Activation of the p38 Mitogen-activated Protein Kinase by Type I Interferons*

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The p38 mitogen-activated protein (Map) kinase plays a critical role in the generation of signals in response to stress stimuli, but its role in interferon (IFN) signaling and its potential regulatory role in the activation of Janus signaling transducer and activator of transcription (Stat) pathway are not known. In the present study, we provide evidence that the p38 Map kinase is rapidly phosphorylated and activated during treatment of cells with Type I interferons (IFNα and IFNβ). Furthermore, the Type I IFN-dependent activation of p38 regulates induction of the catalytic domains of MapKap kinase-2 and MapKap kinase-3, strongly suggesting the existence of an IFNαα signaling cascade activated downstream of the p38 kinase. The engagement of this pathway in interferon signaling plays a critical role in interferon-dependent transcriptional regulation, as evidenced by the fact that inhibition of p38 activation results in abrogation of interferon-dependent gene transcription via interferon-stimulated response elements. Interestingly, inhibition of the kinase activity of the p38 blocks IFNαα-induced gene transcription without inhibiting DNA binding or tyrosine phosphorylation of Stat proteins, suggesting that the p38 pathway acts in cooperation with the Stat pathway. Thus, the p38 kinase signaling cascade is activated by the Type I interferon receptor and plays a critical role in interferon signaling and interferon-dependent transcriptional regulation.

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† The abbreviations used are: IFN, interferon; Stat, signal transducer and activator of transcription; ISRE, interferon-stimulated response element; MPK, mitogen-activated protein; PGE2, polyacrylamide gel electrophoresis; GDAC, genomic DNA affinity chromatography.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The Daudi (lymphoblastoid), Molt-4 (acute T-cell lymphoblastic leukemia), and KG-1 (acute myeloid leukemia) cell lines were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with fetal bovine serum (Life Technologies, Inc.) and antibiotics. Human recombinant IFNo2 was provided by Hoffmann LaRoche. Human recombinant IFNo-consensus was provided by Amgen Inc. Human recombinant IFNγ was provided by Biogen Inc. (Cambridge, MA). A polyclonal antibody against p38 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against the MapKap-kine-2 or MapKap-kine-3 kinases were obtained from Upstate Biotechnol. An anti-Stat-1 antisera was provided by Dr. Andrew Larner (Cleveland Clinic Research Foundation, Cleveland, OH) and was used for immunoprecipitations. A monoclonal antibody against Stat-1 was obtained from Transduction Laboratories (Lexington, KY) and was used...
Type I interferons induce phosphorylation of the p38 Map kinase. A, Molt-4 cells were incubated in the presence (+) or absence (−) of IFNα or IFNβ for 30 min at 37 °C as indicated. Total cell lysates, corresponding to 1 × 10⁶ cells, were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of p38 (left panel). The blot was subsequently stripped and reprobed with an antibody against p38 (right panel). B, Daudi cells were incubated in the presence or absence of IFNα or IFNβ for 30 min at 37 °C as indicated. Total cell lysates, corresponding to 1 × 10⁶ cells, were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of p38 (left panel). The blot was then stripped and reprobed with an antibody against p38 (right panel).

for immunoblotting. An antibody that recognizes specifically the tyrosine-phosphorylated form of Stat-1 at tyrosine 701 was obtained from Upstate Biotechnology and was used for immunoblotting. A polyclonal antibody against the phosphorylated/activated form of ATF-2 was obtained from New England Biolabs and was used for immunoblotting. A polyclonal antibody that recognizes the phosphorylated/activated form of ATF-2 was obtained from New England Biolabs. The SB203580 inhibitor was obtained from Calbiochem Inc.

