A Linear Signal Transduction Pathway Involving Phosphatidylinositol 3-Kinase, Protein Kinase Cε, and MAPK in Mesangial Cells Regulates Interferon-γ-induced STAT1α Transcriptional Activation*\[S]

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Interferon-γ (IFN-γ) exerts a pleiotropic effect in mesangial cells in inflammatory glomerular diseases. The biologic effect of IFN-γ is mediated by STAT1α. The precise mechanism by which IFN-γ stimulates the transcriptional activity of STAT1α is poorly understood. I investigated the role of protein kinase C (PKC) ε in regulating the transcriptional activation of STAT1α in mesangial cells. IFN-γ increased PKCε activity in a time-dependent manner with a concomitant increase in STAT1α transcriptional activity. Expression of constitutively active PKCε mimicked the effect of IFN-γ on STAT1α-dependent transcription. Expression of dominant negative PKCε inhibited IFN-γ-induced STAT1α-dependent transcription. Ly294002, a pharmacological inhibitor of phosphatidylinositol (PI) 3-kinase, blocked IFN-γ-induced PKCε activity and resulted in inhibition of STAT1α transcriptional activity but had no effect on STAT1α tyrosine phosphorylation and STAT1α-DNA complex formation. A PKC inhibitor, H7, also had no effect on STAT1α tyrosine phosphorylation and DNA binding. However, Ly294002 and H7 blocked IFN-γ-induced serine phosphorylation of STAT1α. These data indicate that PI 3 kinase-dependent PKCε regulates STAT1α transcriptional activity in the absence of any effect on its DNA binding capability. In addition to activating PKCε, IFN-γ increased MAPK activity, resulting in transcriptional activation of Elk-1, a nuclear target of MAPK. Ly294002 or a dominant negative PI 3-kinase significantly blocked IFN-γ-induced MAPK activity. On the other hand, expression of constitutively active PKCε significantly increased MAPK activity. IFN-γ-stimulated MAPK phosphorylated STAT1α in vitro. Inhibition of MAPK activity blocked IFN-γ-induced serine phosphorylation of STAT1α; but its tyrosine phosphorylation and DNA binding were partially inhibited. Finally, expression of dominant negative MAPK significantly inhibited IFN-γ-induced STAT1α-dependent transcription. These data provide the first evidence that IFN-γ stimulates PKCε in a PI 3-kinase-sensitive manner to activate MAPK, which regulates STAT1α transcriptional activity.

Interferon-γ (IFN-γ) regulates innate and adaptive immunity, causes cell growth inhibition, and mediates activation of antiviral activity (1–6). IFN-γ also plays an important role in the pathogenesis of many inflammatory diseases involving the kidney (7–10). IFN-γ elicits its biological effects through heterodimeric receptors 1 and 2, which are constitutively associated with two tyrosine kinases JAK1 and JAK2 (11). Binding of the ligand stimulates conformational changes in the receptor membrane proximal region, which juxtaposes these tyrosine kinases to enable their cross-phosphorylation and activation (11, 12). JAKs then phosphorylate IFN-γ receptor 1 at tyrosine 440, creating a docking site for the downstream Src homology 2 domain containing protein, signal transducer and activator of transcription 1 (STAT1α) (13, 14). STAT1α, which is recruited to the JAK-receptor complex, is phosphorylated in the C terminus at a conserved tyrosine residue (tyrosine 701). Tyrosine-phosphorylated STAT1α then dissociates from the receptor complex and undergoes reciprocal dimerization. The dimer then translocates to the nucleus by the nuclear receptors, importin-α5 and Ran (15). In the nucleus, STAT1α dimer associates with other transcription factors and transcriptional activators to result in binding to cognate DNA sequences and to stimulate transcription of target genes (15).

The kidney glomerulus is a target of diverse pathological processes including immune-modulated diseases such as glomerulonephritis. Mesangial cells constitute one-third of the cell types in the glomerulus and contribute to pathogenesis of many forms of glomerulonephritis (16). Administration of IFN-γ modestly inhibited glomerular cell proliferation in vivo in a rat model of mesangial proliferative glomerulonephritis (17). Despite its growth inhibitory effect, however, IFN-γ did not abolish overall extracellular matrix production, which is also part of the disease phenotype (17). In mesangial cells, IFN-γ alone stimulates STAT1α and inhibits basal DNA synthesis (18). Interestingly, we (19) have previously shown that IFN-γ cooperates with platelet-derived growth factor and epidermal growth factor to synergistically stimulate DNA synthesis in mesangial cells. This action of IFN-γ correlates with the activation of STAT1α.

