

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

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**Activation of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) is a prerequisite for the formation of the transcription factor complex interferon-stimulated gene factor 3 (ISGF3) in response to interferon- $\alpha$  (IFN- $\alpha$ ). Here we show that p38 mitogen-activated protein kinase (MAPK), an activator of cPLA<sub>2</sub>, is essential for both IFN- $\alpha$  and IFN- $\gamma$  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- $\alpha$  and IFN- $\gamma$  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- $\gamma$ -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

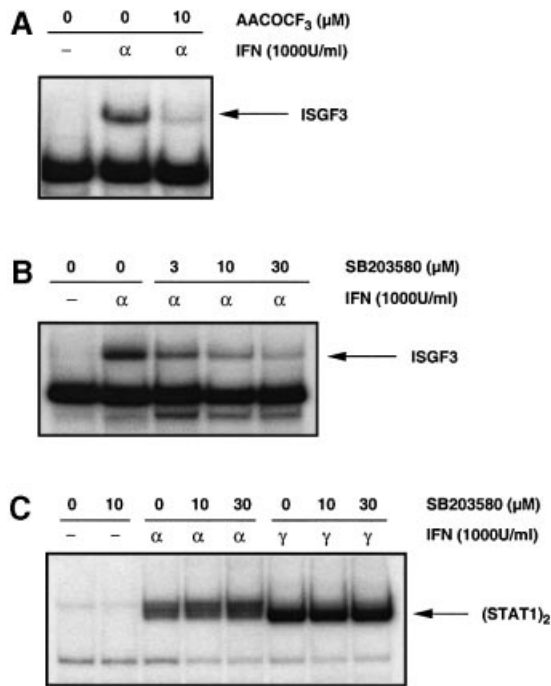
The interferons (IFNs) constitute a family of pleiotropic cytokines that exhibit antiviral, antitumour and immunomodulatory properties in the vertebrates (Stark *et al.*, 1998). They exert their effects by binding to specific surface receptors on target cells to initiate a cytosolic signalling cascade 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 phospho-

tyrosine-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- $\gamma$ , 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- $\beta$  (David *et al.*, 1995), whereas certain T cell receptor components mediate the antiproliferative effects of IFN- $\alpha$  in a STAT-independent manner (Petricoin *et al.*, 1997). SHP-2 (SH2-containing phosphatase-2) becomes tyrosine-phosphorylated upon IFN- $\alpha/\beta$  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- $\alpha$  treatment and recruits p85 (the regulatory subunit of phosphatidylinositol 3-kinase) to the IFN receptor complex (Uddin *et al.*, 1995). However, the IRS pathway is dispensable for the antiviral effects of IFN- $\alpha$ , 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 A<sub>2</sub> (cPLA<sub>2</sub>) activity is required for the formation of ISGF3, the major transcription factor complex induced by IFN- $\alpha$  (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 cPLA<sub>2</sub> activity abrogated ISGF3 formation but had no apparent effect on the STAT1 homodimer binding to DNA induced by IFN- $\alpha$  or IFN- $\gamma$ . Subsequently, cPLA<sub>2</sub> has been shown to be regulated by p38 mitogen-activated protein kinase (MAPK) in various cellular contexts, including neutrophils treated with TNF- $\alpha$  (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- $\alpha$  signalling.