Cell Lysis and Immunoblotting—Cells were stimulated with 1 × 10⁴ units/ml of the indicated interferons for the indicated times, and the cells were lysed as described previously (4, 5). Immunoprecipitations and immunoblotting using an enhanced chemiluminescence method were performed as described previously (4, 5).

p38 map Kinase Assay—Cells were incubated in the presence or absence of the indicated interferons for the indicated times at 37 °C. The cells were subsequently lysed in phosphorylation lysis buffer (11). Cell lysates were immunoprecipitated with an antibody against p38 using protein G-Sepharose (Amersham Pharmacia Biotech). The immunocomplexes were subsequently washed three times with phosphorylation lysis buffer containing 0.1% Triton X-100 and two times with kinase buffer (25 mM Hepes, 25 mM MgCl₂, 25 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na₂VO₃, 20 μM ATP) and resuspended in 30 μl of kinase buffer containing 5 μg of glutathione S-transferase-ATF-2 fusion protein, and 30 μCi of [γ-³²P]ATP was added. The reaction was incubated for 30 min at room temperature and was terminated by the addition of SDS-sample buffer. Proteins were analyzed by SDS-PAGE, and the phosphorylated form of ATF-2 was detected by immunoblotting with an anti-phospho-ATF-2 antibody.

MapKap Kinase-2 and MapKap Kinase-3 Kinase Assays—Cells were serum-starved by overnight incubation in RPMI medium 15% fetal calf serum. They were subsequently incubated in RPMI medium without serum for 2 h and then treated with the indicated IFNs for the indicated times, in the presence or absence of 10 μM SB203580, which was added 30 min prior to IFN treatment. The cells were then lysed in phosphorylation lysis buffer, lysates were immunoprecipitated with antibodies against MapKap kinase-2 or MapKap kinase-3, immunoprecipitated proteins were washed three times in phosphorylation lysis buffer and two times in kinase buffer (25 mM Hepes, pH 7.4, 25 mM MgCl₂, 25 mM β-glycerophosphate 100 μM sodium orthovanadate, 2 mM dithiothreitol, 20 μM ATP), and the immune complex kinase assays were initiated by the addition of 30 μl of kinase buffer containing 5 μg of Hsp-25 protein (Stress Gen Laboratories) as a substrate and 25 μCi of [γ-³²P]ATP. The reaction was incubated for 30 min at room temperature and was terminated by the addition of SDS-sample buffer. Proteins were subsequently analyzed by SDS-PAGE, and the phosphorylated form of Hsp-25 was detected by autoradiography.

Luciferase Reporter Assays—Cells were transfected with a β-galactosidase expression vector and an ISRE-luciferase plasmid using the Superfect transfection reagent as per the manufacturer's recommended procedure (Qiagen). The ISRE-luciferase construct included the wild type ISG15 ISRE (TCGGGAAAAAGACTGCAGCGCC) cloned via cohesive ends into the pZtkLuc vector. Forty-eight hours after transfection, triplicate cultures were either left untreated or treated with 5000 units/ml of IFNα, in the presence or absence of 10 μM SB203580, which was added to the cultures 30 min prior to IFN treatment. In the experiments in which the effects of overexpression of a mutant p38 were determined, the cells were transfected with a mutated dominant-negative p38 DNA subclone in the pcMV5 vector (pcMV-p38AGF) (24) (kindly provided by Dr. R. Davis, Howard Hughes Medical Institute, University of Massachusetts, Worcester, MA) or the pCMVHis vector (pCMV-p38AGF) (24). The measured luciferase activities were normalized for β-galactosidase expression for each sample.

RESULTS AND DISCUSSION

We sought to determine whether the p38 Map kinase is tyrosine-phosphorylated/activated during IFNα treatment of IFNα-sensitive cell lines. Molt-4 or Daudi cells were treated in the presence or absence of IFNα or IFNβ, and after cell lysis, total lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of p38 (New England Biolabs). In both Molt-4 and Daudi cells, we noticed that the phosphorylated form of p38 was induced after IFNα or IFNβ treatment (Fig. 1), suggesting that this member of the MAP family of kinases is a substrate for upstream MKK kinases and possibly is activated by Type I IFNs to transduce downstream signals. Similarly, Type I IFN-dependent tyrosine phosphorylation of p38 was seen in the IFN-sensitive KG-1 myeloid cell line.² The kinetics of this Type I IFN-dependent phosphorylation of p38 were such that peak phosphorylation of the kinase occurred at 30 min, with the signal declining by 60 min, suggesting that the activation/phosphorylation of p38 by interferons is rapid and transient (Fig. 2).