Homozygous deletion of STAT1α in mice demonstrated that this transcription factor mediates the biological function of...
IFN-γ (20, 21). It has recently been shown that macrophages isolated from protein kinase Cε (PKCe) null cells lack the IFN-γ-induced response, indicating a positive regulatory role for PKCe in IFN-γ signaling (22). Signal transduction pathways have been extensively studied in IFN-γ-induced transcriptional activation of STAT1α. In addition to JAK1/ JAK2-mediated tyrosine phosphorylation, phosphorylation of STAT1α on a conserved serine residue (serine 727) also plays an important role in STAT1α activity (23). The specific kinase responsible for phosphorylating STAT1α, however, has not been conclusively identified. Involvement of Erk1/2 (MAPK), c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase has been reported in an agonist-dependent manner in different cell types and a role for MAPK in IFN-γ-induced STAT1α serine phosphorylation has also been argued (23). More recently phosphatidylinositol (PI) 3-kinase/Akt signaling has been shown to regulate STAT1α serine phosphorylation in tumor cells and fibroblasts (24).

In this report, I examined the mechanism of IFN-γ-induced STAT1α-dependent transcription in mesangial cells. I show that IFN-γ stimulates PKCe, which in turn activates STAT1α-mediated transcription. I demonstrate that PI 3-kinase regulates IFN-γ-induced PKCe phosphorylation and activity. Inhibition of PI 3-kinase prevents IFN-γ-induced serine phosphorylation of STAT1α without any effect on its tyrosine phosphorylation and DNA binding, resulting in inhibition of STAT1α-dependent transcription. I demonstrate that, IFN-γ-induced MAPK activity is regulated by PI 3-kinase and PKCe. Finally, I show that MAPK increases STAT1α transcriptional activity with minimal effect on its DNA binding capability. These results suggest that IFN-γ stimulates a linear signal transduction pathway in which PI 3-kinase stimulates PKCe, which in turn activates MAPK to stimulate transcriptional activation of STAT1α.

EXPERIMENTAL PROCEDURES

Materials—Recombinant IFN-γ was obtained from Invitrogen. Phenethylsulfonyl fluoride, Nonidet P-40, and tubulin antibody were purchased from Sigma. Aprotinin was obtained from Bayer. H7 was supplied by Dr. Thomas W. Sturgil, University of Virginia. The cells were maintained with RPMI 1640 medium containing 1% fetal bovine serum as described before (25–27). The cells were made quiescent by incubating in serum-free RPMI 1640 medium for 48 h. 293 cells were grown in Dulbecco’s modified Eagle’s medium with high glucose and made quiescent by incubating in serum-free medium for 48 h. Adenovirus infection of mesangial cells were done essentially as previously described (27, 28).

RESULTS

IFN-γ Increases Novel PKCe Activity in Mesangial Cells—In IFN-γ signaling the nature of the STAT1α kinase is not precisely known. Because macrophages from PKCe null mice display an impaired response to IFN-γ (22), and mesangial cells abundantly express this novel PKC isoform (36, 37), the effect of IFN-γ on activation of PKCe was examined. Lysates of mesangial cells incubated with IFN-γ were immunoprecipitated with PKCe-specific antibody followed by the immune complex kinase assay using a peptide substrate. IFN-γ increased PKCe activity in a time-dependent manner (Fig. 1A). It has recently been shown that phosphorylation of a serine residue (Ser-729) in the C-terminal hydrophobic domain of PKCe is necessary for the activation of this novel PKC isoform (38). To examine this activation in mesangial cells, lysates of cells incubated with IFN-γ were immunoprecipitated with PKCe antibody. The immunoprecipitates were used for the immune complex kinase assay using a PKC assay kit (30, 31). Briefly, The PKCe immunoprecipitates were resuspended in assay buffer containing phosphatidylserine and 12-O-tetradecanoylphorbol-13-acetate in the presence of an acetylated peptide derived from myelin basic protein (MBP) and [γ-32P]ATP. The reaction mixture was incubated at 30 °C for 5 min. An aliquot of the reaction mixture was spotted onto DE-52 filter disks in duplicate. The disks were washed free of unincorporated [γ-32P]ATP and counted by scintillation spectrometry. The specific counts per minute values were converted into picomoles. The PKC activity was expressed as picomoles per minute.

Transient Transfection and Luciferase Assay—The reporter in which the firefly luciferase gene is driven by either four copies of cis-inducible element (SIE) or eight copies of the GAS element and indicated plasmids were cotransfected into mesangial cells with a Renilla luciferase plasmid using LipofectAMINE Plus reagent as described (25–29). Luciferase activity was measured using the dual luciferase assay kit as suggested by the vendor. To correct for transfection efficiency, the ratio of firefly and Renilla luciferase activity was considered as the relative luciferase activity and plotted as arbitrary units as mean of triplicate determinations ± S.E.

