Phosphorylation of the Stat1 transactivating domain is required for the response to type I interferons

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Stat1 (signal transducer and activator of transcription 1) regulates transcription in response to the type I interferons IFN- α and IFN- β , either in its dimerized form or as a subunit of the interferon-stimulated gene factor 3 (Isgf3) complex (consisting of Stat1, Stat2 and interferon-regulating factor 9). Full-length Stat1- α and the splice variant Stat1- β , which lacks the carboxyl terminus and the Ser727 phosphorylation site, are found in all cell types. IFN-induced phosphorylation of Stat1- α on Ser727 occurs in the absence of the candidate kinase, protein kinase C- δ . When expressed in Stat1-deficient cells, Stat1- β and a Stat1-S727A mutant both restored the formation of Stat1 dimers and of the Isgf3 complex on treatment with IFN- β . By contrast, only Stat1- α restored the ability of IFN- β to induce high levels of transcription from target genes of Stat1 dimers and Isgf3 and to induce an antiviral state. Our data suggest an important contribution of the Stat1 C terminus and its phosphorylation at Ser727 to the transcriptional activities of the Stat1 dimer and the Isgf3 complex.

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INTRODUCTION

The type I interferons IFN- α and IFN- β , which are cytokines with essential roles in innate viral immunity (Sen, 2001), induce expression of their target genes through the JAK (Janus kinase)/Stat (signal transducer and activator of transcription) signalling pathway (Darnell *et al.*, 1994; O'Shea *et al.*, 2002). Receptor-associated JAK1 and tyrosine kinase 2 (Tyk2) kinases phosphorylate Stat1 and Stat2 on tyrosine residues, leading to the formation of Stat1 homodimers and Stat1/2 heterodimers. The Stat1 homodimer binds to promoters that contain an interferon- γ -activated site (GAS). The Stat1/2 heterodimer associates with interferon-regulating factor 9 (Irf9) to form the interferon-stimulated gene factor 3 (Isgf3) complex, which binds to interferon-stimulated response elements (ISREs) in target promoters.

Stat1- β is a naturally occurring splice variant of Stat1- α that lacks 38 carboxy-terminal amino acids. Stat1- β dimers bind DNA, but do not activate transcription. Therefore, the transactivating domain (TAD) of Stat1- α is thought to reside in the C terminus of the protein (Shuai *et al.*, 1993), which contains a phosphorylation site at Ser727. Phospho-Ser727 increases the transcriptional activity of Stat1 dimers in response to type II IFN (IFN- γ ; Wen *et al.*, 1995; Kovarik *et al.*, 2001). Type I IFNs also activate a kinase that phosphorylates Stat1 Ser727, which was recently identified as Pkc- δ (Uddin *et al.*, 2002). This suggests that a serine-phosphorylated Stat1 dimer may be required for the induction of gene expression. Several reports suggest that, unlike the Stat1 dimer, the Stat1 TAD and its phosphorylation are not essential for the transcriptional activity of Isgf3, and that the Stat2 TAD provides the Isgf3 transactivation function (Bromberg *et al.*, 1996; Horvath & Darnell, 1996; Qureshi *et al.*, 1996).

We have analysed the effects of expressing Stat1- β and of mutating Ser727 to alanine on the expression of genes that are induced by type I IFNs and are targets of the following: Isgf3 (*Mx*), Stat1 dimers (*Irf1*) or both of these (*Gbp1*). We found that the presence of the Stat1 TAD and its phosphorylation on Ser727 are necessary for the complete induction of all the genes analysed, as well as for the establishment of the antiviral state in response to type I IFNs.

Fig. 1 | Signal transducer and activator of transcription 1 phosphorylation at Ser727 in the absence of protein kinase C- δ . Macrophages derived from wild-type or protein kinase C- δ (Pkc- δ)-deficient mice were treated with interferon (IFN)- β , IFN- γ or lipopolysaccharide (Lps) for 60 min. Stat1 phosphorylation was analysed by western blotting with an antiserum that specifically recognizes the Ser727-phosphorylated molecule (Stat1-pSer727). For normalization, the blot was reprobed with a polyclonal antiserum to the Stat1 carboxyl terminus (Stat1C).