To determine whether the kinase activity of p38 is induced by Type I IFN treatment, KG-1 or Daudi cells were treated with IFNβ; cell lysates were immunoprecipitated with an anti-p38 antibody, and in vitro kinase assays were performed using

² S. Uddin and L. C. Platanias, unpublished observations.
presence or absence of IFNβ for 30 min at 37 °C as indicated. Cell lysates were immunoprecipitated with an antibody against p38, and immunoprecipitates were subjected to an in vitro kinase assay using glutathione S-transferase-ATF-2 as a substrate. Proteins were analyzed by SDS-PAGE, and phosphorylated proteins were detected by immunoblotting with an anti-phospho-ATF-2 antibody (left panel). The blot from the kinase assay was stripped and probed with an antibody against p38 to control for loading (right panel).

A

B

Fig. 3. Type I interferon-dependent induction of the kinase activity of p38. A, KG-1 cells were incubated in the presence or absence (−) of IFNβ for 30 min at 37 °C as indicated. Cell lysates were immunoprecipitated with an antibody against p38, and immunoprecipitates were subjected to an in vitro kinase assay using glutathione S-transferase-ATF-2 as a substrate. Proteins were analyzed by SDS-PAGE, and phosphorylated proteins were detected by immunoblotting with an anti-phospho-ATF-2 antibody (left panel). The blot from the kinase assay was stripped and probed with an antibody against p38 to control for loading (right panel). B, Daudi cells were incubated in the presence or absence of IFNβ for 30 min at 37 °C as indicated. Cell lysates were immunoprecipitated with an antibody against p38, and immunoprecipitates were subjected to an in vitro kinase assay using glutathione S-transferase-ATF-2 as a substrate. Proteins were analyzed by SDS-PAGE, and phosphorylated proteins were detected by immunoblotting with an anti-phospho-ATF-2 antibody (left panel). The blot from the kinase assay was stripped and probed with an antibody against p38 to control for loading (right panel).

determine whether these kinases are also activated downstream of the p38 kinase during engagement of the Type I IFN receptor, lysates from IFNβ-treated or untreated cells were immunoprecipitated with specific antibodies against MapKap kinase-2 or MapKap kinase-3 and an in vitro kinase assay was carried out on the immunoprecipitates using Hsp25 as an exogenous substrate. Proteins were analyzed by SDS-PAGE, and phosphorylated proteins were detected by autoradiography. A, Molt-4 cells were incubated in the presence (+) or absence (−) of IFNβ for 60 min at 37 °C, in the presence or absence of SB203580 as indicated. Cell lysates were immunoprecipitated with an antibody against MapKap kinase-2, and in vitro kinase assays were carried out on the immunoprecipitates using Hsp25 as an exogenous substrate. Proteins were analyzed by SDS-PAGE, and phosphorylated proteins were detected by autoradiography. B, U2OS cells (2 × 10⁵/plate) were transfected with an ISRE luciferase construct. 48 h after transfection the cells were incubated without or with IFNα for 6 h in the absence or presence of 10 μM SB203580 at 37 °C as indicated. The cells were then harvested and assayed for luciferase activity. The data are expressed as fold increase in luciferase activity over background levels, in response to IFNα treatment in the absence or presence of SB203580. The fold increase in each experiment was calculated by dividing the relative luciferase units in IFNα-treated samples with the relative luciferase units in IFNα-untreated samples. Mean values ± S.E. of four independent experiments are shown. B, U2OS cells (2 × 10⁵/plate) were co-transfected with an ISRE luciferase construct and either control vector (pCMV) or the dominant-negative p38 mutant (p38-AGF) construct as indicated. 48 h after transfection, the cells were incubated without or with IFNα for 6 h. The cells were then harvested and assayed for luciferase activity. The data are expressed as fold increase in luciferase activity over background levels, in response to IFNα treatment in the pCMV or p38 AGF transfected cells. The fold increase in each experiment was calculated by dividing the relative luciferase units in IFNα-treated samples with the relative luciferase units in IFNα-untreated samples. Mean values ± S.E. of three independent experiments are shown.