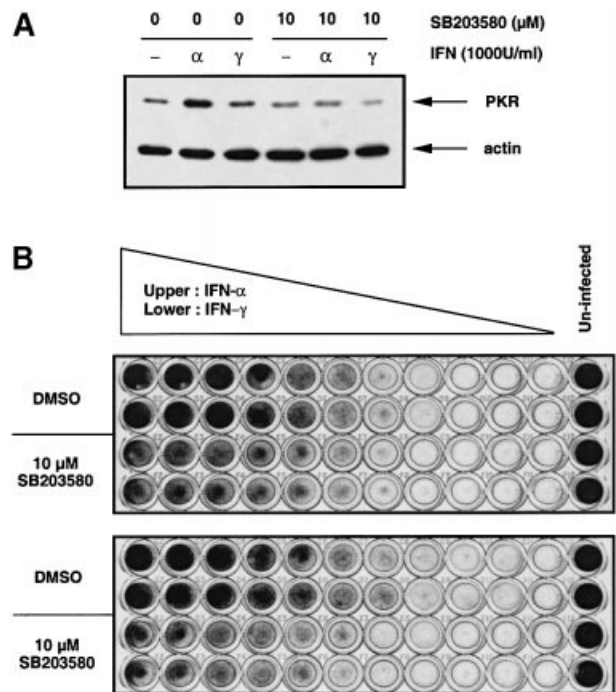
**Fig. 1.** Effect of cPLA<sub>2</sub> and p38 inhibition on ISGF3 formation in HeLa S3. Cells were pretreated with the indicated concentrations of inhibitors for 30 min prior to stimulation with IFN- $\alpha$  or IFN- $\gamma$  for 30 min. WCE were prepared for EMSA with an ISRE-containing probe (A) and (B) or a GAS-containing probe (C) as described in Materials and methods. The data shown is representative of two separate experiments.

In this report, we show that p38 is involved in both IFN- $\alpha$  and IFN- $\gamma$  signalling. Inhibition of p38 activity produces the same effects on ISGF3 as were observed with cPLA<sub>2</sub> 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- $\alpha$  and IFN- $\gamma$ . 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**

cPLA<sub>2</sub> 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 (AACOCF<sub>3</sub>), a novel specific inhibitor of cPLA<sub>2</sub> (Wissing *et al.*, 1997) abrogates ISGF3 formation in response to IFN- $\alpha$  (Figure 1A). Since p38 MAPK is an activator of cPLA<sub>2</sub> 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- $\alpha$  signalling. HeLa S3 cells were pretreated with SB203580, a specific inhibitor of p38, before stimulation with IFN- $\alpha$  or IFN- $\gamma$ . The drug attenuated ISGF3 formation in a dose-dependent manner (Figure 1B) but had no apparent effect on STAT1 homodimer induced by IFN- $\alpha$  or IFN- $\gamma$  (Figure 1C) as judged

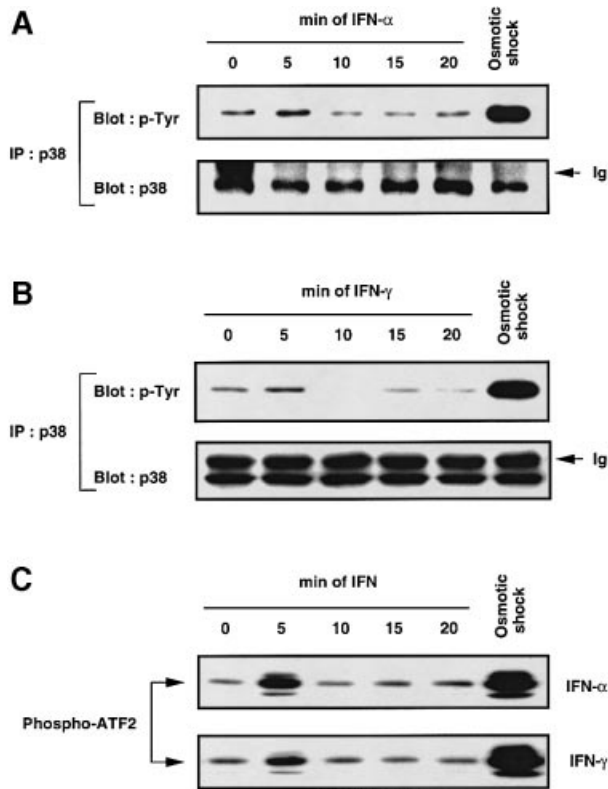


**Fig. 2.** Effect of p38 inhibition on IFN-induced protein and antiviral state in HeLa S3. (A) Cells were pretreated with 10  $\mu$ M SB203580 prior to stimulation with 1000 U/ml IFN- $\alpha$  or IFN- $\gamma$  for 12 h. Lysates containing 30  $\mu$ g of protein were subjected to 8% SDS-PAGE and electroblotting. Membrane was probed with monoclonal antibodies against human PKR and  $\beta$ -actin. (B) CPE assays were performed as described in Materials and methods. The 2-fold serial dilution starts at 250 U/ml for IFN- $\alpha$  and 2000 U/ml for IFN- $\gamma$ . Uninfected cells were not treated with IFNs. Experiments were done in duplicate rows. The data shown is representative of two separate experiments.