Electrophoretic Mobility Shift Assay (EMSA)—Mesangial cells were incubated with IFN-γ in the presence and absence of the indicated kinase inhibitors. The 5′-bp double stranded oligonucleotide probe (SIE) was labeled with [γ-32P]ATP and T4 poly nucleotide kinase as described before (32). The EMSA was performed with the cell lysate essentially as described before (19, 32–34). For supershift analysis, the cell lysates were incubated with the STAT1α antibody before incubation with the probe (19, 33, 34). The protein-DNA complex was separated by 5% polyacrylamide gel electrophoresis.

Northern Analysis—Total RNA was isolated from mesangial cells using RNAzol. Northern analysis was performed as described previously using murine MCP-1 as probe (30, 31).

Immunofluorescence—Immunofluorescence detection of STAT1α was performed essentially as described previously (28). Briefly, the cells were plated in eight-chamber slides. Quiescent cells were infected with an adenovector expressing the myristoylated p110 catalytic subunit of PI-3 kinase for 24 h or treated with IFN-γ for 15 min. Treated cells were fixed in cold methanol for 15 min, blocked by donkey IgG for 15 min. The cells were then incubated with the monoclonal STAT1α antibody for 30 min. Stained cells were then incubated with Cy3-tagged secondary anti-mouse antibody for 30 min. The cells were then visualized using a confocal microscope (Olympus Fluoview 500) and photographed using Fluoview software.

MAPK Assay—Lysates of mesangial cells were immunoprecipitated with MAPK antibody as described above. The immunoprecipitates were resuspended in MAPK assay buffer. The reaction was started with 1 μCi of [γ-32P]ATP in the presence of MBP as substrate (25, 26, 35). Phosphorylated MBP was separated by 15% polyacrylamide gel electrophoresis.

Data Analysis—The significance of the data was determined by analysis of variance.
IFN-γ were immunoblotted with phospho-PKCε antibody that specifically recognizes the phosphorylated serine 729 residue. IFN-γ increased phosphorylation of PKCε, indicating activation of this isozyme (Fig. 1B, lane 2).

PI 3-Kinase Regulates IFN-γ-induced PKCε—Many PKC isoforms have been shown to be associated with the PI 3-kinase target PDK1 (39). Furthermore, in the serum-induced hydrophobic domain, serine phosphorylation of novel PKCs is sensitive to the pharmacological inhibitor of PI 3-kinase Ly294002 (38, 39). Therefore, the role of PI 3-kinase in IFN-γ-induced PKCε phosphorylation was examined. Ly294002 blocked phosphorylation of PKCε resulting in inhibition of its enzymatic activity in response to IFN-γ (Fig. 1, B and C, respectively). These data indicate that IFN-γ increases PKCε activity in a PI 3-kinase-dependent manner.

PKCε Regulates STAT1α-dependent Transcription—IFN-γ elicits its biological activity through activation of STAT1α (20, 21, 40). Because PI 3-kinase regulates IFN-γ-induced PKCε activity, its role in STAT1α-dependent transcription was tested next. We and others (19, 32, 33, 40, 41) have previously used SIE as a STAT1α target DNA element. To study whether PI 3-kinase regulates STAT1α DNA binding, mesangial cells were incubated with the pharmacological inhibitor of PI 3-kinase, Ly294002, followed by incubation with IFN-γ. Cell lysates were analyzed by EMSA using SIE as probe. Ly294002 did not inhibit IFN-γ-induced STAT1α DNA binding (Fig. 3C, compare lane 4 with lane 2). I have shown that PKCε regulates STAT1α-dependent transcription. To test the effect of PKCε on STAT1α DNA binding, a pharmacological inhibitor, H7, which blocks the activity of all isoforms of PKC was used. Treatment of mesangial cells with H7 prior to incubation with IFN-γ did not inhibit STAT1α DNA complex formation (Fig. 3D). These data indicate that IFN-γ-induced DNA binding of STAT1α is not regulated by PI 3-kinase and PKCε.