Fig. 5. p38 is required for IFNα gene transcription via ISREs. A, KG-1 cells were incubated in the presence or absence of IFNβ for 60 min at 37 °C and in the presence or absence of 10 μM SB203580 as indicated. Cell lysates were immunoprecipitated with an antibody against MapKap kinase-2, and an in vitro kinase assay was carried out on the immunoprecipitates using Hsp25 as an exogenous substrate. Proteins were analyzed by SDS-PAGE, and phosphorylated proteins were detected by autoradiography. B, Daudi cells were incubated in the presence or absence of IFNβ for 30 min at 37 °C as indicated. Cell lysates were immunoprecipitated with an antibody against p38, and immunoprecipitates were subjected to an in vitro kinase assay using glutathione S-transferase-ATF-2 as a substrate. Proteins were analyzed by SDS-PAGE, and phosphorylated proteins were detected by immunoblotting with an anti-phospho-ATF-2 antibody (left panel). The blot from the kinase assay was stripped and probed with an antibody against p38 to control for loading (right panel).
cells were subsequently incubated in the presence or absence of IFN-α empty vector (pCMV) or the pCMV-p38AGF construct as indicated. The cells were subsequently lysed, and equal amounts of total lysates (100 μg/lane) were analyzed by SDS-PAGE and immunoblotted with an antibody that recognizes the tyrosine-phosphorylated form of Stat-1 at tyrosine 701 (left panel). The blot was then stripped and reprobed with an antibody against Stat-1 to control for loading (right panel). B, U2OS cells were transfected with either control empty vector (pCMV) or the pCMV-p38AGF construct as indicated. The cells were subsequently incubated in the presence or absence of IFN-α for 20 min at 37 °C as indicated. After cell lysis, equal amounts of total lysates (100 μg/lane) were analyzed by SDS-PAGE and immunoblotted with an antibody that recognizes the tyrosine-phosphorylated form of Stat-1 at tyrosine 701 (left panel). The blot was then stripped and reprobed with an antibody against Stat-1 to control for loading (middle panel). Equal amounts of protein (100 μg/lane) were analyzed in parallel by SDS-PAGE and immunoblotted with an anti-p38 antibody (right panel).

The p38 kinase is also activated by Type I IFNs, and overexpression of a dominant-negative p38-AGF mutant also blocks the IFN-α-dependent induction of ISRE-luciferase activity (Fig. 5A). Treatment of cells with SB203580 clearly reduced such induction (Fig. 5A), suggesting that the p38 pathway mediates signals required for ISRE-regulated gene transcription during activation of the Type I IFN receptor. To further establish the role of p38 in the induction of IFN-α gene transcription via ISREs, we measured IFN-α-dependent induction of luciferase activity in cells overexpressing a p38 kinase that cannot undergo phosphorylation/activation (p38AGF), as the tyrosine and threonine phosphorylation sites have been mutated (24).

As shown in Fig. 5B, overexpression of p38AGF blocked the IFN-α-induced increase in luciferase activity, establishing that a functional p38 kinase is essential for transcriptional regulation via ISREs.

