p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons

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Activation of cytosolic phospholipase A2 (cPLA2) is a prerequisite for the formation of the transcription factor complex interferon-stimulated gene factor 3 (ISGF3) in response to interferon-α (IFN-α). Here we show that p38 mitogen-activated protein kinase (MAPK), an activator of cPLA2, is essential for both IFN-α and IFN-γ signalling. SB203580, a specific inhibitor of p38, was found to inhibit ISGF3 formation but had no apparent effects on signal transducer and activator of transcription (STAT)1 homodimer formation. Regardless of this, the antiviral activities of both IFN-α and IFN-γ were attenuated by SB203580. Treatment with either IFN led to rapid and transient activation of p38. Both IFNs induced STAT1 Ser727 phosphorylation, which was inhibited by SB203580 but not by an extracellular signal related kinase (ERK)1/2 inhibitor (PD98059). In an inducible 3T3-L1 clone, expression of dominant-negative p38 led to defective STAT1 serine phosphorylation and diminished IFN-γ-mediated protection against viral killing. Reporter activity mediated by ISGF3 or STAT1 homodimer was diminished by SB203580 and enhanced by a constitutively active mutant of MKK6, the upstream activator of p38. Therefore, p38 plays a key role in the serine phosphorylation of STAT1 and transcriptional changes induced by both IFNs.

Keywords: interferon/p38/Ser727/STAT1

Introduction

The interferons (IFNs) constitute a family of pleiotropic cytokines that exhibit antiviral, antitumour and immunomodulatory properties in the vertebrates (Stark et al., 1998). They exert their effects by binding to specific surface receptors on target cells to initiate a cytosolic signalling cascade that culminates in the alteration of gene expression in the nucleus. The molecular events of this cascade have been well characterized (Stark et al., 1998). IFN binding causes receptor clustering, transphosphorylation of receptor-associated Janus kinases (Jaks) and phosphorylation of critical tyrosine residues by Jaks on the cytoplasmic domain of the receptors. This allows receptor recruitment and Jak-mediated phosphorylation of STAT (signal transducer and activator of transcription) molecules, which dimerize through mutual phoshorylsen-src homology 2 (SH2) domain interactions and translocate into the nucleus to regulate gene transcription.

While phosphorylation on Tyr701 is mandatory for STAT1 dimerization, nuclear translocation and DNA binding in response to IFN-γ, full transcriptional activity of the homodimer is manifested only when Ser727 in the transcription activation domain (TAD) is also phosphorylated (Wen et al., 1995). This phosphorylation enhances binding of the TAD to several nuclear proteins, including mini-chromosome maintenance 5 (MCM5), leading to full transcriptional activation by STAT1 (Zhang et al., 1998). The identity of the serine kinase for STAT1 has been elusive, but its activity can be detected in partially purified cytoplasmic extracts (Zhu et al., 1997).

Although Jaks and STATs are the primary components of the IFN signalling cascade, other signalling molecules have been shown to be essential for the biological effects of IFNs (Stark et al., 1998). These ancillary signalling proteins support IFN signalling through either a STAT-dependent or a STAT-independent manner. For example, extracellular signal related kinase (ERK)2 is required for the STAT-mediated effects of IFN-β (David et al., 1995), whereas certain T cell receptor components mediate the antiproliferative effects of IFN-α in a STAT-independent manner (Petricoin et al., 1997). SHP-2 (SH2-containing phosphatase-2) becomes tyrosine-phosphorylated upon IFN-α/β stimulation, and expression of a dominant-negative form can suppress reporter activity driven by interferon-stimulated gene factor 3 (ISGF3) (David et al., 1996). Insulin receptor substrate-1 (IRS-1) is tyrosine-phosphorylated after IFN-α treatment and recruits p85 (the regulatory subunit of phosphatidylinositol 3-kinase) to the IFN receptor complex (Uddin et al., 1995, 1998). However, the IRS pathway is dispensable for the antiviral effects of IFN-α, in contrast to the obligatory role of STATs in the antiviral process (Uddin et al., 1997). There is, therefore, compelling evidence for the existence of independent or supportive signalling components in addition to the pivotal role of STATs in IFN biology.

