Peroxisome Proliferator-activated Receptor γ-independent Activation of p38 MAPK by Thiazolidinediones Involves Calcium/Calmodulin-dependent Protein Kinase II and Protein Kinase R

CORRELATION WITH ENDOPLASMIC RETICULUM STRESS*

Oliveia S. Gardner‡§, Chung-Wai Shiau¶, Ching-Shih Chen¶, and Lee M. Graves‡**

From the ‡Curriculum in Toxicology, the ¶Department of Pharmacology, University of North Carolina at Chapel Hill, North Carolina 27599 and the ¶Division of Medicinal Chemistry, College of Pharmacy, The Ohio State University, Columbus, Ohio 43210

The thiazolidinediones (TZDs) are synthetic peroxisome proliferator-activated receptor γ (PPARγ) ligands that promote increased insulin sensitivity in type II diabetic patients. In addition to their ability to improve glucose homeostasis, TZDs also exert anti-proliferative effects by a mechanism that is unclear. Our laboratory has shown that two TZDs, ciglitazone and troglitazone, rapidly induce calcium-dependent p38 mitogen-activated protein kinase (MAPK) phosphorylation in liver epithelial cells. Here, we further characterize the mechanism responsible for p38 MAPK activation by PPARγ ligands and correlate this with the induction of endoplasmic reticulum (ER) stress. Specifically, we show that TZDs rapidly activate the ER stress-responsive pancreatic eukaryotic initiation factor 2α (eIF2α) kinase or PKR (double-stranded RNA-activated protein kinase)-like endoplasmic reticulum kinase/pancreatic eIF2α kinase, and that activation of these kinases is correlated with subsequent eIF2α phosphorylation. Interestingly, PPARγ ligands not only activated calcium/calmodulin-dependent kinase II (CaMKII) 2-fold over control, but the selective CaMKII inhibitor, KN-93, attenuated MKK3/6 and p38 as well as PKR and eIF2α phosphorylation. Although CaMKII was not affected by inhibition of PKR with 2-amino-purine, phosphorylation of MKK3/6 and p38 as well as eIF2α were significantly reduced. Collectively, these data provide evidence that CaMKII is a regulator of PKR-dependent p38 and eIF2α phosphorylation in response to ER calcium depletion by TZDs. Furthermore, using structural derivatives of TZDs that lack PPARγ ligand-binding activity as well as a PPARγ antagonist, we show that activation of these kinase signaling pathways is PPARγ-independent.

The thiazolidinedione (TZD) drug class was created over 20 years ago with the synthesis of ciglitazone, an analog of the hypolipidemic agent clofibrate (1). The TZDs have since grown to include three other members, troglitazone, rosiglitazone, and pioglitazone, which function to improve metabolic control in patients with type II diabetes by increasing insulin sensitivity in adipose tissue, muscle, and liver (2). Following their discovery, it was learned that TZDs are ligands for the gamma isoform of the peroxisome proliferator-activated receptor (PPARγ) (3). From this, it was understood that these agents exert their insulin-sensitizing effects primarily through PPARγ-dependent transcription of genes involved in glucose and lipid metabolism and energy balance.

In addition to their ability to promote effective glycemic control, TZDs have also been shown to exert growth inhibitory effects in multiple cell and animal models (4–7). This is consistent with the observation that PPARγ is both necessary and sufficient to promote adipocyte differentiation (8). However, the sensitivity of various cell lines to TZD-induced growth inhibition does not correlate with levels of PPARγ expression (9). This suggests that TZDs have distinct PPARγ-independent effects that are important for these additional, unanticipated mechanisms of action. In support of this hypothesis, troglitazone was shown to equally inhibit proliferation of both PPARγ−/− and PPARγ+/+ mouse embryonic stem cells (10). In addition, structural derivatives of ciglitazone and troglitazone that lack PPARγ ligand-binding activity were demonstrated to prevent growth of prostate cancer cells.2 Interestingly, these derivatives exhibited more potent growth inhibitory effects

* This work was supported in part by the National Institute of Health with Public Health Service Grants and the Environmental Protection Agency Science to Achieve Results Award R-8292140-1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by the Susan G. Komen Breast Cancer Foundation.

** To whom correspondence should be addressed: Dept. of Pharmacology, University of North Carolina at Chapel Hill, CB# 7365, Chapel Hill, NC 27599-7365. Tel.: 919-966-0915; Fax: 919-966-5640; E-mail: lm@med.unc.edu.

† The abbreviations used are: TZD, thiazolidinedione; PPAR, peroxisome proliferator-activated receptor; ciglitazone, (+)-5-(4-[(1-methylcyclohexyl)oxy]methyl)-5-[4-(1,2,4-thiazolidine-2,4-dione)-3,5-dithiol-2-yl]thiazolidine-2,4-dione; τ2-ciglitazone, (+)-5-[4-(4-chlorobenzyl)-4-thiazolidine-2,4-dione]; troglitazone, (+)-5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-ylmethoxy)benzyl]-2,4-thiazolidinedione; KN-92, troglitazone, (+)-5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-ylmethoxy)benzylidene]-2,4-thiazolidinedione; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; EGFR, epidermal growth factor receptor; CRNK, calcium-dependent tyrosine kinase-related non-kinase; ER, endoplasmic reticulum; PERK, PKR-like endoplasmic reticulum kinase/pancreatic eIF2α kinase; eIF2α, eukaryotic initiation factor 2α; PKC, protein kinase C; CaMKII, calcium/calmodulin-dependent protein kinase II; PKR, double-stranded RNA-activated protein kinase; FBS, fetal bovine serum; Pyk2, proline-rich tyrosine kinase; P2K, 4-amino-5-(4-chlorophenyl)-7-(3-butyl)pyrazolo[3,4-d]pyrimidine; TPA, 12-0-tetradecanoylphorbol-13-acetate; KN-93, 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; KN-92, 2-N-[4-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine-phosphate; GW9662, 2-chloro-5-nitrobenzamide; BAPTA, 1,2 bis(o-aminoethyl)oxysphosphorylethanz-N,N,N’-tetraacetic acid; JNK, c-Jun NH2-terminal kinase; Ad, adenovirus; PACT, PKR-activating protein.