A corollary of the above observation is that tyrosine phosphorylation of STAT1α is not regulated by PI 3-kinase and PKCε. Therefore, the effect of Ly294002 directly on the tyrosine phosphorylation of STAT1α was examined. IFN-γ increased tyrosine phosphorylation of STAT1α as determined by immunoblot analysis using an antibody against phosphotyrosine 701 (Tyr(P)-701). Treatment of mesangial cells with Ly294002 did not have any effect on IFN-γ-induced tyrosine phosphorylation of STAT1α (Fig. 4A, compare lane 4 with lane 2). On the contrary, Ly294002 significantly inhibited serine phosphorylation of STAT1α (Fig. 4B, compare lane 4 with lane 2). To study the role of PKCε on tyrosine phosphorylation, the effect of H7 was examined. Incubation of mesangial cells with H7 did not affect IFN-γ-induced tyrosine phosphorylation of STAT1α (Fig. 4C, upper panel, compare lane 4 with lane 2). In contrast, H7 significantly inhibited serine phosphorylation of STAT1α (Fig.
In mesangial cells, IFN-γ stimulates expression of MCP-1 (44). Therefore, expression of MCP-1 was used as a target of STAT1α to examine the involvement of PI 3-kinase. IFN-γ robustly increased MCP-1 mRNA expression in mesangial cells (Fig. 5A). Incubation of mesangial cells with the PI 3-kinase inhibitor Ly294002 abolished IFN-γ-induced expression of MCP-1 (Fig. 5A, compare lane 4 with lane 2). These data indicate that PI 3-kinase and PKC do not regulate tyrosine phosphorylation of STAT1α. However, serine phosphorylation of STAT1α is regulated by PI 3-kinase/PKC signaling.

In mesangial cells, IFN-γ stimulates expression of MCP-1 (43). It has recently been shown that IFN-γ-induced MCP-1 expression is mediated by STAT1α (44). Therefore, expression of MCP-1 was used as a target of STAT1α to examine the involvement of PI 3-kinase. IFN-γ robustly increased MCP-1 mRNA expression in mesangial cells (Fig. 5A). Incubation of mesangial cells with the PI 3-kinase inhibitor Ly294002 abolished IFN-γ-induced expression of MCP-1 (Fig. 5A, compare lane 4 with lane 2). These data indicate that PI 3-kinase regulates expression of the STAT1α target gene MCP-1 in mesangial cells.

The transactivation domain and DNA binding domain are separate in STAT1α as they are in the other transcription factors. To examine whether there is any effect of PI 3-kinase on the transactivation property of STAT1α, the reporter plasmid 4xSIE-Luc was transfected into mesangial cells. Incubation of the cells with the PI 3-kinase inhibitor Ly294002 blocked the IFN-γ-induced transcriptional activation of the reporter gene (Fig. 5B). The effect of Ly294002 on the transcriptional activation of the GAS-driven reporter construct...
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(8xGas-Luc) in mesangial cells was also tested. Similar to SIE-mediated transcription, the PI 3-kinase inhibitor Ly294002 significantly inhibited IFN-γ-induced transcription from the GAS element (Fig. 5C). To further confirm the involvement of PI 3-kinase, the effect of a dominant negative form of this enzyme was also tested. Cotransfection of dominant negative PI 3-kinase with the 8xGAS-Luc reporter construct into mesangial cells prevented IFN-γ-induced transcription (Fig. 5D). I have shown above that PI 3-kinase regulated PKCε, which increases STAT1α-dependent transcription. These data placed PKCε downstream of PI 3-kinase. Thus expression of dominant negative PI 3-kinase did not inhibit transcription of the reporter gene induced by PKCε (Fig. 5E). Additionally, adenovirus-mediated gene transfer of a constitutively active PI 3-kinase was sufficient to increase transcription of 8xGAS-Luc, similar to IFN-γ (Fig. 6A). To investigate the mechanism of this observation, the nuclear translocation of STAT1α by immunofluorescence was examined. Expression of the constitutively
active subunit of PI 3-kinase increased nuclear localization of STAT1α similar to IFN-γ treatment (Fig. 6B). These data indicate that although PI 3-kinase may not have any effect on DNA binding of STAT1α, it causes the nuclear presence of the transcription factor to stimulate transcription of target genes.

