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Specificity of signaling by STAT1 depends on SH2 and C-terminal domains that regulate Ser727 phosphorylation, differentially affecting specific target gene expression

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Complete activation of signal transducer and activator of transcription 1 (STAT1) requires phosphorylation at both Y701 and a conserved $PMS_{727}P$ sequence. S727 phosphorylation of STAT1 in interferon-γ (IFN-γ)-treated mouse fibroblasts occurred without a need for p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1 and 2 or c-Jun kinases, and required both an intact SH2 domain and phosphorylation of Y701. In contrast, UV irradiation-induced STAT1 phosphorylation on S727 required p38MAPK, but no SH2 domain–phospho-tyrosine interactions. Mutation of S727 differentially affected IFN-γ target genes, at the level of both basal and induced expression. Particularly strong effects were noted for the GBP1 and TAP1 genes. The $PMS_{727}P$ motif of STAT3 was phosphorylated by stimuli and signaling pathways different from those for STAT1 S727. Transfer of the STAT3 C-terminus to STAT1 changed the stimulus and pathway specificity of STAT1 S727 phosphorylation to that of STAT3. Our data suggest that STAT C-termini contribute to the specificity of cellular responses by linking individual STATs to different serine kinase pathways and through an intrinsically different requirement for serine phosphorylation at different target gene promoters.

Keywords: phosphorylation/signal transduction/STAT1/transcription

Introduction

The JAK–STAT paradigm specifies a signaling pathway employed by most cytokine receptors to reprogram gene expression rapidly (Darnell *et al*., 1994; Schindler and Darnell, 1995). Receptor-associated JAKs are activated after binding of ligand, whereupon they phosphorylate receptor chains and thus create docking sites for the STAT SH2 domains. Receptor-associated STATs are phosphorylated by JAKs on a single tyrosine residue. They dimerize by reciprocal phosphotyrosine–SH2 domain interactions, traverse the nuclear pore and bind a class of DNA sequences collectively named GAS elements (Decker *et al*., 1997). The only two firmly established interaction partners for the STAT SH2 domains are cytokine receptors and homo- or heterotypic STATs; in addition, the JAKs may possibly also interact (Gupta *et al*., 1996; Barahmand-Pour *et al*., 1998).

The JAK–STAT pathway is intertwined with other signaling routes (Briscoe *et al*., 1997; Decker and Kovarik, 1999; Ramana *et al*., 2000a). An important signal convergence point was uncovered with the identification of a STAT serine phosphorylation site (Wen *et al*., 1995). With the exception of STATs 2 and 6, the C-termini of all family members contain a conserved PMSP or a PSP motif located between amino acids 720 and 730. Its phosphorylation can be caused by a variety of different stimuli and signaling pathways (Decker and Kovarik, 2000). Mutation of the C-terminal serine residue to alanine decreases the transcription factor activity of STAT1 and STAT4 (Wen *et al*., 1995; Visconti *et al*., 2000), prolongs tyrosine phosphorylation and DNA binding of STAT5a (Beuvink *et al*., 2000), or causes both a decrease in transcriptional activity and sustained tyrosine phosphorylation of STAT3 (Wen *et al*., 1995; Chung *et al*., 1997; Schuringa *et al*., 2000). How these seemingly opposing effects on activity are embedded into STAT3-dependent cytokine responses still needs to be solved.

Type I and type II interferons (IFNs) cause phosphorylation of STAT1 on both Y701 and S727 (Wen *et al*., 1995; Kovarik *et al*., 1998; Goh *et al*., 1999; Uddin *et al*., 2000). Reconstitution of STAT1-deficient U3A fibrosarcoma cells with STAT1-S727A restored biological responses to IFN-α. However, the mutation severely reduced the ability of STAT1 to establish the antiproliferative or antiviral effects after IFN-γ treatment (Bromberg *et al*., 1996; Horvath and Darnell, 1996). Therefore, phosphorylation of the STAT1 dimer on S727 appears to be critical for IFN-γ responses, but not for IFN-α responses. Moreover, the heterotrimeric ISGF3 (STAT1–STAT2–IRF9) complex that binds to a distinct response element, the ISRE (Levy *et al*., 1988), appears to dominate the response to type I IFN without requiring S727-phosphorylated STAT1.

IRF1 is the only target gene analyzed so far for its dependence on STAT1 S727 phosphorylation. In STAT1-S727A-reconstituted U3A cells, transcription was reduced by ~80% (Wen *et al*., 1995). It is unclear whether this result can be extrapolated to STAT1 target genes in general, or whether the effect of the mutation differs for individual genes.