by electrophoretic mobility shift assay (EMSA). IFN- $\alpha$  is also known to activate STAT3 (Yang *et al.*, 1998), thus accounting for the triplet pattern discernible in the lanes for IFN- $\alpha$  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- $\gamma$ . The selective abrogation of ISGF3 by p38 inhibition is consistent with our placement of p38 upstream of cPLA<sub>2</sub> in IFN- $\alpha$  signalling.

### **SB203580 attenuates IFN- $\alpha$ -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- $\alpha$ , 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- $\alpha$  treatment led to a loss of PKR-induction by IFN- $\alpha$  (Figure 2A). To investigate whether the biochemical observations described above translate into a physiological phenotype, cytopathic effect (CPE)

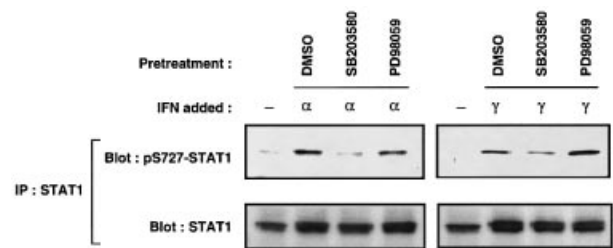


**Fig. 3.** Activation of p38 MAPK by IFNs in HeLa S3. (A) Cells were treated with 1000 U/ml IFN- $\alpha$  for different times or with 0.5 M NaCl for 20 min. Lysates were prepared for p38 immunoprecipitation (IP) and subjected to 12% SDS-PAGE and electroblotting. Membranes were probed for phosphotyrosine with PY20H mAb before stripping and reprobing with p38 pAb. (B) Identical to (A) except for the use of IFN- $\gamma$  as agonist. The data shown is representative of four separate experiments. (C) Cells were treated as in (A) and (B) followed by immune complex kinase assays as described in Materials and methods. The blots were probed with antibody against phospho-ATF2.

assays were used to evaluate whether inhibition of p38 altered the antiviral state conferred by the IFNs. Surprisingly, both IFN- $\alpha$ - and IFN- $\gamma$ -mediated protection of HeLa S3 cells against the CPE of encephalomyocarditis virus (EMCV) was compromised by pretreatment of cells with 10  $\mu$ M SB203580 prior to IFN stimulation (Figure 2B). At this concentration, SB203580 exhibits no effect on the proliferation or viral sensitivity of HeLa S3 cells over the duration of the CPE assay (data not shown). The inhibition of the antiviral activity of IFN- $\alpha$  by SB203580 (Figure 2B, upper panel) indicates the requirement for p38-dependent activation of cPLA<sub>2</sub> to facilitate ISGF3 formation, leading to antiviral gene transcription. Although SB203580 did not inhibit IFN- $\gamma$ -induced STAT1 homodimer formation (Figure 1C), p38 activity appears also to be essential for the antiviral activity of IFN- $\gamma$  (Figure 2B, lower panel). Accordingly, the status of p38 activity was examined after IFN- $\alpha$  or IFN- $\gamma$  stimulation of HeLa S3 cells.