2 C.-W. Shiau, C.-C. Yang, S. K. Kulp, K.-F. Chen, C.-S. Chen, J.-W. Huang, and C.-S. Chen. (2005) Cancer Res. 64, in press.
than their parent analogs. Collectively, these data support the growing list of evidence that TZDs have potentially important PPARγ-independent or “non-genomic” effects.

Both our laboratory and others have shown that TZDs influence mitogen-activated protein kinase (MAPK) activity (11–14). The three best characterized mammalian MAPKs, extracellular signal-regulated kinase (Erk), p38, and c-Jun N-terminal kinase (JNK), are known to play important roles in coordinating a variety of cellular processes, including growth, differentiation, and, in some cases, apoptosis (15). MAPKs are activated via a three-tiered, phospho-relay mechanism whereby a MAPK kinase kinase (MKKK or MEKK) phosphorylates an MAPK kinase (M KK or MEK), which subsequently phosphorylates a MAPK. For example, p38 is selectively phosphorylated by MKK3 and MKK6 (16, 17); on the other hand, multiple MEKs are known to converge on a particular MKK (18). This increases the complexity and selectivity of these signaling pathways such that distinct MAPK cascades can be activated both independently and simultaneously in a stimulus-specific manner. In general, the Erk pathway is activated primarily by mitogens, whereas JNK and p38 are preferentially activated by environmental stresses and inflammatory cytokines. Activation of MAPKs results in phosphorylation of transcription factors that increase the expression of target genes. Thus, in addition to their PPARγ ligand-binding activity, the ability of TZDs to induce MAPK phosphorylation may represent an additional pathway by which these agents affect cell growth and differentiation. It is therefore important to understand how these agents induce MAPK signaling as well as to determine the role of PPARγ in this process.

We previously characterized the mechanism by which ciglitazone and troglitazone acutely activate members of the MAPK family in GN4 rat liver epithelial cells (11). Interestingly, our data suggest that PPARγ is not required for TZD-induced MAPK phosphorylation. In the current study, we have utilized the aforementioned structural derivatives of ciglitazone and troglitazone that are devoid of PPARγ ligand-binding activity in addition to a PPARγ antagonist to provide definitive evidence that MAPK activation by TZDs in GN4 cells is indeed PPARγ-independent. Additionally, we have further characterized the mechanism responsible for p38 activation by TZDs and provide evidence suggesting a link between p38 activation and induction of endoplasmic reticulum stress.

**EXPERIMENTAL PROCEDURES**

**Materials**—The thiazolidinediones ciglitazone and troglitazone, PP2, PP3, and KN-93 were purchased from Biomol. Δ2-Ciglitazone and Δ2-troglitazone, TZD derivatives with attenuated PPARγ ligand-binding activity, were synthesized as described.5 These compounds were prepared as stock solutions in Me2SO and were added to cells in media with a final Me2SO concentration of 0.1% TPA, 2-aminopurine, and thapsigargin were purchased from Sigma, whereas KN-92 was obtained from Calbiochem. GW9662 was from Cayman Chemical. Anti-phospho Erk monoclonal antibody (E-4), anti-Erk polyclonal antibody (C-14), anti-p38 polyclonal antibody (C-20-G), anti-PKR polyclonal antibody (M-515), anti-phosphophorysine (pan) (PT99) monoclonal antibody, and anti-actin polyclonal antibody (I-19) were all from Santa Cruz Biotechnology. Anti-phospho EGFR (Ty r272) anti-EGFR, anti-phospho MKK3/6 (Ser180/Ser181), anti-phospho p38 (Thr180/Tyr182), anti-phospho PERK (Thr381), anti-phospho-p38, and anti-phospho-epiFra (Ser257) polyclonal antibodies were purchased from Cell Signaling. Anti-Pyk2 monoclonal antibody was from BD Transduction Laboratories. Anti-EGFR C-terminal polyclonal antibody (#22) and anti-Pyk2 C-terminal polyclonal antibody (#72) were generated as previously described (19, 20). [γ-32P]ATP was purchased from PerkinElmer Life Sciences. The recombinant adenoviral vector encoding the C terminus of Pyk2, termed CRNK (21), (Ad.CRNK) was constructed and amplified as previously described (22).

**Cell Culture**—GN4 rat liver epithelial cells were grown in Richter’s minimal essential medium supplemented with 10% heat-inactivated FBS and penicillin/streptomycin as detailed earlier (23). A549 human lung carcinoma cells were purchased from ATCC and similarly propagated in Dulbecco’s modified Eagle’s medium/Nutrient mixture F-12 (1/1) supplemented with serum and antibiotics as above as well as 1.5 g/L bicarbonate and 2 mmL-glutamine. Prior to experiments, cells at 70–80% confluency were serum-starved overnight in the appropriate medium containing 0.1% FBS.

**Cell Lysate Preparation**—Following the stimulation for the times indicated, media was aspirated and the cells were rinsed twice with ice-cold phosphate-buffered saline. The cells were then scraped into ice-cold radioimmuno precipitation assay buffer (150 mM NaCl, 9.1 mM Na2HPO4, 1.7 mM NaH2PO4, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS, pH 7.4) with freshly added 200 μM Na3VO4, 250 μM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 10 μM microcystin. To analyze changes in PERK, PKR, and eIF2α phosphorylation, cells were lysed in ice-cold HEPEs buffer (20 mM HEPEs, pH 7.3, 50 μM NaF, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA) supplemented with 1 mM Na3VO4, 500 μM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin. The cell lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4 °C. Protein concentration was determined using the Coomassie protein assay reagent (Pierce).

**Immunoblotting**—In a typical experiment, 10 μg of cell lysate was resuspended in SDS-PAGE sample buffer (0.5 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.1% bromphenol blue) and heated at 95 °C for 5 min to denature proteins. Lysates were then resolved by SDS-PAGE on Novex pre-cast 10% Tris-glycine gels (Invitrogen) and transferred to polyvinylidene fluoride (Immobilon-P, Millipore). Immunoblots were incubated with the appropriate primary antibody overnight at 4 °C, washed 3× with TBST, and probed with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoblots were then developed with ECL (Amershams Pharmacia Biotech) according to the manufacturer’s instructions and visualized by autoradiography (X-Omat Blue film, Eastman Kodak). In certain instances, membranes were stripped in buffer (62.5 μM Tris, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol) at 55 °C for 30 min and reprobed with another antibody.