It is well established that IFN regulation of gene transcription in the interferon system is dependent on phosphorylation of Stat proteins and the formation of DNA binding complexes by activated Stat proteins (1–3). IFN-induced complexes include Stat 1:2 heterodimers that participate in the formation of the active ISGF3 complexes that regulate gene transcription via ISREs (1–3). As SB203580 and overexpression of a dominant-negative p38 construct inhibited induction of IFN-α-dependent gene transcription, we sought to determine whether the IFN-α activation of p38 affects tyrosine phosphorylation and activation of the DNA binding activity of Stat proteins that form the ISGF3 complex. We determined whether treatment of cells with SB203580 inhibits detection of the IFN-α-induced tyrosine-phosphorylated/activated form of Stat-1. Daudi cells were treated with IFN-α in the presence or absence of SB203580, and total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated/activated form of Stat-1. As shown in Fig. 6A, SB203580 had no effect on the IFN-α-dependent tyrosine phosphorylation of Stat-1 on tyrosine 701.

We next performed GDAC studies to determine whether nuclear translocation and DNA binding activity of ISGF3 are regulated by p38. Nuclear extracts from IFN-α-treated cells, incubated in the presence or absence of SB203580, were analyzed using GDAC and the high salt eluate fractions were resolved by SDS-PAGE and transferred to nitrocellulose. Anti-Stat immunoblotting revealed that after IFN-α treatment, nuclear extracts from U2OS cells contained inducible DNA binding factors that correspond to the Stat proteins, Stat-1 and Stat-2, the induction of which was not affected by treatment of cells with SB203580 (Fig. 7D). To further characterize the IFN-α-inducible Stat-containing DNA binding activities, we performed gel mobility shift assays, using the ISRE recognition sequence for the ISGF3 complex.
element. Cells were incubated in the presence or absence of SB203580, and the formation of ISGF3-ISRE complexes in response to IFNα was determined. As shown in Fig. 7E, the formation of DNA binding Stat-complexes was not blocked by inhibition of the kinase activity of p38. Thus, although the function of the p38 kinase is essential for Type I IFN-dependent gene transcription, its activation is not required for Stat-tyrosine phosphorylation and DNA binding.

In the present study, we provide the first evidence for the existence of a Type I IFN-dependent signaling pathway involving activation of the p38 kinase and downstream regulation of the MapKap-2 and Mapkap-3 kinases. This pathway is apparently regulated upstream by a member of the MKK family of kinases, as evidenced by the rapid Type I IFN-dependent phosphorylation of p38. Previous studies have established that MKK3 and MKK6 are selective activators of p38 (17), whereas MKK4 activates both p38 and JNK. It remains to be seen whether any of the known MKK family members, or a novel MKK, regulate the Type I IFN-dependent activation of this pathway.

Our findings also provide direct evidence that the p38 pathway acts in coordination with the Jak-Stat pathway to regulate IFN-dependent gene transcription. It is well known that tyrosine phosphorylation of Stats is required for their translocation to the nucleus and DNA binding. As inhibition of p38 activation blocks IFN gene transcription without affecting Stat DNA binding, our data establish that the p38 pathway does not affect Jak kinase activity and tyrosine phosphorylation of Stats.

It has been reported that maximal activation of transcription by Stat1 in response to IFNα requires serine phosphorylation of Stat-1 in addition to tyrosine phosphorylation (31–33) but the serine kinase regulating Stat-1 phosphorylation is unknown. Although there is no direct evidence so far that serine phosphorylation of Stat-1 and/or Stat-2 also occurs in the Type I IFN system, it is possible that such phosphorylation occurs and is regulated by a serine kinase downstream of p38, therefore modulating the transcriptional activity of Stat1, Stat2, or both. Another explanation, however, is that the p38 pathway converges with the Stat pathway further downstream, possibly at the nucleus, and cooperates with it to regulate transcription of interferon sensitive genes. Such a model for a synergism between these two pathways is similar to the previously described effects of p38 on NF-κB dependent pathways, where pharmacological inhibition of p38 has been shown to block NF-κB-dependent gene transcription, without affecting NF-κB-dependent binding activity (34). Viewed together, these data strongly suggest that the p38 pathway regulates gene transcription without affecting the DNA binding activity of transcription factors. The results presented herein provide the first evidence for such effects on gene products regulated by the IFN-activated Jak-Stat pathway.

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