**IFN-γ Increases MAPK Activity in PI 3-Kinase- and PKCe-dependent Mammals**—It is established that PI 3-kinase-dependent PKCe regulates IFN-γ-induced STAT1α transcriptional activity. It is known that various PKC isotypes regulate MAPK activity in different cells (45–47). With some agonists, PI 3-kinase has also been shown to be an upstream regulator of MAPK (35, 48, 49). IFN-γ is known to stimulate MAPK activity to a lesser extent compared with that induced by other growth factors (50). The precise mechanism of IFN-γ-induced MAPK activation is not clear. One possibility is that PI 3-kinase-coupled PKCe may regulate MAPK activity. To examine this possibility, IFN-γ-induced MAPK activity was tested. Lysates of mesangial cells incubated with IFN-γ were immunoprecipitated with a MAPK antibody followed by the immune complex kinase assay using MBP as substrate. IFN-γ increased activation of MAPK in a time-dependent manner in mesangial cells (Fig. 7A). One of the targets of MAPK is the ternary complex factor Elk-1. Serine phosphorylation at the C-terminal domain of Elk-1 by MAPK is necessary for transcriptional activation (51, 52). To test whether IFN-γ stimulates transcriptional activation of Elk-1, cotransfection assays were performed in mesangial cells with an expression vector encoding the Elk-1 C-terminal transactivation domain fused to the GAL-4 DNA binding domain and a reporter plasmid under the control of GAL-4 DNA element. Transiently transfected cells were incubated with IFN-γ (Fig. 7B). These data indicate that IFN-γ stimulates the transcriptional event that results from activation of MAPK.

Next I tested whether PI 3-kinase regulates MAPK activity. Mesangial cells were treated with Ly294002 prior to incubation with IFN-γ. MAPK immunoprecipitates from the lysates of mesangial cells were used in immune complex kinase assays with MBP as substrate. The PI 3-kinase inhibitor completely blocked IFN-γ-induced MAPK activity (Fig. 8A, compare lane 4 with lane 2). To confirm this observation, mesangial cells were cotransfected with Myc-tagged Erk2 MAPK and dominant negative PI 3-kinase expression vectors. Transiently transfected cells were incubated with IFN-γ. Cell lysates were immunoprecipitated with anti-Myc antibody followed by immune complex kinase assay using MBP as substrate. IFN-γ increased Erk2 activity (Fig. 8B, lane 2). Expression of dominant negative PI 3-kinase abolished IFN-γ-induced Erk-2 activity (Fig. 8B, compare lane 4 with lane 2). These data for the first time demonstrate that PI 3-kinase is necessary for IFN-γ-induced MAPK activity. Because it was shown that PI 3-kinase regulates IFN-
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MAPK Regulates STAT1α Transcriptional Activity—Our data demonstrate that PI 3-kinase and PKCe regulate STAT1α transcriptional activity and that both PI 3-kinase and PKCe are required for MAPK activation. Therefore, the effect of MAPK on STAT1α activation in mesangial cells was examined. Incubation of mesangial cells with U0126, a pharmacological inhibitor of MEK, the upstream activating kinase of MAPK, abolished IFN-γ-induced MAPK activity with a partial inhibition of STAT1α tyrosine phosphorylation (Fig. 9, A, compare lane 4 with lane 2 and B, upper panel, compare lane 4 with lane 2). However, inhibition of MAPK completely inhibited serine phosphorylation of STAT1α (Fig. 9B, middle panel, compare lane 4 with lane 2). To address the role of MAPK in STAT1α transcriptional activity, the effect of this novel PKC isoform on MAPK activation was tested next. Mesangial cells were transfected with Myc-Erk2 and constitutively active PKCe expression vectors. Immune complex kinase assays on the anti-Myc immunoprecipitates using MBP as substrate showed increased Erk2 activity in constitutively active PKCe-transfected mesangial cells, similar to IFN-γ-treated cells (Fig. 8C, lanes 2 and 3). These data for the first time indicate that PI 3-kinase regulates IFN-γ-induced MAPK in a PKCe-dependent manner.

Fig. 8. PI 3-kinase and PKCe regulate MAPK activity. A, PI 3-kinase inhibitor blocks IFN-γ-induced MAPK activity. Quiescent mesangial cells were treated with Ly294002 prior to incubation with IFN-γ. MAPK immunoprecipitates from the cell lysates were assayed for MAPK activity as described in the legend to Fig. 7. The bottom panel shows the immunoblot analysis of the same samples with anti-MAPK antibody. B, dominant negative PI 3-kinase inhibits IFN-γ-induced MAPK activity. Mesangial cells were cotransfected with Myc-tagged Erk2 MAPK and vector or dominant negative PI 3-kinase (pSBαp85). Serum-deprived, transiently transfected cells were incubated with IFN-γ. The cell lysates were immunoprecipitated with anti-Myc antibody followed by immune complex kinase assay with MBP as substrate. The middle panel shows an anti-p85 immunoblot to demonstrate the dominant negative PI 3-kinase expression. The bottom panel shows Myc-Erk2 expression in the samples. C, constitutively active PKCe increases MAPK activity. Mesangial cells were cotransfected with Myc-Erk2 and vector or cPKCe plasmids. Selected serum-deprived transfected cells were incubated with IFN-γ. The cell lysates were immunoprecipitated with anti-Myc antibody followed by immune complex kinase assay for MAPK as described above. Middle panel shows anti-PKCα immunoblot to show expression of PKCe in the samples. Bottom panel shows anti-Myc immunoblot of the same samples.