**Immunoprecipitation**—Following stimulation, cells were rinsed as described above and scraped into ice-cold radioimmuno precipitation assay buffer without SDS. The lysates were cleared by centrifugation. Fifteen microliters of Protein A-agarose beads (Santa Cruz Biotechnology) were added to each sample, which were then incubated an additional hour at 4 °C. Immune complexes were collected by brief centrifugation and washed four times in ice-cold lysis buffer. Remaining wash buffer was carefully removed with a Hamilton syringe. Immune complexes were then resuspended in SDS-PAGE sample buffer and resolved by SDS-PAGE as described above.

**In Vitro CaMKII Kinase Assay**—CaMKII kinase activity in stimulated GN4 cells was measured using a commercial CaMKII assay kit (Upstate Biotechnology) according to the manufacturer’s instructions with slight modification. Briefly, CaMKII was immunoprecipitated from 400 μg of cell lysate in HEPEs binding buffer (20 mM HEPEs, pH 7.7, 50 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 0.5% Triton X-100) by overnight incubation with an anti-CaMKII antibody (H-300, Santa Cruz Biotechnology). CaMKII activity present in immune complexes was assessed by measuring the transfer of [γ-32P]ATP to a specific CaMKII substrate peptide for 10 min at 30 °C. Phosphorylated substrate was then separated from residual [γ-32P]ATP using F-81 phosphocellulose paper (Whatman) and quantified with a scintillation counter.

**RESULTS**

**Src-dependent Activation of EGFR and Erk by Cigitazone Does Not Require PPARγ**—Previously, we have shown that ciglitazone-induced Erk phosphorylation in rat liver epithelial (GN4) cells required Src-dependent EGFR transactivation (11). Interestingly, Src kinase activity was unaffected by a PPARγ antagonist suggesting that ciglitazone activates this signaling pathway via a non-genomic mechanism. We recently obtained derivatives of two thiazolidinediones, Δ2-ciglitazone and Δ2-troglitazone (Fig. 1), which completely lack PPARγ ligand-binding activity. Because pharmacological inhibitors often have nonspecific effects, we tested whether Δ2-ciglitazone retained the ability to activate EGFR and Erk in GN4 cells. Similar to ciglitazone, treatment of GN4 cells with Δ2-ciglitazone...
zone induced rapid, robust EGFR phosphorylation that correlated with Erk activation (Fig. 2, A and B). In contrast, Δ2-troglitazone failed to significantly activate EGFR and Erk, consistent with our previous finding that troglitazone was a much weaker Erk activator than cigitazone in GN4 cells (11). These data demonstrate that the Δ2-derivatives act similarly to their parent compounds with respect to EGFR and Erk phosphorylation.

Because Src is required for EGFR transactivation and downstream Erk phosphorylation by cigitazone (11), we tested whether this kinase played a similar, necessary role in signaling events following exposure to Δ2-cigitazone. Using an antibody that recognizes the EGFR when phosphorylated at Tyr845, a putative Src-specific phosphorylation site (24), we observed that Δ2-cigitazone, like cigitazone, induced Tyr845 phosphorylation (Fig. 3). Pretreatment of cells with PP2, a selective Src kinase inhibitor, completely blocked Δ2-cigitazone-induced EGFR phosphorylation at Tyr845 as well as downstream Erk activation. In contrast, pretreatment of cells with PP3, a pharmacologically inactive analog of PP2, did not affect the ability of Δ2-cigitazone to induce either EGFR or Erk phosphorylation. These data suggest that EGFR transactivation as well as Erk phosphorylation by Δ2-cigitazone requires Src. Collectively, these findings corroborate our earlier studies using a PPARγ antagonist and, importantly, provide additional evidence that TZDs activate PPARγ-independent kinase signaling pathways in GN4 cells.

**Δ2-Thiazolidinediones Are Less Effective Activators of the p38 MAPK Pathway**—In addition to their effects on the EGFR and Erk, we previously showed that both cigitazone and troglitazone rapidly activate p38 in GN4 cells (11). PPARγ ligand-induced p38 phosphorylation occurred in an EGFR-independent manner and was instead correlated with increases in intracellular calcium and activation of the calcium-dependent tyrosine kinase Pyk2. Here, we tested whether Δ2-cigitazone and Δ2-troglitazone retained the ability to similarly activate Pyk2 and p38. Interestingly, both of the Δ2-derivatives showed significantly reduced efficacy in activating p38 when compared with the parent compounds (Fig. 4A). Examination of MKK3/6, the MAP kinase kinase immediately upstream of p38, and Pyk2 phosphorylation (Fig. 4B) revealed similar findings; Δ2-cigitazone and Δ2-troglitazone failed to activate these kinases as strongly as cigitazone and troglitazone, respectively.

To further evaluate the role of PPARγ in TZD-dependent p38 activation, we pretreated GN4 cells with the PPARγ antagonist GW9662 prior to stimulation with cigitazone, troglitazone, or their respective Δ2-derivatives. GW9662 failed to inhibit the ability of cigitazone or troglitazone to activate MKK3/6 and p38 (Fig. 4C). Importantly, co-treatment of GN4 cells with GW9662 and either of the Δ2-TZDs did not potentiate the lack of effect on MKK3/6 and p38 phosphorylation in response to these derivatives alone. Together, these findings suggest that TZD-induced p38 phosphorylation, like Erk activation, is also PPARγ-independent. In support of this hypothesis, structur-
ally related TZDs and more potent PPARγ agonists, rosiglitazone and pioglitazone, were unable to activate p38 MAPK in GN4 cells (data not shown).

Role of Pyk2 as an Upstream Activator of p38—Because our previous studies suggested a link between Pyk2 and p38 MAPK, we further examined if activation of Pyk2 was necessary for the effects of PPARγ ligands on p38 phosphorylation. Specifically, GN4 cells were infected with a recombinant adenovirus encoding either bacterial β-galactosidase (Ad.lacZ) or a C-terminal inhibitory form of Pyk2 (Ad.CRNK) prior to stimulation with PPARγ ligands. CRNK represents an alternative potential splice variant of Pyk2 (21), which functions to negatively regulate endogenous Pyk2 autophosphorylation (25). Angiotensin II-dependent Pyk2 phosphorylation in GN4 cells was reduced to near basal levels following infection with increasing amounts of Ad.CRNK with maximal inhibition observed using 4 × 10⁶ plaque-forming units/ml Ad.lacZ or Ad.CRNK, cells were serum-starved overnight in media supplemented with 0.1% FBS prior to stimulation with ciglitazone (Cig, 50 μM), troglitazone (Tro, 50 μM), or Me2SO (DMSO, 0.1%) for 10 min. Cell lysates were prepared as detailed under “Experimental Procedures.” 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 assessed using an anti-Pyk2 antibody. B, lysates were resolved directly by SDS-PAGE, and immunoblotting was performed as described in the legend to Fig. 4 to detect changes in MKK3/6 and p38 phosphorylation. To verify equal protein loading, the blots were stripped and reprobed with an anti-p38 antibody.