#### Activation of p38 MAPK by IFN- $\alpha$ and IFN- $\gamma$

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 a MAPK and its level of tyrosine



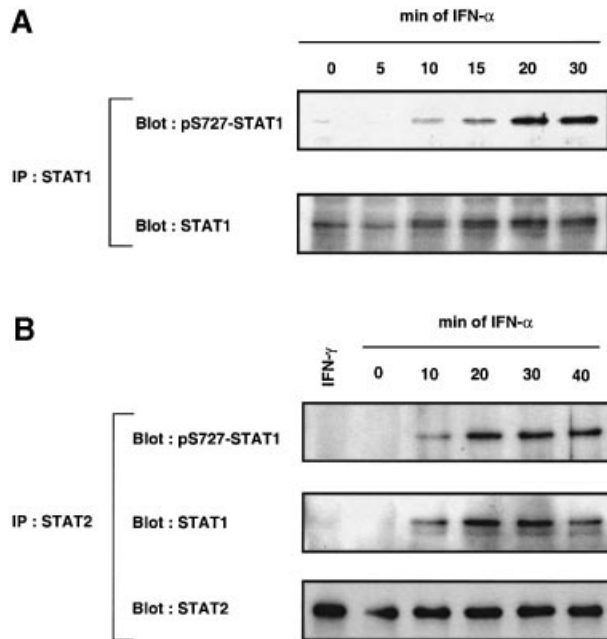
**Fig. 4.** Effect of p38 inhibition on IFN-induced pS727-STAT1 in HeLa S3. Cells were pretreated with 10  $\mu$ M SB203580, 30  $\mu$ M PD98059 or carrier for 30 min prior to stimulation with 1000 U/ml IFN- $\alpha$  or IFN- $\gamma$  for 30 min. Lysates were prepared for STAT1 immunoprecipitation (IP) and subjected to 8% SDS-PAGE and electroblotting. Membranes were probed with pS727-STAT1 antiserum before stripping and reprobing with STAT1 mAb. The data shown is representative of two separate experiments.

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- $\alpha$  and IFN- $\gamma$  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- $\alpha$  and IFN- $\gamma$  signalling pathways.

#### STAT1 Ser727 phosphorylation requires p38 activity

Ser727 phosphorylation on STAT1 is absolutely required for IFN- $\gamma$ -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- $\gamma$  even though STAT1 homodimers are formed in both cases. The inhibition of the antiviral activity of IFN- $\gamma$  by SB203580 (Figure 2B, lower panel) suggests that the ligand may require p38 activity to mediate its antiviral effect, perhaps by facilitating STAT1 Ser727 phosphorylation. Since previous reports implicate a role for ERK2 MAPK in STAT serine phosphorylation (David *et al.*, 1995; Chung *et al.*, 1997), we examined whether p38 or ERK2 activity is required for STAT1 Ser727 phosphorylation in our system.

STAT1 serine phosphorylation was analyzed in extracts from HeLa S3 cells treated with IFN- $\alpha$  or IFN- $\gamma$  after exposure to SB203580 or PD98059, a specific inhibitor of the ERK2 pathway. The results clearly show that SB203580 but not PD98059 causes attenuation of STAT1 serine phosphorylation induced by both IFNs in HeLa S3 cells (Figure 4). Therefore, p38 activity is required for