Fig. 9. MAPK partially regulates STAT1α DNA binding. A, effect of MEK inhibitor U0126 on IFN-γ-induced MAPK activity. Quiescent mesangial cells were treated with U0126 for 1 h before incubation with IFN-γ. Cell lysates were used for immune complex kinase assay for MAPK with MBP as substrate. The bottom panel shows an anti-MAPK immunoblot of the same samples. B, effect of U0126 on tyrosine and serine phosphorylation of STAT1α. Quiescent mesangial cells were treated with U0126 prior to incubation with IFN-γ. The cell lysates were immunoblotted with STAT1α phosphotyrosine 701 (top panel) or phosphoserine 727 (middle panel) antibody. The bottom panel shows immunoblot analysis of the same samples with tubulin antibody. C, effect of MEK inhibitor on STAT1α DNA binding. Quiescent mesangial cells were incubated with U0126 followed by IFN-γ. The cell lysates were used in an EMSA using SIE as probe.
DNA binding, mesangial cells were treated with U0126 followed by incubation with IFN-γ. Cell lysates were then used in EMSA using SIE as probe. U0126 partially prevented the STAT1α DNA binding (Fig. 9C, compare lane 4 with lane 2), similar to its effect on STAT1α tyrosine phosphorylation.

Next, whether STAT1α is a direct substrate for MAPK isolated from IFN-γ-stimulated mesangial cells was examined. Recombinant STAT1α was used as substrate with immunoprecipitated MAPK from mesangial cells. MAPK immunoprecipitated from lysates of mesangial cells incubated with IFN-γ increased phosphorylation of STAT1α as compared with that from immunoprecipitated control cells (Fig. 10A, compare lane 2 with lane 1). To examine directly the role of MAPK on trans-activation of STAT1α, mesangial cells were cotransfected with the 8xGAS-Luc reporter plasmid and dominant negative Erk2 followed by incubation with IFN-γ. As expected, IFN-γ increased transcription of the reporter gene. However, expression of dominant negative Erk-2 significantly inhibited IFN-γ-induced transcription of the reporter gene (Fig. 10B). These data indicate that although MAPK has a partial effect on STAT1α DNA binding, it directly regulates the transcriptional activity of STAT1α.

DISCUSSION

The present study shows that IFN-γ activates PKCε in mesangial cells. I also show that activation of PI 3-kinase is necessary for IFN-γ-induced serine phosphorylation of STAT1α. I demonstrate that PI 3-kinase does not affect the DNA binding and tyrosine phosphorylation of STAT1α. Additionally, for the first time, I show that IFN-γ-induced PI 3-kinase activity is necessary for PKCε phosphorylation and activation. Furthermore, these data provide the first evidence that IFN-γ-induced PKCε regulates MAPK activity, which in turn phosphorylates STAT1α and stimulates its transcriptional activity. On the basis of my data, I propose a linear signal transduction pathway for IFN-γ-induced STAT1α activation in mesangial cells (Fig. 11).

PKC plays an important role in activation of macrophages and endothelial cells in response to IFN-γ (53–56). Three closely related subgroups (classical, novel, and atypical) of PKCs containing at least 11 different isozymes have been identified (38, 56). Among classical PKCs, IFN-γ has recently been shown to activate PKCa, which stimulates STAT1α to increase transcription of ICAM-1 from a GAS element present in its promoter (57). In neuronal cells, IFN-γ increases expression of the novel PKCε (58). Also a role of PKCε has been suggested in inflammation-induced pain (59). More recently, it is reported that macrophages from the PKCε null mouse display a severely impaired response to IFN-γ (22), suggesting a pivotal role of this novel PKC isozyme in IFN-γ signaling. In the present paper, I demonstrate that IFN-γ stimulates PKCε activity in mesangial cells (Fig. 1A). Furthermore, I show that the PKC inhibitor H7 and PI 3-kinase inhibitor Ly294002 do not inhibit IFN-γ-induced STAT1α DNA binding and tyrosine phosphorylation (Figs. 3, C and D, and 4, A and C). These data contrast with observations in fibroblasts and 293 cells demonstrating that PKCε is necessary for JAK-dependent tyrosine phosphorylation of STAT1α (60). In this study, Ivaska et al. (60) transfected the green fluorescent protein-STAT1α fusion protein into 293 cells to detect STAT1α tyrosine phosphorylation (60). In the present study, the effect of the PKC inhibitor H7 on the endogenous STAT1α tyrosine phosphorylation in 293 cells was examined. H7 inhibited IFN-γ-induced tyrosine phosphorylation of STAT1α (Supplementary Materials Fig. 1, compare lane 4 with lane 2). These data indicate that PKCε may be involved in tyrosine phosphorylation in 293 cells as observed by Ivaska et al. (60). However, I demonstrate that PKCε does not regulate tyrosine phosphorylation of STAT1α in mesangial cells; but it plays an important role in STAT1α transcriptional activity in the absence of any effect on its DNA binding capability.