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Fig. 2 | Expression and activation of mutant signal transducer and activator of transcription 1. (A) Isolation of fibroblast clones expressing signal transducer and activator of transcription 1- β (Stat1- β). Stat1-deficient fibroblasts were transfected with a plasmid driving the expression of Stat1- β , selection was carried out, and cells were cloned by a limiting dilution procedure. Levels of Stat1- β were analysed by western blotting using a monoclonal antibody that recognizes the amino terminus (Stat1N). For normalization, the blot was reprobed with an antibody (pan-ERK) against extracellular signal-regulated protein kinases 1 and 2 (ERKs 1 and 2). The results from two clones that expressed Stat1- β (clones β 1 and β 8) and, for comparison, clones that expressed Stat1- α or the Stat1-S727A mutant are shown. (B) Stat1 tyrosine phosphorylation in response to interferon- β (IFN- β). Fibroblasts that expressed Stat1- α , Stat1- β or Stat1-S727A were stimulated with IFN- β for the durations indicated, and Stat1 phosphorylation at Tyr701 (pY701) was analysed by western blotting using a phospho-specific antiserum. For normalization, the blots were reprobed with a monoclonal antibodies that recognize either the N terminus or the carboxyl terminus of Stat1. (C) Formation of the interferon-stimulated gene factor 3 (Isgf3) complex. Fibroblasts expressing Stat1- α , Stat1- β or Stat1-S727A were treated for the durations indicated with IFN- β . The presence of Isgf3 in cell extracts was analysed by an electrophoretic mobility-shift assay using DNA containing an interferon-stimulated response-element sequence as a probe.

RESULTS

Stat1 phosphorylation in *Pkc- δ* -deficient cells

Pkc- δ is activated by IFN- α and IFN- β and catalyses Stat1 Ser727 phosphorylation *in vitro* (Uddin *et al.*, 2002). We tested whether *Pkc- δ* is essential for the phosphorylation on Ser727 that occurs after treatment with IFN- β and, as controls, with IFN- γ and lipopolysaccharide (Lps) (Fig. 1). When normalized to Stat1 expression levels, the differences in Stat1 phosphorylation on Ser727 observed in macrophages from wild-type and *Pkc- δ* -deficient mice were not significant. The same results were obtained using fibroblasts (data not shown).

Transcriptional activity of Stat1- β and Stat1-S727A

The importance of the Stat1 C terminus and its phosphorylation at Ser727 was studied in reconstituted fibroblasts derived from Stat1-deficient mice. Cells that express Stat1- α or Stat1-S727A have been described recently (Kovarik *et al.*, 2001). Similar stable cell lines

that express Stat1- β were produced, and two clones (β 1 and β 8) were selected for further investigation, as the levels of Stat1- β expression in these cells closely matched those reported for Stat1- α and Stat1-S727A in the two cell lines described recently (Fig. 2A; Kovarik *et al.*, 2001).

Levels of Stat1 tyrosine phosphorylation were similar in cells that express Stat1- α , Stat1- β and Stat1-S727A after treatment with IFN- β for various durations. One of the Stat1- β -expressing clones (β 8) showed a slightly more transient response (Fig. 2B). All of the variants of Stat1 that were expressed localized to the nucleus after IFN treatment, as visualized by immunofluorescence (data not shown). Similar amounts of the Isgf3 complex were formed in all lines after treatment with IFN- β (Fig. 2C). Therefore, signalling from the type I IFN receptor was intact in all of the clonal lines that were used in subsequent experiments.

To study gene expression, *Stat1*^{-/-} cells and cells that express Stat1- α , Stat1- β or Stat1-S727A were treated with IFN- β for 4 h or 6 h.

Complementary DNAs were synthesized from total RNA, which was normalized by agarose gel electrophoresis and used for real-time PCR analysis using TaqMan technology. Fig. 3 summarizes the effects of the absence of the Stat1 C terminus and of the mutation of its Ser727 phosphorylation site on the inducibility of the *Mx1*, *Gbp1* and *Irf1* genes after 4 h or 6 h of treatment with IFN- β . Consistent with previous studies, none of the genes were induced in Stat1-deficient cells. Cells that express Stat1- β or Stat1-S727A showed residual inducibility, but, for both, the reduction in induction of gene expression compared to cells that express Stat1- α was ~75–90%. Importantly, a requirement for the phosphorylation of the Stat1 C terminus was shown for the *Isgf3* target gene, *Mx1*, as well as for the Stat1-dimer target gene, *Irf1*, and for the *Gbp1* gene, the promoter of which contains both a GAS and an ISRE sequence (Hug et al., 1988; Pine et al., 1994; Briken et al., 1995).