IFN-γ-stimulated phosphorylation of STAT1 on S727 and Y701 was reported to occur without a mechanistic interdependence because mutation of one residue did not

affect phosphorylation of the other (Zhu *et al*., 1997). However, in IFN-γ-treated murine macrophages, most of the S727-phosphorylated STAT1 was contained in the fraction of molecules also phosphorylated on Y701 (Kovarik *et al*., 1998). This result suggested some positive influence of one phosphate on the presence of the other. The IFN-γ-stimulated STAT1 serine kinase remains to be identified. IFN-γ stimulates S727 phosphorylation independently of p38 mitogen-activated protein kinase (MAPK; Kovarik *et al*., 1999). JAK2 or protein kinase R (PKR) deficiency, or expression of a dominant-negative allele of the Pyk2 tyrosine kinase, abrogates IFN-γ-induced STAT1 S727 phosphorylation (Zhu *et al*., 1997; Takaoka *et al*., 1999; Ramana *et al*., 2000b). A link between these transducers, if any, has not been established, and their position with respect to STAT1 is unclear.

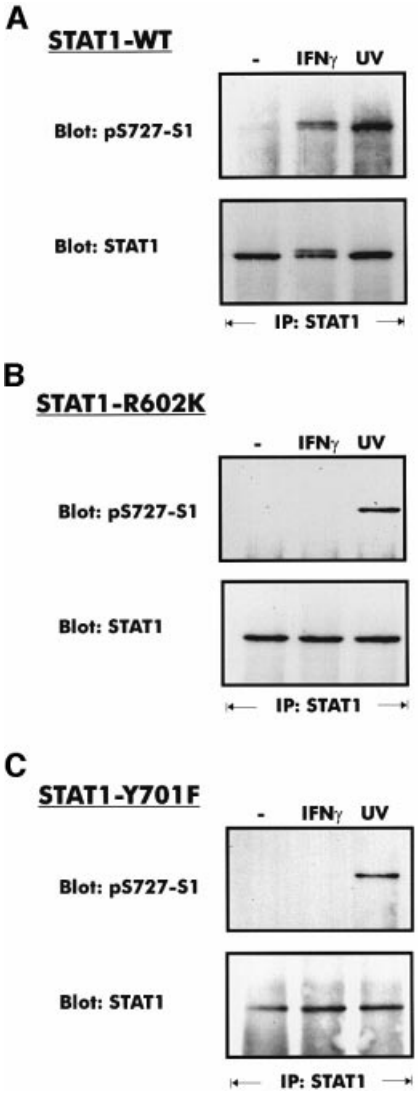

Fig. 1. Phosphorylation of S727 in fibroblast clones from STAT1-deficient mice after stable introduction of STAT1-WT (**A**), STAT1-R602K (**B**) or STAT1-Y701F (**C**). Where indicated, the cells were treated with IFN-γ for 30 min, or irradiated with UV light. STAT1 was precipitated from cellular extracts with a C-terminal antiserum. Western blots of the precipitated proteins were stained with phosphospecific antiserum for S727 (upper panels) and reprobed with a STAT1 C-terminal antiserum (lower panels).

Phosphorylation of STAT1 on S727 in response to signals of stress, inflammation or infection (hereafter collectively referred to as stress) occurs in the absence of tyrosine phosphorylation. The biological relevance is to prime STAT1 for an increased transcriptional response once IFN-γ provides the stimulus for tyrosine phosphorylation (Kovarik *et al*., 1998, 1999; Stoiber *et al*., 1999). Stress signals to STAT1 through a p38MAPK pathway. p38MAPK specifically phosphorylates STAT1 on S727 *in vitro* and may be the stress-induced STAT1 kinase, rather than a signaling intermediate. It is an unresolved question whether stress- and IFN-γ-mediated phosphorylation of STAT1 on S727 involve common steps and signal transducers.

The biological activities of STAT1 and STAT3 differ strikingly (Levy, 1999; Akira, 2000; Bromberg and Darnell, 2000). In simple terms, STAT1 activity is correlated with pro-inflammatory and antiproliferative responses, whereas STAT3 activity is linked to proliferation and mostly anti-inflammatory cytokines. This suggests that in spite of the identical position of the PMSP motif in their C-termini, serine phosphorylation of STAT1 and STAT3 might be regulated by different cell surface stimuli and via different signaling pathways. The structural basis for phosphorylation by different kinases *in vivo* is not known. Compared with the STAT1 C-terminus, the STAT3 C-terminus appears to be a far better substrate for extracellular signal-regulated kinases (ERKs; Chung *et al*., 1997).