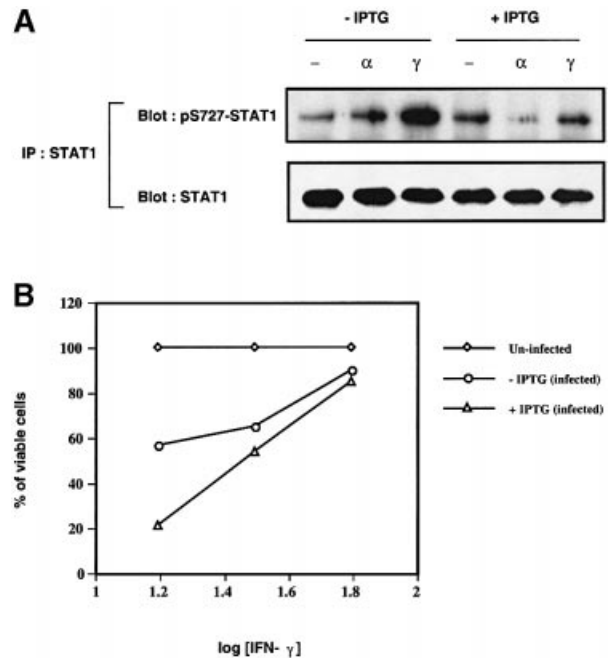


**Fig. 5.** Induction of pS727-STAT1 by IFN- $\alpha$  and its presence in ISGF3 in HeLa S3. (A) Cells were treated for different times with 1000 U/ml IFN- $\alpha$  before lysates were prepared for STAT1 immunoprecipitation (IP) and subjected to 8% SDS-PAGE and electroblotting. Membranes were probed with pS727-STAT1 antiserum before stripping and reprobing with STAT1 mAb. (B) Cells were treated for different durations with 1000 U/ml IFN- $\alpha$  before lysates were prepared for STAT2 immunoprecipitation (IP) and subjected to 8% SDS-PAGE and electroblotting. Membranes were probed with pS727-STAT1 antiserum before stripping and reprobing with STAT1 mAb. A third Western blot was performed to check for equal STAT2 loading. The data shown is representative of two separate experiments.

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- $\gamma$  stimulation, but there is only an isolated report related to IFN- $\alpha$  (Kovarik *et al.*, 1998). To examine in greater detail the induction of Ser727 phosphorylation upon IFN- $\alpha$  treatment, HeLa S3 cells were treated for different times with IFN- $\alpha$  and lysates prepared for STAT1 immunoprecipitation. IFN- $\alpha$  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- $\alpha$ , since the STAT1 S727A mutant is capable of conferring IFN- $\alpha$ -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- $\gamma$  treatment as a control. IFN- $\gamma$ , which induces Ser727 phosphorylation consistently, did not give a signal due to its inability to induce ISGF3 (Figure 5B, extreme left lane). Thus, IFN- $\alpha$  induces Ser727 phosphorylation on STAT1, and it is likely that pS727-STAT1 is a normal component of ISGF3.

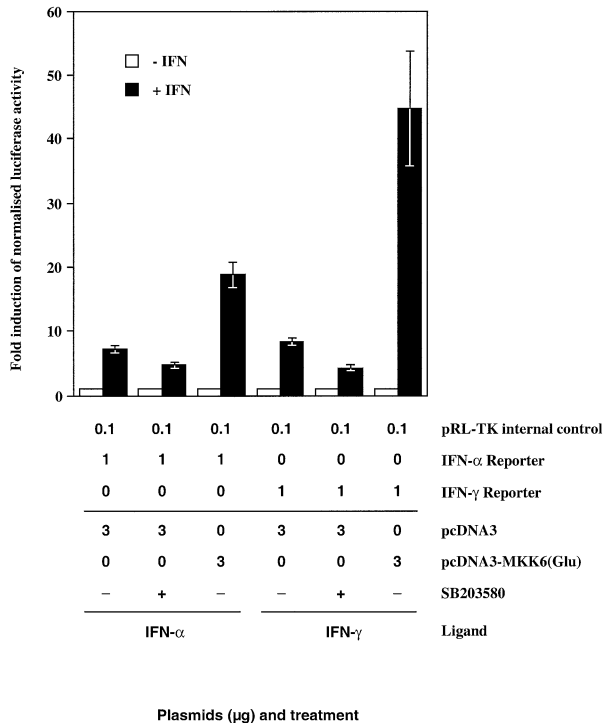


**Fig. 6.** Effect of dominant-negative p38 expression on IFN-induced pS727-STAT1 formation and correlation with a diminished antiviral state in 3T3-L1. (A) Cells were treated with 5 mM IPTG or carrier for 48 h before treating with 1000 U/ml IFN- $\alpha$  or IFN- $\gamma$  for 30 min. Lysates were prepared for STAT1 immunoprecipitation (IP) and subjected to 8% SDS-PAGE and electroblotting. Membranes were probed with pS727-STAT1 antiserum before stripping and reprobing with STAT1 mAb. (B) CPE assays were performed as described in Materials and methods. Data is presented for cells treated with three different concentrations of IFN- $\gamma$  (15, 30 and 60 U/ml). The data shown is representative of two separate experiments.