To rule out any effects of clonal variation, a second pair of clones, which expressed similar levels of Stat1 variants to each other, but higher levels of Stat1- α and Stat1-S727A than in the previous clones, was analysed and gave similar results (data not shown). Furthermore, a transient transfection assay was carried out to analyse the effects of Stat1- α and Stat1-S727A expression in a non-clonal population of Stat1-deficient cells. Comparable gene-transfer efficiency was controlled for by using a co-transfected β -galactosidase expression vector. Half of each transfected population was treated with IFN- β for 4 h and used for real-time quantitative PCR analysis of the endogenous *Mx1*, *Gbp1* and *Irf1* genes. Compared to cells that express Stat1- α , an ~50% reduction in IFN- β -mediated induction of the *Mx1* and *Gbp1* genes was seen in cells that express Stat1-S727A (Fig. 4). The reduced effect of the S727A mutant protein compared with that seen in the stable lines is probably due to high levels of expression of the Stat1 proteins during transient transfection, and has also been seen for the response to IFN- γ (K.R. and T.D., unpublished data). Any induction of *Irf1* in the transient transfection experiments was below detectable levels.

Antiviral response in cells that express mutant Stat1

To study the biological consequences of mutations in the Stat1 TAD, Stat1-deficient fibroblasts and lines that express Stat1- α , Stat1- β or Stat1-S727A were treated with serial dilutions of IFN- β and were infected with vesicular stomatitis virus (VSV). The cytopathic effect (CPE) of viral infection was evaluated by staining surviving cells with crystal violet, followed by taking an absorbance measurement. As expected, cells that express Stat1- α were protected from CPE

◀ **Fig. 3** | Requirement of the signal transducer and activator of transcription 1 transactivating domain and its phosphorylation at Ser727 for the expression of interferon- β -induced genes. Stat1-deficient fibroblasts, and cells that express Stat1- α , Stat1- β or Stat1-S727A were treated with interferon- β (IFN- β) for 4 h or 6 h. Expression of the *Irf1*, *Gbp1* and *Mx1* genes was determined by quantitative real-time PCR of reverse-transcribed messenger RNA. For normalization to input quantities, the amount of *Hprt* mRNA was determined. (A) Inducibility in response to treatment with IFN- β (that is, the ratio of expression in IFN- β -treated and untreated cells) after 4 h and 6 h in one of three independent experiments. (B) The results of three independent experiments are shown, expressed as the percentage induction $\pm$ s.d. in cells that express Stat1- β , Stat1-S727A or that express no Stat1, compared with the values measured in cells that express Stat1- α (100%).

Fig. 4 | Effect of the signal transducer and activator of transcription 1-S727A mutation on the expression of the endogenous *Mx1* and *Gbp1* genes during transient transfection. Stat1-deficient fibroblasts were co-transfected with Stat1- α or Stat1-S727A and a plasmid that drives expression of the β -galactosidase gene (β -Gal). Some of the transfected cells were used for measuring β -Gal activity in cell extracts to ensure comparable transfection efficiencies. The remaining cells were either left untreated or were treated with interferon- β (IFN- β) for 4 h, after which total RNA was isolated and processed for real-time quantitative PCR analysis of the expression of the *Mx1* and *Gbp1* genes. Inducibility is calculated as the ratio of expression measured in IFN- β -treated and untreated cells.

even at low levels of IFN- β and Stat1-deficient cells were not protected from viral lysis at all. Stat1- β - and Stat1-S727A expressing cells showed a reduction in the ability to establish the antiviral state (Fig. 5). At high IFN- β levels, a residual antiviral response was seen in cells that express Stat1-S727A, whereas it was almost abolished in cells that express Stat1- β .

DISCUSSION

We, and others, have recently reported serine phosphorylation of the Stat1 TAD during responses to type I IFNs (Kovarik *et al.*, 1998; Goh *et al.*, 1999). Here, we show that phosphorylation occurs in the absence of Pkc- δ , a kinase that is reported to be stimulated by type I IFNs and that is able to phosphorylate Stat1 at Ser727 *in vitro* (Uddin *et al.*, 2002). Our results do not rule out a function for Pkc- δ in the phosphorylation of Stat1, but they do show that Pkc- δ is not the only kinase able to do this in cells stimulated with type I IFN. Potential candidates for other enzymes that can carry out this function are other Pkc isoforms or calcium/calmodulin-dependent protein kinase II, which is thought to phosphorylate Stat1 at Ser727 in response to IFN- γ (Nair *et al.*, 2002).