We have shown previously that cytosolic phospholipase A2 (cPLA2) activity is required for the formation of ISGF3, the major transcription factor complex induced by IFN-α (Hannigan and Williams, 1991; Flati et al., 1996). ISGF3 is a heterotrimeric complex of STAT1, STAT2 and a member of the IRF (interferon regulatory factor) family, p48. Inhibition of cPLA2 activity abrogated ISGF3 formation but had no apparent effect on the STAT1 homodimer binding to DNA induced by IFN-α or IFN-γ. Subsequently, cPLA2 has been shown to be regulated by p38 mitogen-activated protein kinase (MAPK) in various cellular contexts, including neutrophils treated with TNF-α (Waterman et al., 1996), and platelets treated with thrombin or collagen (Kramer et al., 1996; Borsch-Haubold et al., 1997). We therefore set out to explore the possible involvement of p38 in IFN-α signalling.
In this report, we show that p38 is involved in both IFN-α and IFN-γ signalling. Inhibition of p38 activity produces the same effects on ISGF3 as were observed with cPLA2 inhibition. Furthermore, p38 inhibition, via pharmacological or genetic means, leads to diminished Ser727 phosphorylation on STAT1, reduces reporter activity mediated by ISGF3 or STAT1 homodimer and compromises the antiviral activities of IFN-α and IFN-γ. Our data suggest an essential role for p38 MAPK in regulating the serine phosphorylation and transcriptional activity of STAT1.

**Results**

**SB203580 attenuates ISGF3 but not STAT1 homodimer formation**

cPLA2 activity is essential for the formation of ISGF3 (Hannigan and Williams, 1991; Flati et al., 1996). In accordance with this, pretreatment of HeLa S3 cells with arachidonyltrifluoromethyl ketone (AACOCF3), a novel specific inhibitor of cPLA2 (Wissing et al., 1997) abrogates ISGF3 formation in response to IFN-α (Figure 1A). Since p38 MAPK is an activator of cPLA2 in different cellular contexts (Kramer et al., 1996; Waterman et al., 1996; Borsch-Haubold et al., 1997), we examined the possible involvement of p38 in IFN-α signalling. HeLa S3 cells were pretreated with SB203580, a specific inhibitor of p38, before stimulation with IFN-α or IFN-γ. The drug attenuated ISGF3 formation in a dose-dependent manner (Figure 1B) but had no apparent effect on STAT1 homodimer induced by IFN-α or IFN-γ (Figure 1C) as judged by electrophoretic mobility shift assay (EMSA). IFN-α is also known to activate STAT3 (Yang et al., 1998), thus accounting for the triplet pattern discernible in the lanes for IFN-α in Figure 1C. The triplet is likely to comprise STAT3 homodimer in the top band, STAT1–STAT3 heterodimer in the middle band and STAT1 homodimer in the bottom band that co-migrates with the intense STAT1 homodimer complex induced by IFN-γ. The selective abrogation of ISGF3 by p38 inhibition is consistent with our placement of p38 upstream of cPLA2 in IFN-α signalling.

**SB203580 attenuates IFN-α-induction of double-stranded RNA-dependent protein kinase (PKR) and the antiviral activities of both IFNs**

ISGF3 is a major mediator of the signalling pathway of IFN-α, which triggers an antiviral response in cells by inducing several endogenous enzymes that antagonize viral replication (Stark et al., 1998). One such enzyme is the double-stranded RNA-dependent protein kinase, PKR, whose promoter contains the classical IFN-stimulated response element (ISRE) recognized by ISGF3 (Xu and Williams, 1998). Consistent with the attenuation of ISGF3 formation by SB203580, pretreatment with the drug prior to a 12 h IFN-α treatment led to a loss of PKR-induction by IFN-α (Figure 2A). To investigate whether the biochemical observations described above translate into a physiological phenotype, cytopathic effect (CPE)
between the activity of an MAPK and its level of tyrosine phosphorylation (Widmann et al., 1999). To determine whether p38 is activated by IFNs, the protein was immunoprecipitated from HeLa S3 cell extracts prepared after different times of IFN treatment and assayed for tyrosine phosphorylation. Both IFN-α and IFN-γ induced rapid and transient tyrosine phosphorylation of p38 (Figure 3A and B). An ~2-fold increase in p38 phosphorylation was evident at 5 min, which dipped below basal levels at 10 min before returning to basal levels at 20 min. An independent immune complex kinase assay using recombinant ATF-2 as substrate gave a good correlation between tyrosine phosphorylation of p38 and its activity (Figure 3C). Although much weaker than the tyrosine phosphorylation induced by osmotic shock (positive control in Figure 3), the weak induction caused by the IFNs is comparable to that induced by lipopolysaccharide (data not shown), a well known activator of p38 (Lee et al., 1994; Raingeaud et al., 1995). The above data suggests that p38 is activated in both IFN-α and IFN-γ signalling pathways.