Fig. 5. Ad.CRNK blunts Pyk2 activation by PPARγ ligands but has no affect on MKK3/6 or p38 phosphorylation. Following infection of confluent GN4 cells with 4 × 10⁶ plaque-forming units/ml Ad.lacZ or Ad.CRNK, cells were serum-starved overnight in media supplemented with 0.1% FBS prior to stimulation with ciglitazone (Cig, 50 μM), troglitazone (Tro, 50 μM), or Me2SO (DMSO, 0.1%) for 10 min. Cell lysates were prepared as detailed under “Experimental Procedures.” 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 assessed using an anti-Pyk2 antibody. B, lysates were resolved directly by SDS-PAGE, and immunoblotting was performed as described in the legend to Fig. 4 to detect changes in MKK3/6 and p38 phosphorylation. To verify equal protein loading, the blots were stripped and reprobed with an anti-p38 antibody.
that classic PKCs are not involved in PPARγ phosphorylation was not affected (Fig. 6, data not shown), ciglitazone and troglitazone-induced p38 completely prevented transient, TPA-dependent Erk activation chronic exposure of GN4 cells to TPA. Although PKC depletion MKK3/6 as well as p38 activation by PPARγ agonists, classic PKCs were depleted by chronic exposure of GN4 cells to TPA. Although PKC depletion completely prevented transient, TPA-dependent Erk activation (data not shown), ciglitazone and troglitazone-induced p38 phosphorylation was not affected (Fig. 6A). This result suggests that classic PKCs are not involved in PPARγ ligand-dependent p38 activation.

To determine whether CaMKII is necessary for p38 phosphorylation in response to PPARγ agonists, the ability of ciglitazone and troglitazone to activate p38 in the presence of the selective CaMKII inhibitor, KN-93 (26), was evaluated. Interestingly, pretreatment of GN4 cells with KN-93 blocked both MKK3/6 and p38 activation by PPARγ ligands (Fig. 6B). These effects were specific for KN-93, because KN-92, a pharmacologically inactive analog of KN-93, did not prevent activation of these kinases.

Further, the ability of ciglitazone and troglitazone to directly stimulate CaMKII activity was examined. CaMKII was immunoprecipitated from cell lysates, and in vitro kinase assays were performed using a specific substrate peptide. Ciglitazone and troglitazone significantly increased CaMKII activity 2-fold over vehicle control within 10 min, a time that coincides with maximal p38 phosphorylation (Table I). Consistent with its documented ability to directly inhibit CaMKII (26), KN-93 significantly blunted PPARγ ligand-induced CaMKII activation. In contrast to its effects on CaMKII, MKK3/6, and p38, KN-93 failed to prevent TZD-induced Pyk2 phosphorylation (Fig. 6C). Collectively, these findings support our hypothesis that CaMKII, and not Pyk2, acts upstream of MKK3/6 and p38 in this signaling pathway.

Δ2-Ciglitazone and Δ2-troglitazone were less effective MKK3/6 and p38 activators than their parent TZDs in GN4 cells (Fig. 4). Because MKK3/6 and p38 phosphorylation in response to ciglitazone and troglitazone was KN-93-sensitive, we assessed whether the Δ2-derivatives failed to potently activate CaMKII. Indeed, CaMKII kinase assays revealed that Δ2-ciglitazone and Δ2-troglitazone were significantly weaker CaMKII activators than their parent compounds (Table I). These data further support our finding that CaMKII is necessary for p38 activation in response to PPARγ ligands and is consistent with the inability of Δ2-TZDs to effectively induce MKK3/6 and p38 phosphorylation.

PPARγ Ligands Induce ER Stress and eIF2α Phosphorylation in GN4 Cells—Ciglitazone and troglitazone were reported to rapidly increase cytosolic calcium by inhibiting capacitative calcium entry into the endoplasmic reticulum (ER) (10). Calcium is actively involved in proper protein folding and transport within the ER such that its depletion leads to the accumulation of misfolded proteins and subsequent ER stress (27, 28). To test whether PPARγ ligands induce ER stress in GN4 cells, we assessed the ability of these compounds to rapidly phosphorylate PERK, a recently discovered ER-resident serine/threonine kinase whose activity is increased selectively in response to chemical-induced ER stress (29, 30). Immunoblotting with a phospho-PERK antibody revealed that treatment of GN4 cells with ciglitazone and troglitazone induced a time-dependent increase in PERK activation (Fig. 7A). We also ob-

| CaMKII activity | Treatment | -Fold × MeSO | p |
|-----------------|-----------|--------------|---|
| Cig 1.5         | 1.09 ± 0.22<sup>a</sup> | 0.002        |
| Cig 5           | 1.40 ± 0.05  | 0.06         |
| Cig 10          | 1.97 ± 0.44  | 0.02         |
| Cig + KN-93     | 1.38 ± 0.22<sup>b</sup> | 0.09         |
| Δ2-Cig          | 1.28 ± 0.24<sup>b</sup> | 0.009        |
| Cig + 2-AP      | 1.64 ± 0.44  | 0.22         |
| Tro 1.5         | 1.38 ± 0.42<sup>b</sup> | 0.03         |
| Tro 5           | 1.50 ± 0.30  | 0.04         |
| Tro 10          | 1.97 ± 0.31  | 0.013        |
| Tro + KN-93     | 1.46 ± 0.21  | 0.026        |
| Δ2-Tro          | 1.43 ± 0.30<sup>b</sup> | 0.17         |
| Tro + 2-AP      | 1.65 ± 0.34  |              |

<sup>a</sup> Denotes statistical significant differences from ciglitazone or troglitazone (10-min treatments) as appropriate (p < 0.01; one-way ANOVA).