Previous reports have suggested that the Stat1 TAD and its phosphorylation are important for the function of Stat1 dimers (Shuai *et al.*, 1993; Kovarik *et al.*, 2001); however, their impact on Isgf3 activity has

Fig. 5 | Antiviral activity. Fibroblasts that express Stat1- α , Stat1- β , Stat1-S727A or no form of Stat1 were treated with serial dilutions of interferon- β (IFN- β) for 14 h. The cells were then infected with vesicular stomatitis virus (VSV) at an MOI (multiplicity of infection) of 0.1. After 24 h, remaining viable cells were stained with crystal violet and staining intensity was determined by absorbance measurements at 620 nm.

not been recognized previously. Consistent with our findings, serine-phosphorylated Stat1 participates in Isgf3 formation (Goh *et al.*, 1999). Phospho-Ser727 greatly enhanced the ability of Stat1 to interact with the transcriptional machinery. One explanation for this might be an increased association of Ser727-phosphorylated Stat1 with the minichromosome maintenance 5 (Mcm5) protein, the variation in expression of which during the cell cycle correlates with the ability of IFN- γ to induce the expression of a Stat1 reporter gene (Zhang *et al.*, 1998). An interaction of Mcm5 with the Isgf3 complex has not been tested; however, the data suggest that Mcm5 functions as a transcriptional coactivator in the context of the Stat1 dimer. Alternatively, or in addition, phosphorylation might influence the association of Stat1 complexes with nuclear chromatin, a possibility that is not ruled out by the apparent lack of requirement for Stat1 phosphorylation on Ser727 for its association with DNA *in vitro* (Wen *et al.*, 1995).

The prevailing view in the literature is that Stat2 provides the essential TAD for the Isgf3 complex. This is based on genetic experiments in human fibrosarcoma cells made deficient for either Stat1 (U3A cells) or Stat2 (U6A cells) by chemical mutagenesis (Darnell *et al.*, 1994). The importance of the Stat2 TAD for Isgf3 activity was established in experiments using U6A cells that express a Stat2 protein with a truncated C terminus (Qureshi *et al.*, 1996), and was proven further using GAL4-based reporter systems (Park *et al.*, 1999; Paulson *et al.*, 1999) and by the demonstration of coactivator recruitment (Paulson *et al.*, 1999, 2002). Expression of Stat1- β in U3A cells in the presence of wild-type Stat2 had variable effects on IFN-induced gene expression. Most genes analysed, notably *Gbp1*, were significantly affected by the absence of the Stat1 TAD (Muller *et al.*, 1993). The latter study is therefore consistent with our results in showing a function of the Stat1 TAD in IFN-type-I-induced transcription, but emphasizes that Isgf3 complexes that contain Stat1- β retain some transcriptional activity.

Our study provides evidence for an important function of a phosphorylated Stat1 TAD in the establishment of the antiviral state in response to IFN- β . U3A cells that express Stat1- β or Stat1-S727A have been reported to establish an antiviral state in response to treatment with saturating concentrations of type I IFNs (Horvath & Darnell, 1996). In our study, the already reduced ability of Stat1-S727A to induce the antiviral state in the presence of saturating concentrations

of IFN- β decreased rapidly to even lower levels at lower concentrations of IFN- β . As seen for the IFN- γ response (Kovarik *et al.*, 2001), discrepancies between results from reconstituted U3A cells and those from reconstituted fibroblasts from Stat1-deficient mice might be due to the origin of the cells (human versus mouse; transformed versus immortalized), or to previously unnoticed effects of the chemical mutagenesis that preceded the selection of U3A cells. In the 11.1 cell line, which is like the U3A mutagenized progeny of 2fTGH human fibrosarcoma cells, Tyk2 deficiency abolishes responsiveness to IFN- α (John *et al.*, 1991; Velazquez *et al.*, 1992). By contrast, cells derived from Tyk2-deficient mice show reduced IFN- α signalling, but establish an antiviral state comparable to that of wild-type cells (Karaghiosoff *et al.*, 2000).