#### **Dominant-negative p38 inhibits STAT1 serine phosphorylation and the IFN- $\gamma$ -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- $\beta$ -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).

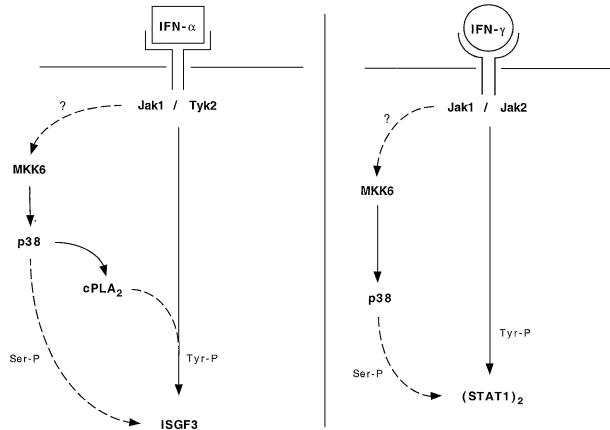
The 3T3-L1 cells harbouring mutant p38 were induced with IPTG or carrier for 48 h before treatment with IFNs and assayed for STAT1 Ser727 phosphorylation. Both IFN- $\alpha$ - and IFN- $\gamma$ -dependent serine phosphorylation of STAT1 were attenuated after cells were induced to express the dominant-negative 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- $\gamma$  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- $\gamma$  via a pathway involving STAT1 serine phosphorylation.



**Fig. 7.** Regulation of IFN-induced gene expression by MKK6 and p38 in HT-1080. Cells in 10-cm dishes were transiently transfected with the indicated amounts of plasmids. Twenty hours later, cells were pretreated for 30 min with SB203580 or carrier before treatment with 1000 U/ml IFN- $\alpha$  or IFN- $\gamma$  for 6 h. Firefly luciferase activities were normalized against *Renilla* luciferase activities to obtain a relative light unit, which is then presented as fold-induction relative to untreated sample for each construct. Error bars indicate standard deviation in triplicate samples. The data shown is representative of two separate experiments.

### 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- $\alpha$ -responsive reporter was constructed (see Materials and methods), which yielded an ~7-fold induction of reporter activity in HT-1080 cells upon IFN- $\alpha$  treatment (Figure 7). Pretreatment with SB203580 reduced the fold induction to 4.7, consistent with the involvement of p38 and cPLA<sub>2</sub> in ISGF3 formation. In contrast, co-transfection with a construct overexpressing constitutively activated MKK6 resulted in a >100% increase in IFN- $\alpha$ -induced reporter activity, to ~18-fold. This dramatic enhancement can be explained by an increase in p38 activation over that normally achieved by IFN- $\alpha$ , 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 (Enslin *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 (Raignaud *et al.*, 1996) used in the above experiment.



**Fig. 8.** Schematic representation of the divergent roles of p38 in IFN signalling. p38 is required for both serine and tyrosine phosphorylation events during STAT activation in IFN- $\alpha$  signalling, but plays a role only in serine phosphorylation during STAT1 activation in IFN- $\gamma$  signalling. MKK6 is a potential activator of p38 in both pathways.

Similar experiments were performed with an IFN- $\gamma$ -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- $\alpha$  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 cPLA<sub>2</sub>, the effects observed with IFN- $\gamma$  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- $\alpha$  and IFN- $\gamma$ .