**STAT1 Ser727 phosphorylation requires p38 activity**

Ser727 phosphorylation on STAT1 is absolutely required for IFN-γ-mediated antiviral activity (Horvath and Darnell, 1996). Reconstitution of STAT1-null cells with wild-type STAT1 protein, but not a STAT1 S727A mutant protein, can restore antiviral protection by IFN-γ even though STAT1 homodimers are formed in both cases. The inhibition of the antiviral activity of IFN-γ by SB203580 (Figure 2B, upper panel) indicates the requirement for p38-dependent activation of cPLA₂ to facilitate ISGF3 formation, leading to antiviral gene transcription. Although SB203580 did not inhibit IFN-γ-induced STAT1 homodimer formation (Figure 1C), p38 activity appears also to be essential for the antiviral activity of IFN-γ (Figure 2B, lower panel). Accordingly, the status of p38 activity was examined after IFN-α or IFN-γ stimulation of HeLa S3 cells.

**Activation of p38 MAPK by IFN-α and IFN-γ**

The activation of p38 is strictly dependent on dual phosphorylation on Thr180 and Tyr182 (Raingeaud et al., 1995). Many reports have shown a direct correlation between the activity of an MAPK and its level of tyrosine phosphorylation (Winsor et al., 2001). The above data suggests that p38 is activated in both IFN-α and IFN-γ signalling pathways.
the serine phosphorylation of STAT1 in HeLa S3 cells, whereas ERK2 is dispensable for this function.

**ISGF3 complex contains STAT1 phosphorylated on Ser727**

STAT1 Ser727 phosphorylation has been studied extensively in connection with IFN-γ stimulation, but there is only an isolated report related to IFN-α (Kovarik et al., 1998). To examine in greater detail the induction of Ser727 phosphorylation upon IFN-α treatment, HeLa S3 cells were treated for different times with IFN-α and lysates prepared for STAT1 immunoprecipitation. IFN-α stimulation results in increasing serine phosphorylation of STAT1 over a 30 min time course (Figure 5A). Furthermore, by performing a STAT2 immunoprecipitation, it is possible to detect the presence of pS727-STAT1 in the ISGF3 complex (Figure 5B). This result was unexpected, as serine phosphorylation has been deemed unnecessary for the antiviral and antiproliferative effects of IFN-α, since the STAT1 S727A mutant is capable of conferring IFN-α-responsiveness on STAT1-null cells (Bromberg et al., 1996; Horvath and Darnell, 1996). Non-specific inclusion of pS727-STAT1 in STAT2 immunocomplexes was excluded by using IFN-γ treatment as a control. IFN-γ, which induces Ser727 phosphorylation consistently, did not give a signal due to its inability to induce ISGF3 (Figure 5B, extreme left lane). Thus, IFN-α induces Ser727 phosphorylation on STAT1, and it is likely that pS727-STAT1 is a normal component of ISGF3.

**Dominant-negative p38 inhibits STAT1 serine phosphorylation and the IFN-γ-induced antiviral state**

Recently, it was suggested that SB203580 might inhibit targets other than p38 MAPK, although these were not identified (Hunt et al., 1999). Therefore, to provide independent confirmation that p38 is required for IFN signaling, we used a stable 3T3-L1 pre-adipocyte clone harbouring an isopropyl-β-D-galactopyranoside (IPTG)-inducible dominant-negative p38 mutant. In this mutant, the critical Thr180 and Tyr182 residues have been altered to Ala and Phe, respectively, and expression of this mutant protein blocks differentiation of the 3T3-L1 cells into adipocytes (Engelman et al., 1998).

The 3T3-L1 cells harbouring mutant p38 were induced with IPTG or carrier for 48 h before treatment with IFNs and assayed for STAT1 Ser727 phosphorylation. Both IFN-α- and IFN-γ-dependent serine phosphorylation of STAT1 were attenuated after cells were induced to express the dominant-negative p38 (Figure 6A). In accordance with this, when the 3T3-L1 pre-adipocytes were induced to express dominant-negative p38 during a CPE assay with EMCV, the antiviral state induced by IFN-γ was compromised (Figure 6B). Preliminary experiments showed that IPTG induction of dominant-negative p38 does not sensitize these cells towards viral killing in the absence of IFN (data not shown). Our data suggest a requirement for p38 in the establishment of an antiviral state by IFN-γ via a pathway involving STAT1 serine phosphorylation.
Effects of p38 and MKK6 on IFN-responsive reporter activity