In this study, we pursued three major goals related to open questions outlined above. First, we investigated STAT1 S727 phosphorylation by either IFN-γ or UV irradiation and report that the former requires an intact SH2 domain and tyrosine phosphorylation of STAT1 whereas the latter does not. Secondly, we show that the C-termini of STATs 1 and 3 specify the interaction with different MAPKs. Thirdly, we report striking differences for IFN-inducible genes in their requirement for STAT1 serine phosphorylation.

Results

Phosphotyrosine–SH2 domain interactions are required for S727 phosphorylation of STAT1 in response to IFN-γ, but not in response to UV irradiation

To determine whether stress- and IFN-γ-induced phosphorylation of STAT1 on S727 might differ in their requirement for SH2 domain–phosphotyrosine interactions, we transfected 3T3 fibroblasts derived from STAT1-deficient mice (Durbin *et al*., 1996; Marie *et al*., 1998) with plasmids expressing either wild-type STAT1 (STAT1-WT) or the R602K mutant with a binding-deficient SH2 domain (Gupta *et al*., 1996). Pools of stable transfectants were selected and clonal lines expressing comparable amounts of STAT1 were derived by a limiting dilution protocol. As expected, STAT1-R602K was not phosphorylated on tyrosine when isolated from IFN-γ-treated cells (data not shown). Western blots using phosphospecific antiserum demonstrated the expected S727 phosphorylation of STAT1-WT by both IFN-γ and UV irradiation (Figure 1A). In contrast, STAT1-R602K was phosphorylated only in the case of UV irradiation, but

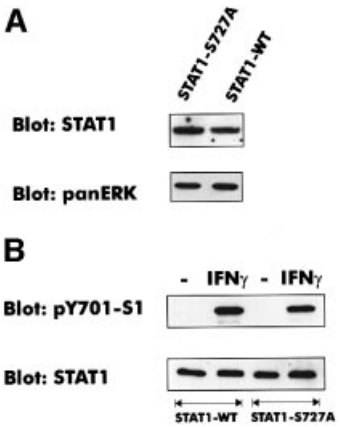

Fig. 2. Expression and tyrosine phosphorylation of STAT1-S727A. (**A**) Cellular lysates of fibroblasts from STAT1-deficient mice, reconstituted with either STAT1-WT or STAT1-S727A, were analyzed by western blotting with a STAT1 N-terminal monoclonal antibody. For loading control, the blot was reprobed with a panERK monoclonal antibody. (**B**) Tyrosine phosphorylation in cellular lysates was determined using a phosphotyrosine 701-specific antiserum. Equal loading was confirmed with a STAT1 N-terminal monoclonal antibody.

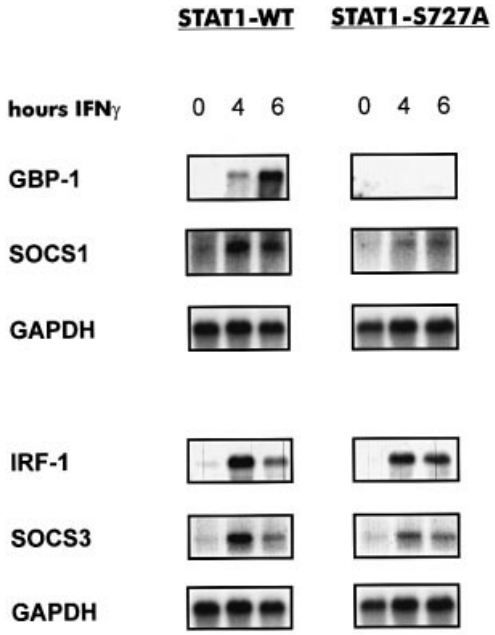

Fig. 3. Northern blot analysis of IFN-inducible mRNAs from STAT1-deficient fibroblasts after reconstitution with STAT1-WT or STAT1-S727A. A 15 μg aliquot of total RNA was blotted to membranes and hybridized to labeled cDNAs corresponding to the indicated mRNAs.

not in the case of IFN-γ treatment (Figure 1B). To test whether STAT1 tyrosine phosphorylation is needed for S727 phosphorylation in response to IFN-γ, we reconstituted Stat1-deficient cells with the Y701F mutant of STAT1. Again, S727 phosphorylation was triggered by UV irradiation, but not by IFN-γ treatment (Figure 1C). Therefore, lack of S727 phosphorylation in the case of the STAT1-R602K mutant most probably reflects the SH2 domain requirement for tyrosine phosphorylation.