Phosphorylations of PKCε at three residues play important roles in full activation of this kinase (38). These sites include the catalytic loop (Thr-566), autophosphorylation site (Thr-710), and the hydrophobic C-terminal site (Ser-729). Phosphorylations at Thr-566 and Ser-729 are sensitive to PI 3-kinase activity (38). In fact, the PI 3-kinase target PDK1 has been shown to be the catalytic loop of the Thr-566 kinase (38, 39). Phosphorylation at Ser-729 increases the activity of PKCε (38). In accordance with this notion, I demonstrate that IFN-γ increases phosphorylation of Ser-729 of PKCε in mesangial cells, thus providing a mechanism for its increased activity (Fig. 1, A and B). Furthermore, Ivaska et al. (60) have recently shown, in detached fibroblasts in suspension, that the PI 3-kinase target PDK1 binds to the hydrophobic site of PKCε, thus protecting the phosphorylation at Ser-729 and retaining PKCε activity.
Inhibition of PI 3-kinase by Ly294002 also results in dissociation of PDK1 from this complex, rendering it sensitive to dephosphorylation and to loss of PKCe activity (60). However, our results in mesangial cells show that the PI 3-kinase inhibitor Ly294002 blocked IFN-γ-induced PKCe Ser-729 phosphorylation and activity (Fig. 1, B and C). It is important to note that in a recent report, Nguyen et al. (24) demonstrated a role for PI 3-kinase/Akt signaling in IFN-γ-mediated serine phosphorylation of STAT1α and its transcriptional activation. The data demonstrating sensitivity of STAT1α-dependent transcription of the reporter gene to the PI 3-kinase inhibitor also confirms this notion (Fig. 5). Although a role of the PI 3-kinase target Akt was described to induce serine phosphorylation of STAT1α and transcriptional activity, involvement of an unidentified kinase was suggested in the absence of any Akt phosphorylation site in STAT1α (24). In mesangial cells, the data indicate that PI 3-kinase/PKCε signaling regulates transcriptional activation of STAT1α without any effect on tyrosine phosphorylation and its specific DNA binding property (Figs. 1–5). It should be emphasized that tyrosine phosphorylation of STAT1α is a requirement for its DNA binding (11, 15, 61). Thus our results contrast with the data demonstrating that PKCe regulates tyrosine phosphorylation of STAT1α in 293 cells (Ref. 60 and Supplementary Materials Fig. 1).

Serine phosphorylation of STAT1α has been suggested to be necessary for full transcriptional activation (23, 61). This postulate is also supported by the observation that the transcriptional activator BRCA1 binds to the C-terminal domain only when STAT1α is phosphorylated (62). Furthermore, the transcriptional activity of STAT1α during the cell cycle correlates with the level of nuclear minichromosome maintenance 5 (mcm5), which also binds to the serine-phosphorylated STAT1α only and activates its transcription (63). Our data show that in mesangial cells, PI 3-kinase regulates serine phosphorylation of STAT1α without any effect on tyrosine phosphorylation (Fig. 4). It is further supported by our observation that PI 3-kinase alone is sufficient to stimulate transcription (Fig. 6A), demonstrating the importance of serine phosphorylation for the transcription. Several recent reports indicate that tyrosine phosphorylation of STAT1α is not required for its nuclear localization (64, 65). Thus overexpression of STAT1α has been shown to activate transcription of the reporter gene in the absence of JAK activation by IFN-γ (66, 67). I show that PI 3-kinase increases the nuclear presence of STAT1α, thus providing a mechanism of increased transcription in mesangial cells (Fig. 6B).

The serine 727 residue phosphorylated in STAT1α is a MAPK consensus site (PMSP) (61). A 28-amino acid long peptide spanning this consensus site in STAT1α was efficiently phosphorylated by purified MAPK (61). Although data exist to support a role for MAPK in phosphorylating STAT1α, several lines of evidence argue against this notion (23). First, IFN-γ is a weak inducer of MAPK activity. Second, the MEK inhibitor does not block IFN-γ-induced STAT1α serine phosphorylation in certain cells (23, 68). Third, down-regulation of the MAPK activity did not affect STAT1α serine phosphorylation in response to IFN-γ (69). Similar to MAPK, positive and negative involvement of p38 has also been reported (68, 70). I show evidence in mesangial cells that IFN-γ significantly increased MAPK activity with concomitant activation of a MAPK-dependent transcription factor Elk-1 (Fig. 7). These data indicate that IFN-γ-induced MAPK is biologically functional in mesangial cells.