The data described above relate to early biochemical changes and late phenotypic outcome that we seek to connect with more intermediate effects. To link these observations to transcriptional events, we assayed reporter gene expression in transient co-transfection experiments in HT-1080 cells. HeLa S3 cells yielded very low transfection efficiency and HT-1080 cells have been used extensively in IFN signalling studies (Stark et al., 1998). An IFN-α-responsive reporter was constructed (see Materials and methods), which yielded an ~7-fold induction of reporter activity in HT-1080 cells upon IFN-α treatment (Figure 7). Pretreatment with SB203580 reduced the fold induction to 4.7, consistent with the involvement of p38 and cPLA2, in ISGF3 formation. In contrast, co-transfection with a construct overexpressing constitutively activated MKK6 resulted in a >100% increase in IFN-α-induced reporter activity, to ~18-fold. This dramatic enhancement can be explained by an increase in p38 activation over that normally achieved by IFN-α, which is much less than that obtainable by osmotic shock (Figure 3A). MKK6 is a potent upstream activator of p38, and is itself activated by phosphorylation on Ser207 and Thr211 (Enslen et al., 1998; Kessler et al., 1998). Mutation of these two residues to Glu has the same effect as dual phosphorylation, generating a constitutively active form of MKK6 (Raingeaud et al., 1996) used in the above experiment.

Similar experiments were performed with an IFN-γ-responsive promoter containing eight tandem copies of a consensus gamma activated sequence (GAS) element (Horvai et al., 1997). SB203580 pretreatment or co-transfection with the activated MKK6 construct yielded the same pattern of changes observed with the IFN-α experiments (Figure 7), and can again be explained by the inhibition or increased extent of p38 activation relative to vector alone. Without the involvement of cPLA2, the effects observed with IFN-γ are probably due solely to changes in serine phosphorylation of STAT1. From the above data, we conclude that the p38 MAPK pathway is involved in gene expression regulated by both IFN-α and IFN-γ.

Discussion

Both type I and type II IFNs are multifunctional cytokines that elicit many similar biological responses from target cells, yet each of them is also capable of inducing many unique responses (Stark et al., 1998). This is a direct outcome of the distinct but overlapping signalling pathways activated by the ligands, leading to the transcriptional regulation of different sets of genes with considerable overlap (Der et al., 1998). The data presented here, in conjunction with previous work on cPLA2, are a vindication of this salient point. Whereas cPLA2 activity is necessary only in the case of IFN-α signalling (Flati et al., 1996), we have shown here that p38 MAPK is a component shared by both IFN-α and IFN-γ signalling pathways. It is not known if p38 also activates cPLA2 during IFN-γ stimulation, but cPLA2 inhibition apparently has no effect on IFN-γ signalling. Thus, p38 can be viewed as playing a dual role in IFN-α signalling to facilitate both STAT1 Ser727 phosphorylation and ISGF3 formation, but performs a more specific role in IFN-γ signalling, mediating only Ser727 phosphorylation (Figure 8). It has been demonstrated that phosphorylation of Tyr701 is necessary and sufficient for STAT1 homodimer to bind the cognate DNA elements, whereas Ser727 phosphorylation has no effect on DNA binding (Wen and Darnell, 1997). As a result, STAT1–DNA binding as detected by EMSA would...
appear normal when IFN-γ-induced Ser727 phosphorylation is inhibited by SB203580 treatment, as in Figure 1C. Ser727 in STAT1 lies in a proline-flanked consensus motif for phosphorylation by MAPKs (Wen et al., 1995). Indeed, its phosphorylation has been correlated with activation of ERK2 (David et al., 1995) and p38 (Gollob et al., 1999), but no evidence has been presented for a direct kinase role for these enzymes. Our results are consistent with the work by Gollob et al. (1999), where they observed a correlation between p38 activation and Ser727 phosphorylation of STAT1 during co-treatment of T cells with interleukin-2 and -12. Beyond that casual link, we have provided the first evidence that p38 MAPK components can regulate IFN-induced gene transcription and phenotypic changes.

Apart from STAT1, four other STAT proteins (STAT3, 4, 5A and 5B) contain a serine residue at the homologous position that is phosphorylated in response to different stimuli (Cho et al., 1996; Wen and Darnell, 1997; Yamashita et al., 1998). Identification of the serine kinase(s) that act directly on these STATs in vivo is important in clarifying the intricacies of the Jak–STAT pathway, but this has proved elusive. Although we have shown that p38 activity is required for the serine phosphorylation of STAT1, the different kinetics of p38 activation and Ser727 phosphorylation (compare Figures 3A and 5A) suggest an indirect role for p38. It is conceivable that while p38 is switched off rapidly by a negative regulatory component, a more downstream kinase leading closer to Ser727 phosphorylation remains active for as long as the phosphorylation is needed.