<sup>b</sup> Denotes statistical significant differences from ciglitazone or troglitazone (10-min treatments) as appropriate (p < 0.05; one-way ANOVA).
PKR is not only involved in ER stress signaling, but was also recently shown to contribute to activation of the p38 pathway in response to both endotoxin and pro-inflammatory cytokines. Similar to our observations with PERK, PKR is also phosphorylated at Ser51 by blocking translation initiation (31). Phosphorylation of the eIF2α subunit of eukaryotic initiation factor 2 (eIF2α) when phosphorylated at Ser51, we observed that eIF2α was also phosphorylated. Using a phospho-specific antibody that detects eIF2α when phosphorylated at Ser51, we observed that ciglitazone and troglitazone induced eIF2α phosphorylation at this site as early as 15 min after treatment and that phosphorylation was sustained for at least 1 h (Fig. 8A). Phosphorylation of eIF2α was also observed in response to thapsigargin (data not shown). Similar to our earlier data, Δ2-cigitazone and Δ2-troglitazone were unable to activate PERK to the same extent as the parent compounds (Fig. 8B).

Role of PKR in PPARy Ligand-dependent p38 Activation—Depletion of ER calcium can also lead to activation of double-stranded RNA-activated protein kinase or PKR (36). Interestingly, PKR is not only involved in ER stress signaling, but was also recently shown to contribute to activation of the p38 pathway in response to both endotoxin and pro-inflammatory cytokines (37). Because PPARy ligand-induced p38 phosphorylation is accompanied by activation of ER stress signaling pathways, we tested whether PKR played a role in TZD-dependent MKK3/6 and p38 phosphorylation. Pretreatment of GN4 cells with 2-aminopurine, an adenine analog inhibitor of PKR activity (38), reduced MKK3/6 and p38 phosphorylation in response to PPARy ligands (Fig. 9A). This inhibitory effect was selective for the p38 pathway, because the EGFR and Erk were still activated by ciglitazone in the presence of 2-aminopurine (data not shown). In addition to MKK3/6 and p38, inhibition of PKR with 2-aminopurine also blunted PPARy agonist-dependent eIF2α phosphorylation (Fig. 9B), a finding consistent with the established role of PKR as an ER stress-induced eIF2α kinase (39).

Because a PKR inhibitor abolished TZD-dependent activation of p38 MAPK and ER stress signaling pathways, we assessed whether these compounds affected PKR activity. Because commercially available phospho-specific PKR antibodies are marketed for detection of human or mouse PKR, and GN4 cells are not amenable to transfection, we determined the effect of TZDs on PKR activity in A549 human lung carcinoma cells. PPARy ligands were recently reported to rapidly induce ER stress in these cells (40), suggesting that pathways similar to those we have demonstrated here in GN4 are also activated in A549. Indeed, stimulation of serum-starved A549 cells with ciglitazone and troglitazone led to a time-dependent increase in MKK3/6 and p38 phosphorylation (Fig. 9C). Interestingly, both TZDs also increased PKR phosphorylation at times that paralleled MAPK activation. Similar to our findings in GN4 cells, troglitazone was a stronger activator of MKK3/6 and p38 than ciglitazone. Consistent with a role for PKR as an upstream mediator of TZD-dependent MAPK phosphorylation, troglitazone also induced greater PKR activation than ciglitazone. Furthermore, pretreatment of A549 cells with 2-aminopurine prior to stimulation with PPARy ligands not only blunted PKR phosphorylation, as expected, but also decreased TZD-induced MKK3/6 and p38 activation (Fig. 9D).

CaMKII Is Correlated with PKR Activation in Response to PPARy Ligands—Collectively, the data presented here suggest a role for both CaMKII and PKR as upstream regulators of PPARy ligand-dependent p38 activation. To determine if

---

**Fig. 7.** PERK is activated by PPARy ligands but not their Δ2-derivatives. A, serum-starved GN4 cells were exposed to ciglitazone (Cig, 50 μM), troglitazone (Tro, 50 μM), or Me2SO (DMSO, 0.1%) as a vehicle control for 1.5, 5, or 10 min as indicated. B, cells were treated with PPARy ligands as described in A or with their Δ2-derivatives (50 μM) for 10 min. Lysates were prepared in HEPES buffer (see “Experimental Procedures”) and resolved by 10% SDS-PAGE. Changes in PERK phosphorylation were detected by immunoblotting (IB) with an anti-phospho PERK antibody. Immunoblotting with an anti-actin antibody was used to verify equal protein loading.

**Fig. 8.** Phosphorylation of eIF2α at Ser51 in response to PPARy ligands and their Δ2-derivatives. A, GN4 cells were stimulated as described in the legend to Fig. 7 for 15, 30, 45, or 60 min as indicated. B, cells were treated with PPARy agonists or their respective Δ2-derivatives (50 μM) for 60 min. Increases in eIF2α phosphorylation at Ser51 were determined by immunoblotting (IB) with an anti-phospho eIF2α (p-eIF2α) antibody. Blots were then stripped and reprobed with an anti-actin antibody.
activation of these two kinases are correlated in response to PPARγ agonists, we tested whether PKR inhibition could influence TZD-induced CaMKII activation. In the presence of 2-aminopurine, PPARγ ligand-dependent CaMKII activity was not affected (Table I), suggesting that CaMKII is activated independently of PKR. We then performed the converse of this experiment and assessed whether CaMKII inhibition influenced PKR activity. Interestingly, the CaMKII inhibitor KN-93, but not the structurally inactive analog KN-92, significantly blunted PPARγ ligand-dependent PKR phosphorylation (Fig. 10A). Similar results were observed for eIF2α; eIF2α phosphorylation in response to ciglitazone and troglitazone was blunted only in the presence of KN-93 (Fig. 10B). Because activation of p38 in response to ciglitazone and troglitazone was also KN-93- and 2-aminopurine-sensitive, we determined whether p38 was necessary for eIF2α phosphorylation. In contrast to KN-93, inhibition of p38 with SB203580 had no affect on PPARγ ligand-induced eIF2α phosphorylation (data not shown). Because PERK is not only known to phosphorylate eIF2α in response to ER stress but is also activated in GN4 cells by ciglitazone and troglitazone, we tested whether KN-93 affected PPARγ ligand-induced PERK activation. Pretreatment of cells with KN-93 was unable to prevent PERK phosphorylation in response to TZDs (data not shown). Collectively, these data suggest that CaMKII-dependent activation of PKR is critical for both p38 and eIF2α phosphorylation by PPARγ ligands.