In most cells, the amounts of Stat1- α present exceed those of Stat1- β , but the ratio of these proteins varies between cell types. Whether, and how, the splicing of alternative Stat1- α or Stat1- β mRNAs is regulated is still unknown. Our results suggest that the variable Stat1- α :Stat1- β ratio might pre-set the level at which IFN- α - and IFN- β -inducible genes are transcribed. A further implication of our study is that the extent to which Stat1 molecules are phosphorylated at Ser727 determines the transcriptional output in cells responding to type I IFNs. Because stress and inflammatory signals cause Stat1 phosphorylation on Ser727 (Decker & Kovarik, 2000), an incoming virus might increase the proportion of serine-phosphorylated Stat1 that is available for a response to IFN. The potential function of the Ser727 phosphorylation site as an environmental sensor for the biological context of an IFN response will be addressed in future research.

METHODS

Cells and cytokines. 3T3 fibroblasts from Stat1-deficient mice (Durbin *et al.*, 1996), either untransfected or stably transfected with Stat1 variants, were cultured in DMEM containing 10% FCS. IFN- β (Calbiochem) was used at a concentration of 500 units ml⁻¹. Bone marrow was isolated from femurs of wild-type or isogenic Pkc- δ -deficient mice (Leitges *et al.*, 2001). Macrophages were obtained by culturing bone marrow in L-cell-derived colony-stimulating factor 1.

Plasmids. A Stat1- β cDNA was inserted into pEF-Zeo, as described recently for Stat1- α and Stat1-S727A (Kovarik *et al.*, 2001).

Cell transfection. For transient transfection, Effectene reagent (Qiagen) was used in accordance with the manufacturer's instructions. Stable transfectants were isolated as described in Kovarik *et al.* (2001).

Antibodies. Antisera against the Stat1 C terminus and against phospho-S727-Stat1 have been described recently (Kovarik *et al.*, 1998). Rabbit antiserum against Tyr701-phosphorylated Stat1 and a monoclonal antibody against the Stat1 amino terminus were purchased from New England Biolabs and Transduction Laboratories, respectively. The monoclonal antibody against extracellular-signal-regulated protein kinases (ERKs) (pan-ERK) was purchased from Transduction Laboratories.

Western blot analysis. A protocol for this procedure has been described recently in Kovarik *et al.* (1998).

Electrophoretic mobility-shift assay. The conditions used for this assay have been described previously (Eilers *et al.*, 1994); this was carried out using a double-stranded oligonucleotide corresponding to the ISRE sequence from the interferon-stimulated gene 5 promoter as a probe.

RNA preparation, complementary DNA synthesis and real-time PCR.

Total RNA extraction and cDNA synthesis have been described in Kovarik *et al.* (2001). TaqMan real-time PCR was carried out using a light cycler (Roche). For normalization, the housekeeping gene that encodes hypoxanthine phosphoribosyltransferase (Hprt) was amplified (Heid *et al.*, 1996). TaqMan probes labelled with the reporter dye 6-carboxy-fluorescein (FAM) and the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA) were obtained from Metabion. The sequences of the probes and the forward (f) and reverse (r) primers were as follows: Hprt/FAM, 5'-TGGGAGGCCATCACATTGTGGC-3'; Hprt-f, 5'-TTGCTCGAGATGT-CATGAAGGA-3'; Hprt-r, 5'-TGAGAGATCATCTCCACCAATAACTT-3'; Irf1/Fam, 5'-CAGTCTGAGTGGCAGCCGACACACA-3'; Irf1-f, 5'-CCGAAGACCTTATGAAGCTCTTTG-3'; Irf1-r, 5'-GCAAGTATCC-CTTGCCATCG-3'; Gbp1/Fam, 5'-AAAATCAAGAACATGCCTCCAC-CTCG-3'; Gbp1-f, 5'-ATCATATCCCTTAACTTCAGGAACAG-3'; Gbp1-r, 5'-GTGGAACAGGGTAGAGAGCTTTAGT-3'; Mx1/Fam, 5'-AGCCTACTACGAGGTGCAGACGGAA-3'; Mx1-f, 5'-GACTAC-CACTGAGATGACCCAGC-3'; Mx1-r, 5'-ATTTCCTCCCCAAAT-GTTTCA-3'.

Antiviral assay. 2 × 10⁴ fibroblasts were seeded into microtitre wells containing serial dilutions of IFN- β . After overnight incubation with the cytokine, the cells were infected with the Indiana strain of vesicular stomatitis virus (VSV) at an MOI (multiplicity of infection) of 0.1. Twenty-four hours after infection, remaining viable cells were stained with a 0.2% solution of crystal violet in 20% methanol. After repeated washing, cell-associated dye was dissolved in methanol and the absorbance was measured at 620 nm.

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