STAT2 contains a highly potent TAD at the C-terminus, which probably accounts for the apparent lack of contribution from STAT1 TAD in ISGF3-mediated antiviral and antiproliferative effects of IFN-α (Bromberg et al., 1996; Horvath and Darnell, 1996). In this respect, we can only speculate on the functional significance of pS727-STAT1 being detected in the ISGF3 complex (Figure 5B). One possibility is simply that pS727-STAT1, generated for the purpose of fully activating STAT1 homodimer, redistributes to a certain degree between ISGF3 and the homodimer. This serendipitous inclusion of pS727-STAT1 in ISGF3 might serve no bona fide function. Another, more enticing postulate would be that the phosphorylated serine might play a major transactivational role in circumstances where ISGF3 binds to a promoter in such a way that STAT2 transcriptional activity is ablated by as yet unidentified elements. These promoters would not constitute part of an antiproliferative or antiviral gene. Indeed, recent work in our laboratory using DNA microarrays revealed many IFN-α-regulated genes that might mediate novel physiological roles apparently unrelated to proliferation or antiviral defence (Der et al., 1998). The concept that a STAT protein can exhibit varying or even opposite modes of transcriptional regulation on different promoters is increasingly evident. For example, STAT1 is required for the constitutive expression of certain caspases (Kumar et al., 1997) where the STAT1 binding element in the promoters does not require STAT1 homodimerization to effect binding and activation. In the case of STAT1-dependent regulation of c-myc by IFN-γ, the GAS element from the c-myc promoter mediates repression by STAT1 but the same GAS element behaves normally as a positive element in a minimal promoter (C.V.Ramana, N.Grammatikakis, M.Chernov, K.C.Goh, H.Nguyen, B.R.G.Williams and G.R.Stark, manuscript submitted). The context-dependent influence of a promoter on STAT activity seems to be the general rule.

Undoubtedly, the data presented here raise further questions about the complex network of signalling components activated by the IFNs. For example, does IFN activate the classical three-kinase MAPK module involving a MAPK kinase kinase, then MKK6 and finally p38? If so, is this activation dependent on JAKs? The answers to these questions should provide further insight into the complexity of cytokine signal transduction.

The IFN-inducible, double-stranded PKR plays a pivotal role in establishing the antiviral state by inhibiting viral protein synthesis (Stark et al., 1998). However, it is also a signal-transducing molecule, mediating the activation of NF-kB and IRF-1 in response to stimuli such as dsRNA and IFN-γ (Williams, 1997). Interestingly, p38 is also widely reported to regulate NF-kB-dependent transcription after its translocation into the nucleus (Schulze-Osthoff et al., 1997). Recently, it has been shown that IFN-γ-induced STAT1 Ser727 phosphorylation is defective in PKR knockout cells (C.V.Ramana, N.Grammatikakis, M.Chernov, K.C.Goh, H.Nguyen, B.R.G.Williams and G.R.Stark, manuscript submitted), suggesting that PKR might act upstream of p38. This completes an interesting tripartite relationship among the three molecules, PKR, p38 and NF-kB, where we can envisage PKR activating NF-kB for nuclear translocation through one pathway and simultaneously activating p38 to fine-tune the activity of NF-kB in the nucleus. This scenario provides an interesting parallel to the Jak–STAT pathway, where STATs are tyrosine-phosphorylated for nuclear translocation and serine-phosphorylated for transcriptional fine-tuning. Further investigation into the potential link between p38 and PKR should prove rewarding.

Finally, it is important to note the potential impact of our findings on some of the other biological effects of IFNs. We have clearly shown that p38 plays a key role in IFN signalling and thus contributes towards the antiviral and potentially antiproliferative effects of IFNs that underlie their clinical application as antiviral and antitumour agents. However, p38 is also known to mediate the production of pro-inflammatory cytokines, and was discovered by virtue of this fact (Lee et al., 1994). As such, our work suggests a molecular link between IFN-α and some of its side effects in therapy, in particular cytokine production and the concomitant symptoms of inflammation (Borden and Parkinson, 1998). Ongoing efforts to improve the clinical tolerance of IFNs to mediate their biological effects.

