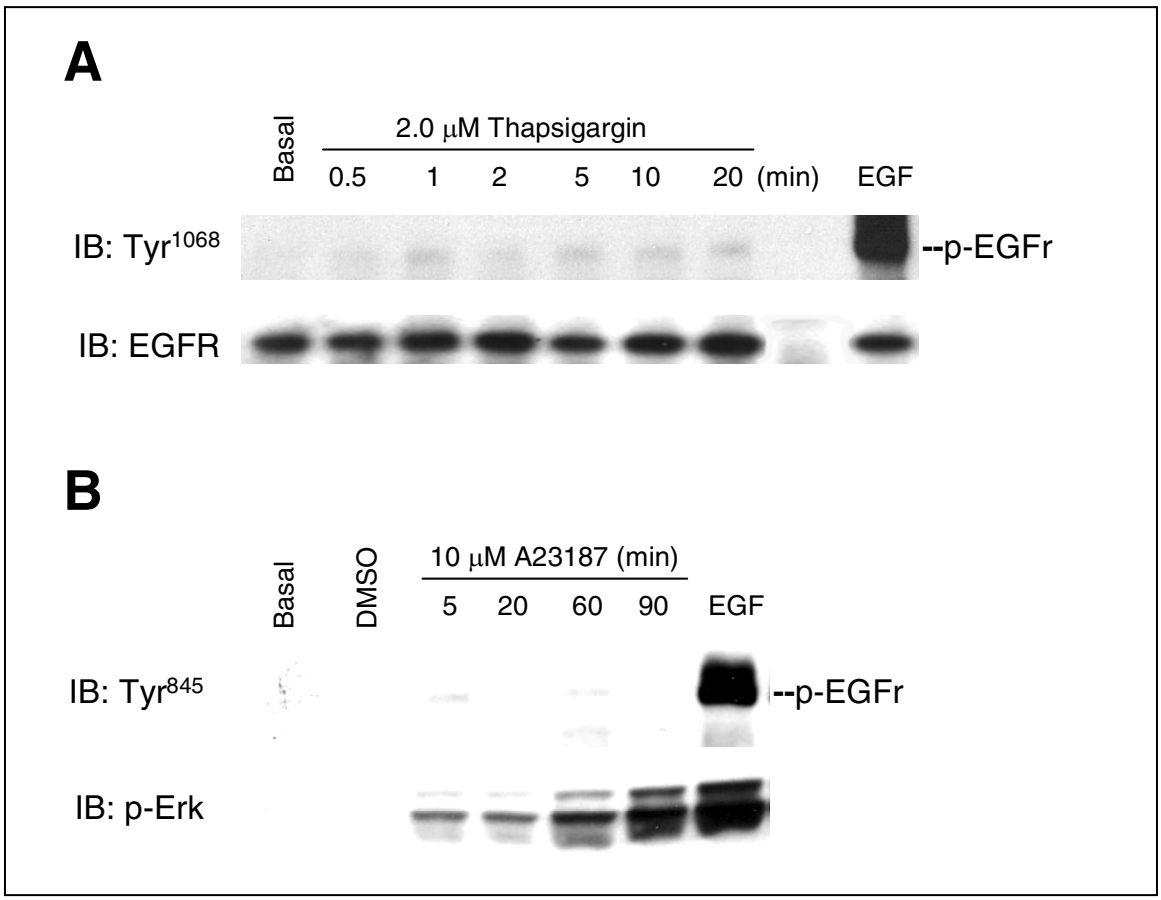

Figure 5.2. EGFR transactivation in response to thapsigargin and ionophore. Confluent serum-starved GN4 cells were treated with (**A**) 2.0 μM thapsigargin or (**B**) 10 μM A23187 (ionophore) for the time indicated in min. Cells were also treated with 100 ng/mL EGF for 5 min as a positive control. Cell lysates were prepared and subjected to SDS-PAGE. EGFR phosphorylation was determined by immunoblotting (IB) using anti-phospho-EGFR Tyr^{845} or Tyr^{1068} antibodies. The blots were stripped and reprobed using antibodies directed against total EGFR, respectively to determine equal protein loading.

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