**DISCUSSION**

The observation that PPARγ ligands rapidly activate MAPKs by our laboratory and others (11–14) raises three important questions: 1) What is the mechanism for activation of MAPKs by these compounds? 2) Is PPARγ ligand-binding activity required for MAPK phosphorylation? and 3) Does PPARγ...
ligand-induced MAPK phosphorylation contribute to the pharmacological effects of these agents? Our previous work, which focused on how PPARγ agonists activated MAPKs in a rat liver epithelial cell line, showed that Erk phosphorylation required Src-dependent EGFR transactivation (11). Moreover, we found that p38 activation occurred independently of the EGFR and instead was correlated with Pyk2 phosphorylation and increases in intracellular calcium. In the current study, we have elaborated on the mechanism of p38 activation by PPARγ ligands and also determined the role of PPARγ in this process.

To directly address the question of whether PPARγ is necessary for TZD-induced MAPK activation in GN4 cells, we have utilized chemical derivatives of these agents that are devoid of ligand-binding activity. The rationale for synthesizing the Δ2-derivatives came from the observation that insertion of a double bond adjoining the terminal thiazolidine-2,4-dione ring abolished PPARγ ligand-binding activity (41), presumably due to increased structural rigidity surrounding the heterocyclic system. Here, we provide evidence that Δ2-ciglitazone retained the ability to induce Src-dependent EGFR phosphorylation and downstream Erk activation. This clearly shows that PPARγ is not required for TZD-induced Erk phosphorylation in GN4 cells and supports our earlier studies with a PPARγ antagonist (11).

Interestingly, Δ2-ciglitazone and Δ2-troglitazone failed to activate CaMKII, Pyk2, and p38 with the same efficacy as their parent compounds. Based on our earlier data examining the effects of ciglitazone and Δ2-ciglitazone on EGFR/Erk signaling, these findings were both unexpected and intriguing. Although this observation suggests a role for PPARγ in these signaling events, the PPARγ antagonists GW9662 had no effect on TZD-dependent p38 phosphorylation. Moreover, structurally related TZDs with higher PPARγ binding affinity do not activate p38 in this cell model. Together, these observations suggest that activation of the p38 MAPK pathway in GN4 cells by ciglitazone and troglitazone is non-genomic. As opposed to a requirement for PPARγ, we propose that the failure of the Δ2-TZDs to strongly activate p38 results instead from an inability of these compounds to efficiently mobilize calcium from the ER. This hypothesis is discussed in more detail below.

While investigating the mechanism of PPARγ ligand-induced p38 phosphorylation, we observed that p38 and Pyk2 are activated by parallel but independent pathways (Fig. 11). Although the role of Pyk2 as an upstream activator of p38 MAPK has been reported in response to several different stimuli (11, 22, 42), the lack of a highly selective Pyk2 inhibitor has made this difficult to substantiate. CRNK (a C-terminal inhibitory form of Pyk2) is a Pyk2-targeted inhibitor thought to compete with the full-length protein for binding partners necessary for activation (25). Adenovirus expression of CRNK significantly reduced Pyk2 activation by ciglitazone and troglitazone as expected, yet had no effect on either MKK3/6 or p38 phosphorylation by PPARγ ligands. Although these data do not refute our earlier finding that Pyk2 and p38 activations are correlated, it further defines this mechanism. Specifically, the current studies illustrate that Pyk2 and p38 phosphorylations in response to PPARγ ligands occur via calcium-dependent pathways that may be parallel but independent.

The data presented here identify a role for CaMKII in TZD-induced p38 phosphorylation. CaMKII has previously been implicated as an activator of the p38 MAPK pathway in response to calcium signals in neurons (43). In the current study, both ciglitazone and troglitazone time dependently increased CaMKII kinase activity ~2-fold over vehicle control at times that coincide with maximal p38 phosphorylation. Moreover, the selective CaMKII inhibitor KN-93, but not its structurally inactive analog KN-92, reduced PPARγ ligand-dependent MKK3/6 and p38 activation to near basal levels yet had no affect on Pyk2 activation. Collectively, these data not only support our findings with CRNK, but also further suggest that Pyk2 and p38 are activated by separate pathways. Instead, CaMKII is a critical upstream activator of p38 phosphorylation in GN4 cells to response to PPARγ ligands. Although PKC is also activated by increases in intracellular calcium, we found no evidence (by TPA down-regulation) for involvement of this kinase family in PPARγ agonist-dependent p38 activation. Similar to our previous findings with BAPTA-AM (11), neither CRNK overexpression nor KN-93 prevented EGFR and Erk phosphorylation in response to these compounds (data not shown). Importantly, this provides further evidence that PPARγ ligands activate two distinct kinase pathways in GN4 cells.

We hypothesize that the intracellular calcium required for activation of these kinases in GN4 cells is derived from the endoplasmic reticulum (ER). Ciglitazone and troglitazone were previously shown to promote calcium release from the ER leading to PKR-dependent phosphorylation of eIF2α, translation inhibition, and ultimately growth arrest (10). Together, these observations suggest that PPARγ ligands cause ER stress. In support of this idea, we show here that TZDs not only induce eIF2α phosphorylation but also activate PERK and PKR. Although TZD-dependent PKR activation was previously reported (10), this is the first evidence that these compounds also influence PERK activity; our finding is significant because PERK phosphorylation was previously observed only in response to ER and not cytoplasmic stress (30). Moreover, PERK and PKR are important for ER stress-induced eIF2α phospho-
rlation (39, 44). Indeed, a PKR-selective inhibitor attenuated PPARγ ligand-dependent eIF2α phosphorylation in GN4 cells. Collectively, these data suggest that intracellular calcium mobilization by PPARγ ligands in GN4 cells interferes with proper protein folding in the ER thus promoting ER stress.

These findings have led us to hypothesize that disruptions in ER calcium homeostasis are not only important for ER stress, but also provide a mechanistic link identifying the intracellular source of calcium required for PPARγ ligand-induced CaMKII, Pyk2, and p38 activation (Fig. 11). Evidence for p38 as a transducer of ER stress has been documented previously, because this MAPK was shown to phosphorylate multiple ER stress-induced transcription factors (e.g. activating transcription factor 2, activating transcription factor 6, and growth arrest and DNA damage-inducible gene 153), leading to increases in transcriptional activity (45–47). In addition, we provide evidence here that ER stress-responsive eIF2α kinases are not only activated by PPARγ ligands, but that PKR contributes to PPARγ agonist-dependent MKK3/6 and p38 activation.