### 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 cPLA<sub>2</sub>, are a vindication of this salient point. Whereas cPLA<sub>2</sub> activity is necessary only in the case of IFN- $\alpha$  signalling (Flati *et al.*, 1996), we have shown here that p38 MAPK is a component shared by both IFN- $\alpha$  and IFN- $\gamma$  signalling pathways. It is not known if p38 also activates cPLA<sub>2</sub> during IFN- $\gamma$  stimulation, but cPLA<sub>2</sub> inhibition apparently has no effect on IFN- $\gamma$  signalling. Thus, p38 can be viewed as playing a dual role in IFN- $\alpha$  signalling to facilitate both STAT1 Ser727 phosphorylation and ISGF3 formation, but performs a more specific role in IFN- $\gamma$  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- $\gamma$ -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- $\alpha$  (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- $\alpha$ -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- $\gamma$ , 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- $\kappa$ B and IRF-1 in response to stimuli such as dsRNA and IFN- $\gamma$  (Williams, 1997). Interestingly, p38 is also widely reported to regulate NF- $\kappa$ B-dependent transcription after its translocation into the nucleus (Schulze-Osthoff *et al.*, 1997). Recently, it has been shown that IFN- $\gamma$ -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- $\kappa$ B, where we can envisage PKR activating NF- $\kappa$ B for nuclear translocation through one pathway and simultaneously activating p38 to fine-tune the activity of NF- $\kappa$ B 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- $\alpha$  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 IFN- $\alpha$  by ameliorating these side effects need to take into account the impact of applying anti-inflammatory agents on the signalling pathways used by IFNs to mediate their biological effects.

## Materials and methods

### Reagents

IFNs were obtained from the following sources: human IFN- $\alpha$  (Schering-Plough), human IFN- $\gamma$  (R&D Systems), murine IFN- $\alpha$  (Hoffman La-Roche) and murine IFN- $\gamma$  (Boehringer Mannheim). SB203580, PD98059 and AACOCF<sub>3</sub> 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 antiserum) and Dr Ara Hovanessian (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- $\gamma$ -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 *Xma*I and *Xho*I linkers (forward primer: 5'-TAACCCGGGATCTATCATGATGGCCAC-3' and reverse primer: 5'-CCCCCTCGAGAATAAAGGGGATTCTAAA-3'). The digested PCR product was cloned into the *Xma*I-*Xho*I site of pGL2-Basic (Promega) to generate an IFN- $\alpha$ -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  $\mu$ g/ml streptomycin. Unless otherwise specified, cells were grown in 10-cm dishes to ~80% confluence before being placed 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, 10 mM  $\beta$ -glycerophosphate, 0.1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate and 2  $\mu$ g/ml each of pepstatin, aprotinin and leupeptin. Extracts were clarified by centrifugation (10 000 g) for 20 min at 4°C. Protein concentrations were determined by the Bradford or bicinchoninic acid assays. Extracts containing normalized amounts of protein were incubated with appropriate concentrations of primary antibodies (as recommended by manufacturers) for 1–2 h at 4°C before a further 1–2 h incubation with protein G-Sepharose beads. Immunocomplexes were washed three times with lysis buffer and boiled in Laemmli sample buffer for 3 min. Eluted proteins were resolved by SDS-PAGE (gel % depends on protein of interest) and electroblotted onto PVDF membrane. Membranes were blocked with blocking buffer (PBS containing 0.1% Tween-20 and 2% bovine serum albumin) before incubation for 1–3 h with the primary antibody diluted in blocking buffer. After washing three times with PBS containing 0.5% Tween-20, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1–2 h before three further washes. Enhanced chemiluminescence (ECL) was performed according to manufacturer's protocol (Amersham). For a second Western, the membrane was agitated in stripping buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM  $\beta$ -mercaptoethanol) for 30 min at 50°C. The membrane was blocked prior to reprobing.

### 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  $\mu$ l of kinase reaction buffer containing 25 mM HEPES pH 7.5, 10 mM magnesium acetate, 50  $\mu$ M ATP and 1  $\mu$ g of recombinant ATF2. The mixture was incubated at 30°C for 20 min, mixed with 30  $\mu$ l of 2 $\times$  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  $4 \times 10^4$  cells/well onto 96-well plates and incubated overnight. Cells were pretreated with either 10  $\mu$ M 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  $1 \times 10^4$  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- $\gamma$  for 18 h, then infected with EMCV at m.o.i. of  $1 \times 10^4$ . 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 uninfected EMCV-infected cells.

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