It has been (35, 48, 49) shown that in certain cell backgrounds, one of the upstream regulators of MAPK is the PI 3-kinase. Using pharmacological inhibition and dominant negative PI 3-kinase, I showed that IFN-γ-induced MAPK activity in mesangial cells is regulated by PI 3-kinase (Fig. 8). I described above that a target of PI 3-kinase is the novel PKCe isoform (38, 60). Recently PKCe has been shown to modulate the MAPK activity in cardiomyocytes and T-cells (71, 72). Furthermore, in endothelial cells and glioma cells, PKCe is capable of activating MAPK in an adhesion-dependent manner (73, 74). Our observations that IFN-γ activates PKCe and MAPK in a similar kinetics demonstrate that the former kinase may regulate IFN-γ-induced MAPK activity (Figs. 1 and 7). Indeed, I demonstrate in mesangial cells that expression of constitutively active PKCe increases MAPK activity (Fig. 8C).

Contradictory results have been reported for STAT serine phosphorylations (23). For example, MAPK phosphorylates serine 727 of STAT3 in response to growth factors, resulting in inhibition of DNA binding (50). On the other hand, the same serine phosphorylation in STAT1α and STAT3 affects transcriptional activation in the absence of any effect on their DNA binding (61). More recently, Nguyen et al. (75) reported that inhibition of MAPK potentiates IFN-γ-induced STAT1α DNA binding (75). Here I demonstrate that pharmacological inhibition of the MAPK activity induced by IFN-γ had a partial inhibitory effect on STAT1α tyrosine phosphorylation and DNA binding (Fig. 9). Thus our data argue against a complete negative regulatory role of MAPK in STAT1α DNA binding in mesangial cells. In contrast, the transcriptional activity of STAT1α was significantly blocked when a dominant negative MAPK was expressed (Fig. 10B). These data conclusively indicate in mesangial cells that MAPK positively regulates the STAT1α-dependent transcription with a partial effect on its tyrosine phosphorylation and DNA binding. Whether this partial effect on DNA binding has consequences on the observed transcriptional inhibition is not clear. However, inhibition of MAPK completely blocked serine phosphorylation of STAT1α (Fig. 9B). Therefore, I prefer the notion that MAPK regulation...
of transcription observed in Fig. 10B may be the result of regulation of serine phosphorylation by the kinase.

Similar to the effects in many mesenchymal cells, IFN-γ inhibits basal DNA synthesis in mesangial cells (18). This inhibition was correlated with the activation of STAT1α. In inflammatory diseases of the kidney, cross-talk between IFN-γ signaling and other cytokine actions regulate the pathology (76). For example, many growth factors and cytokines, including platelet-derived growth factor, epidermal growth factor, and IFN-γ, are present in the microenvironment of glomerulus in proliferative glomerulonephritis (77, 78). Interaction of the signal transduction pathways initiated by these polypeptide factors affects the disease process. In fact, it was shown previously (19) that IFN-γ treatment of mesangial cells makes them more sensitive to growth factor-induced proliferation. This effect of IFN-γ correlated with the synergistic activation of STAT1α. Identification of the precise signaling pathways that mediate the activation of STAT1α, which regulates the biological activities of IFN-γ, will allow selective therapeutic targeting of mesangial cell injury in kidney diseases.

In summary, I have shown that IFN-γ stimulates PKCe in mesangial cells in a PI 3-kinase-dependent manner. I have also shown that IFN-γ-induced tyrosine phosphorylation and DNA binding of STAT1α are not affected by PI 3-kinase, whereas both PI 3-kinase and PKCe regulate STAT1α serine phosphorylation and transcriptional activity. Our data demonstrate that IFN-γ stimulates MAPK resulting in Elk-1-mediated transcription. Furthermore, our data for the first time show that IFN-γ-induced MAPK activation is regulated by PI 3-kinase and PKCe. Finally I have provided the first evidence that PKCe-activated MAPK regulates IFN-γ-induced STAT1α-dependent transcription. Taken together, the data indicate a linear signaling pathway involving PI 3-kinase, PKCe, and MAPK sequentially, in IFN-γ-induced STAT1α transcriptional activation (Fig. 11).

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