Although PKR has been implicated as an upstream activator of p38 in the innate immune response (37), this is the first evidence that PKR can influence ER stress-induced p38 phosphorylation. Although the mechanism by which PKR activates p38 remains unclear, it was recently shown that PKR physically interacts with MKK6 in response to double-stranded RNA, forming a catalytic complex and thus facilitating p38 phosphorylation (48). These previous studies along with the current data using 2-aminopurine suggest that PKR acts as an important upstream mediator of TZD-dependent p38 activation. However, our findings do not discount the potential role of additional, unknown MKK kinases that could also contribute to p38 phosphorylation in this model. Specifically, 2-aminopurine completely blunts PKR activation in response to PPARγ ligands, but modest MKK3/6 and p38 phosphorylation is still observed. Furthermore, the time course for PKR activation in A549 cells closely parallels rather than precedes that of MKK3/6 and p38. Although this could be due to a lack of sensitivity in the immunoblot assay, additional studies in the current model are necessary to clarify a direct relationship between PKR and p38 as previously reported in different cell types. Importantly, these data identify the ER as a potential key mediator in TZD-induced signaling in GN4 cells (Fig. 11); however, future studies are needed to determine a causal role for the ER in this mechanism.

Interestingly, the data presented here support a role for both CaMKII and PKR as regulators of the p38 MAPK and eIF2α pathways in response to PPARγ ligands. Although double-stranded RNA is the classic PKR stimulator, the activity of this kinase is also increased in double-stranded RNA-independent conditions by a novel protein activator PKR-activating protein (also known as RAX) (49, 50). In response to cellular stresses such as thapsigargin, PACT is phosphorylated on serine residues and associates with PKR leading to increases in kinase activity and eIF2α phosphorylation (50, 51). Although serine phosphorylation of PACT occurs at putative CaMKII consensus sites, the kinase responsible for PACT phosphorylation has not been identified. Based on our current findings, we propose that CaMKII via PACT activates PKR in response to PPARγ ligands; PKR then facilitates downstream MKK3/6 and p38 as well as eIF2α phosphorylation (Fig. 11).

Consistent with their inability to potently activate CaMKII, Pyk2, and p38, Δ2-TZDs also failed to induce PERK phosphorylation to the same extent as their parent compounds. Thus, in the pathways we examined in GN4 cells, Δ2-cigitazone and Δ2-troglitazone differ from cigitazone and troglitazone only in their ability to activate the "calcium-regulated" kinases. Although we have shown that PPARγ ligand-induced Pyk2 and p38 phosphorylation in GN4 cells is calcium-dependent (11), activation of PERK, PKR, and CaMKII in response to intracellular calcium release has been documented extensively in the literature. On the other hand, BAPTA-AM, Ad.CRNK, KN-93, and 2-aminopurine failed to significantly inhibit PPARγ ligand-dependent EGFR and Erk phosphorylation (data not shown). This observation suggests that activation of this latter signaling pathway is calcium-independent and may therefore explain why Δ2-cigitazone, which does not potently activate p38, induces EGFR and Erk phosphorylation to the same extent as cigitazone. We therefore hypothesize that the failure of the Δ2-derivatives to potently induce phosphorylation of certain kinases in GN4 cells is related to their inability to promote ER calcium release. Consistent with this hypothesis, Δ2-cigitazone and Δ2-troglitazone were unable to induce eIF2α phosphorylation to the same extent as the parent compounds.

In summary, we provide evidence that the PPARγ ligands cigitazone and troglitazone activate CaMKII and that this calcium-activated kinase, as opposed to Pyk2, is essential for downstream MKK3/6 and p38 phosphorylation. Activation of MAPKs is correlated with phosphorylation of the ER stress-sensitive kinase PERK and subsequent eIF2α phosphorylation. We identify CaMKII as a potential novel regulator of PKR in response to ER stress and demonstrate that PKR plays a necessary role in activation of the p38 pathway in response to ER calcium depletion. The ability of cigitazone and troglitazone to induce ER stress as well as activation of MAPKs may play an important role in their effects on cell growth and maturation. Consistent with earlier studies, we found here that both cigitazone and troglitazone significantly reduced GN4 cell viability (data not shown). In contrast, rosiglitazone and pioglitazone not only failed to induce MAPKs and eIF2α phosphorylation in this cell model, but also were unable to significantly affect GN4 cell viability suggesting that these kinases play a necessary role in PPARγ-ligand-induced cell inhibition. Collectively, these observations suggest that TZDs induce PPARγ-independent signaling events that have potential relevance to the mechanism responsible for their antitumor activity in a variety of cancers.

REFERENCES

1. Sohda, T., Mizuno, K., Imamiya, E., Sugiyama, Y., Fujita, T., and Kawamatsu, Y. (1982) Chem. Pharm. Bull. 30, 3580–3600

2. Olefsky, J. M. (2000) J. Clin. Invest. 106, 467–472

3. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkinson, W. O., Wilson, T. M., and Kiehler, S. A. (1995) J. Biol. Chem. 270, 12953–12956

4. Kubota, T., Koshizuka, K., Williamson, E. A., Asou, H., Said, J. W., Holden, S., Miyoshi, I., and Koeffler, H. P. (1998) Cancer Res. 58, 3344–3352

5. Tsubouchi, Y., Sano, H., Kawahito, Y., Muki, S., Yamada, R., Kehno, M., Inoue, K., Hla, T., and Kondo, M. (2000) Biochem. Biophys. Res. Commun. 270, 400–406

6. Kistner, E., Muller, C., Koshizuka, K., Williamson, E. A., Park, D., Asou, H., Shintaku, P., Said, J. W., Heber, D., and Koeffler, H. P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8806–8811

7. Demetri, G. D., Fletcher, C., Mueller, E., Sarraf, P., Naouktsis, R., Campbell, N., Spiegelman, B. M., and Singer, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3951–3956

8. Tontezon, P., Hu, E., and Spiegelman, B. M. (1994) Cell 79, 1147–1156

9. Mueller, E., Sarraf, P., Tontezon, P., Evans, R. M., Martin, R. J., Zhang, M., Fletcher, C., Singer, S., and Spiegelman, B. M. (1998) Mol. Cell 1, 465–470