Materials and methods

Reagents
IFNs were obtained from the following sources: human IFN-α (Schering-Plough), human IFN-γ (R&D Systems), murine IFN-α (Hoffman-LaRoche) and murine IFN-γ (Boehringer Mannheim). SB203580, PD98059 and AACOCF₁ were from Calbiochem. Antibodies were obtained from Santa Cruz Biotechnology (p38 mAb), Transduction Laboratories (PY20H, STAT1 N-terminus mAb and STAT2 mAb), New England
Biolabs (phospho-ATF2 pAb), Dr David Frank (pS727-STAT1 antisera) and Dr Ara Hovanesian (PKR mAb). Full-length recombinant ATF2 was obtained from Santa Cruz Biotechnology.

**Plasmids**
The Renilla luciferase plasmid for normalizing transfection efficiency, pRL-TK, was obtained from Promega. The IFN-γ-responsive promoter has been reported (Horvai et al., 1997). The human 6-16 promoter was amplified from a CAT reporter construct (Porter et al., 1988) by PCR with primers containing Xmtal and Xhol linkers (forward primer: 5′-TAACCCGGGATCTATCATGATGCCCC-3′; reverse primer: 5′-CCCCGGGATCAGATAAAGGGGATCTAA-3′). The digested PCR product was cloned into the Xmtal–Xhol site of pGL2-Basic (Promega) to generate an IFN-α-responsive luciferase reporter plasmid. The plasmids pcDNA3 and pcDNA3-MKK6(Glu) were gifts of Dr Roger Davis.

**Cell culture**
HeLa S3, HT-1080 (from ATCC) and 3T3-L1 cells (from Dr Philipp Scherer) were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Gibco-BRL), 100 U/ml penicillin and 100 μg/ml streptomycin. Unless otherwise specified, cells were grown in 10-cm dishes to ~80% confluence before being plated in low-serum media (0.3%) for 12–18 h. Serum-deprived cells were treated as specified in the figure legends.

**Cell lysis, immunoprecipitation and immunoblotting**
After treatment with appropriate agonists, cells were washed with phosphate-buffered saline (PBS) at room temperature and lysed in lysis buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 50 mM NaF, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate and 2 μg/ml each of pepstatin, aprotinin and leupeptin. Extracts were clarified by centrifugation (10 000 g; 30 min). Proteins were resolved by SDS–PAGE, transferred onto PVDF membranes and probed with anti-phospho-ATF2 antibody.

**Immune complex kinase assay**
After treatment with appropriate agonists, immunoprecipitation for p38 was performed as above. Instead of boiling in sample buffer, the immunocomplexes were placed in 30 μl of kinase reaction buffer containing 25 mM HEPES pH 7.5, 10 mM magnesium acetate, 50 μM ATP and 1 μg of recombinant ATF2. The mixture was incubated at 30°C for 20 min, mixed with 30 μl of 2× Laemmli sample buffer and boiled. The proteins were resolved by SDS-PAGE, transferred onto PVDF membrane and probed with anti-phospho-ATF2 antibody.

**DNA transfection and luciferase assay**
Transient transfections of HT-1080 cells were performed in triplicates on 60–80% confluent cells in 10-cm dishes using the LipofectAMINE PLUS™ protocol (Gibco-BRL). Twenty hours after transfection, cells were treated with appropriate stimuli for 6 h or left untreated. Luciferase assays were performed using the protocol for the Dual-Luciferase™ Reporter Assay System (Promega).

**EMSA**
Whole-cell extract (WCE) preparation and EMSA were performed according to published protocols (Flati et al., 1996).

**CPE assay**
For HeLa S3 experiments, cells were plated at 4 × 10^5 cells/well onto 96-well plates and incubated overnight. Cells were pretreated with either 10 μM SB203580 or carrier (dimethylsulfoxide) for 30 min before addition of a 2-fold serial dilution of human IFNs. After 18 h, cells were infected with EMCV at multiplicity of infection (m.o.i.) of 1. After 24 h of infection, cells were washed with PBS and stained with 0.1% crystal violet in PBS.

For 3T3-L1 experiments, cells were plated at 1 × 10^5 cells/well onto 96-well plates and induced with 5 mM IPTG or carrier. After 40–48 h, cells were treated with a 2-fold serial dilution of murine IFN-γ for 18 h, then infected with EMCV at m.o.i. of 1 × 10^4. After 24 h, cell viability was quantitated using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit from Promega. A viability of 100% corresponds to the difference between the absorbance of uninfected cells and the unprotected EMCV-infected cells.

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**References**


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