Promoters of IFN-inducible genes can be distinguished by a different requirement for STAT phosphorylation on S727

IFN-inducible promoters contain the GAS or ISRE sequences, or both elements (Darnell *et al*., 1994; Stark *et al*., 1998). The IRF1 promoter, whose activation requires STAT1 S727 phosphorylation, contains only the GAS (Pine *et al*., 1994). No data are available on how the other promoter types are affected by the S727A mutation. To address the importance of STAT1 serine phosphorylation for different target genes, STAT1-deficient 3T3 fibroblasts were reconstituted with the STAT1-S727A mutant as described above. Clonal lines expressing levels of STAT1-S727A comparable to STAT1-WT-reconstituted cells (shown in Figure 2A) were of particular importance, because vast overexpression tended to reduce the penetrance of the S727A mutation.

Following IFN-γ treatment, STAT1-WT and STAT1-S727A were phosphorylated on tyrosine with almost identical efficiency (Figure 2B). This confirms that S727 phosphorylation has no detectable effect on STAT1 tyrosine phosphorylation by the JAKs (Zhu *et al*., 1997; Kovarik *et al*., 1998) and excludes the transcriptional differences between STAT1-S727A and STAT1-WT resulting from different levels of tyrosine phosphorylation.

The IFN-inducible IFP53 promoter contains a GAS, but no ISRE (Strehlow *et al*., 1993). In transient transfections, the S727A mutant caused an average reduction in IFN-γ-stimulated IFP53 reporter gene transcription of 50%, whereas in U3A cells the reduction was ~80%, consistent with published data obtained with a Ly6 reporter gene (data not shown; Wen *et al*., 1995).

Expression of the genomic IRF1 gene, analyzed by northern blotting (Figure 3), was reduced in STAT1-S727A cells by ~20–50% in independent experiments. In contrast, GBP1 mRNA was virtually undetectable in IFN-γ-treated STAT1-S727A cells. Since the same RNA preparation was used to analyze IRF1 and GBP1, misinterpretations based on variations in the IFN-γ response can be excluded. SOCS1 and SOCS3 mRNAs encoding IFN-induced feedback inhibitors of cytokine signaling (Hilton, 1999) showed an intermediate dependence on S727 phosphorylation. The reduction in IFN-γ-induced expression was more pronounced than in the case of IRF1, but less than in the case of the GBP gene. Thus, the extent to which serine phosphorylation of STAT1 is required for function appears to be determined largely by the target promoter.

To investigate the effect of the S727A mutation on the GBP promoter beyond the sensitivity of the northern blot experiment, we employed real-time PCR of reverse-transcribed mRNA. Parameters were adjusted to obtain a linear relationship between input mRNA (cDNA) copy number and the number of PCR cycles required to reach a fixed threshold of DNA synthesis (C_T value). In this range, the gene of interest can be normalized to the housekeeping HPRT gene product and a quantitative comparison of mRNA amounts in different situations is possible. Figure 4A shows the standard curves obtained for GBP and HPRT; however, similar standard curves were obtained for all genes analyzed below (data not shown).

Real-time PCR analysis of reverse-transcribed, IFN-γ-induced GBP1 mRNA confirmed the strong decrease caused by the lack of STAT1-S727 phosphorylation