10. Palakurthi, S. S., Aktas, H., Grubissich, L. M., Mortensen, R. M., and Halperin, J. A. (2001) Cancer Res. 61, 6213–6218

11. Gardner, O. S., Dewar, B. J., Earp, H. S., Samet, J. M., and Graves, L. M. (2003) J. Biol. Chem. 278, 46261–46269

12. Baek, S. J., Wilson, L. C., Hsi, L. C., and Eling, T. E. (2003) J. Biol. Chem. 278, 5845–5853

13. Turel, T., Hernandez, R., Benito, M., and Lorenzo, M. (2003) J. Biol. Chem. 278, 263–269

14. Islam, A. M., Ramage, M., Dessouroux, A., and Pierre, M. (2002) J. Biol. Chem. 277, 29681–29685

15. Johnson, G. L., and Lapadat, R. (2002) Science 298, 1911–1912

16. Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) Science 267, 682–685

17. Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gothy, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E., and Hagwara, H. (1996) J. Biol. Chem. 271, 13675–13679
18. Kyriakis, J. M., and Avruch, J. (2001) *Physiol. Rev.* 81, 807–869
19. Li, X. L., Lee, J. W., Graves, L. M., and Earp, H. S. (1998) *EMBO J.* 17, 2574–2583
20. Yu, H., Li, X. L., Marchetto, G., Dy, R., Hunter, D., Calvo, B., Dawson, T. L., Wilm, M., Anderigg, R., Graves, L. M., and Earp, H. S. (1996) *J. Biol. Chem.* 271, 29993–29998
21. Schaller, M. D., and Sasaki, T. (1997) *J. Biol. Chem.* 272, 25319–25325
22. Yu, H., Li, X. L., Marchetto, G., Dy, R., Hunter, D., Calvo, B., Dawson, T. L., Wilm, M., Anderigg, R., Graves, L. M., and Earp, H. S. (1996) *J. Biol. Chem.* 271, 29993–29998
23. Earp, H. S., Huckle, W. R., Dawson, T. L., Li, X. L., Graves, L. M., and Dy, R. (1995) *J. Biol. Chem.* 270, 28440–28447
24. Biscardi, J. S., Maa, M.-C., Tice, D. A., Cox, M. E., Leu, T.-H., and Parsons, S. J. (1999) *J. Biol. Chem.* 274, 8335–8343
25. Li, X., Dy, R., Cance, W., Graves, L. M., and Earp, H. S. (1999) *J. Biol. Chem.* 274, 8917–8924
26. Sumi, K., Kiuchi, K., Ishikawa, T., Ishii, A., Hagiwara, M., Nagatsu, T., and Hidaka, H. (1991) *Biochem. Biophys. Res. Commun.* 181, 968–975
27. Lodish, H. F., and Kong, N. (1990) *J. Biol. Chem.* 265, 10893–10899
28. Kozutsumi, Y., Segal, M., Normington, K., Gething, M. J., and Sambrook, J. (1988) *Nature* 332, 462–464
29. Shi, Y., Vattem, K. M., Sood, R., An, J., Liang, J., Stramm, L., and Wek, R. C. (1998) *Mol. Cell. Biol.* 18, 7499–7509
30. Harding, H. P., Zhang, Y., and Ron, D. (1999) *Nature* 397, 271–274
31. Kaufman, R. J. (2004) *Trends Biochem. Sci.* 29, 152–158
32. Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S., and Kaufman, R. J. (2001) *Mol. Cell.* 7, 1165–1176
33. Berlanga, J. J., Herrera, S., and de Haro, C. (1998) *J. Biol. Chem.* 273, 32340–32346
34. Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. F., and Hinnenbusch, A. G. (1992) *Cell* 68, 585–596
35. Chong, K. L., Feng, L., Schappert, K., Meurs, E., Donahue, T. F., Friesen, J. D., Hovanesian, A. G., and Williams, B. R. (1992) *EMBO J.* 11, 1553–1562
36. Prostko, C. R., Dholakia, J. N., Broström, M. A., and Broström, C. O. (1995) *J. Biol. Chem.* 270, 6211–6215
37. Geh, K. C., de Veer, M. J., and Williams, B. R. (2000) *EMBO J.* 19, 4292–4297
38. Tiwari, R. K., Kasai, J., Kumar, R., and Sen, G. C. (1998) *Mol. Cell. Biol.* 8, 4289–4294
39. Srivastava, S. P., Davies, M. V., and Kaufman, R. J. (1995) *J. Biol. Chem.* 270, 16619–16624
40. Fan, Y. H., Chen, H., Natarajan, A., Gus, Y., Harhinski, F., Iyasa, J., Christ, W., Aktas, H., and Halperin, J. A. (2004) *Bioorg. Med. Chem. Lett.* 14, 2547–2550
41. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) *Cell* 83, 803–812
42. Pandey, P., Avraham, S., Kumar, S., Nakazawa, A., Place, A., Ghanem, L., Rana, A., Kumar, V., Majumder, P. K., Avraham, H., Davis, R. J., and Kharbanda, S. (1999) *J. Biol. Chem.* 274, 10140–10144
43. Takeda, K., Matsuoka, A., Nishitoh, H., Tobiume, K., Kishida, S., Ninomiya-Tsuji, J., Matsuzato, K., and Ichijo, H. (2004) *EMBO Rep.* 5, 161–166
44. Harding, H. P., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000) *Mol. Cell* 5, 897–904
45. Wang, X. Z., and Ron, D. (1996) *Science* 272, 1347–1349
46. Luo, S., and Lee, A. S. (2002) *Biochem. J.* 366, 787–795
47. Chen, K. D., Chen, L. Y., Huang, H. L., Lieu, C. H., Chang, Y. N., Chang, M. D., and Lai, Y. K. (1998) *J. Biol. Chem.* 273, 749–755
48. Silva, A. M., Whitmore, M., Xu, Z., Jiang, Z., Li, X., and Williams, B. R. (2004) *J. Biol. Chem.* 279, 37670–37676
49. Patel, R. C., and Sen, G. C. (1998) *EMBO J.* 17, 4379–4390
50. Bo, T., Yang, M., and May, W. S. (1999) *J. Biol. Chem.* 274, 15427–15432
51. Bennett, R. L., Bialock, W. L., and May, W. S. (2004) *J. Biol. Chem.* 279, 42687–42693