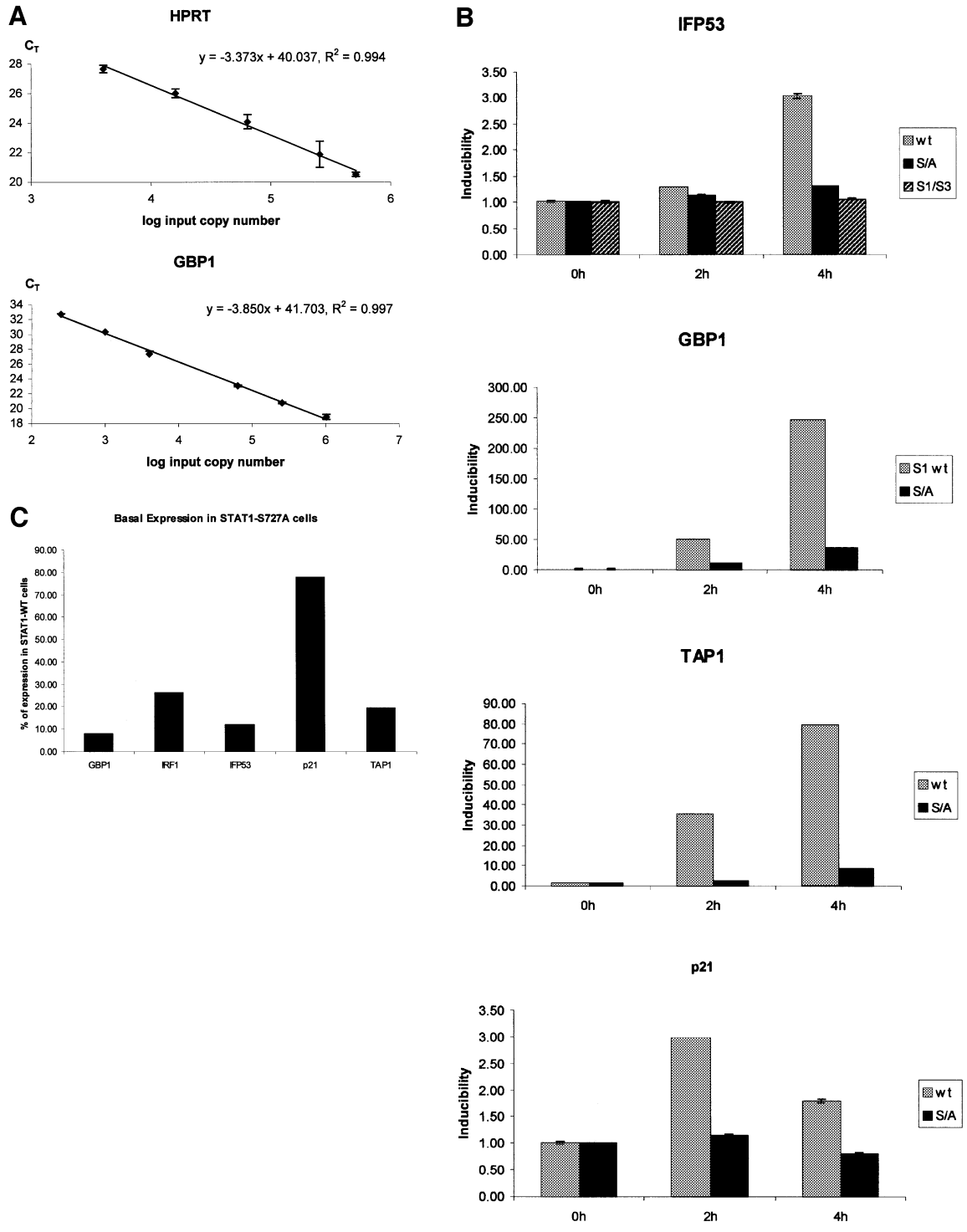

Fig. 4. Real-time PCR analysis of reverse-transcribed IFN-γ-induced mRNAs. (**A**) Standard curves for HPRT and GBP1. The input cDNA templates (log input copy number) are plotted against the C_T values. The HPRT standard curve was used to normalize the target gene expression for differences in the amount of cDNA added to each reaction. (**B**) mRNA inducibility of the indicated genes determined in fibroblasts reconstituted with STAT1-WT- (light gray), STAT1-S727A- (dark gray) or STAT1–STAT3 chimera-reconstituted fibroblasts (hatched, IFP53 only). mRNA quantities were determined in samples from untreated cells or after 2 and 4 h of IFN-γ treatment. Inducibility was calculated as the difference of HPRT-normalized C_T values in control and IFN-γ-treated cells. (**C**) Quantitative comparison of the basal expression levels of the indicated genes in STAT1-WT- and STAT1-S727A-reconstituted cells. The numbers of mRNA molecules in each sample were calculated from HPRT-normalized standard curves and the percentage reduction caused by the S727A mutation was calculated.

(Figure 4B). However, a second effect of S727A mutation became apparent: the severe reduction of basal GBP expression to 8% of the amount found in STAT1-WT cells (Figure 4C). Compared with STAT1-WT cells, the combined effect of reduced basal and induced expression caused an ~85-fold decrease in the number of mRNA molecules found in IFN-γ-treated STAT1-S727A cells. This explains why the low residual GBP inducibility in

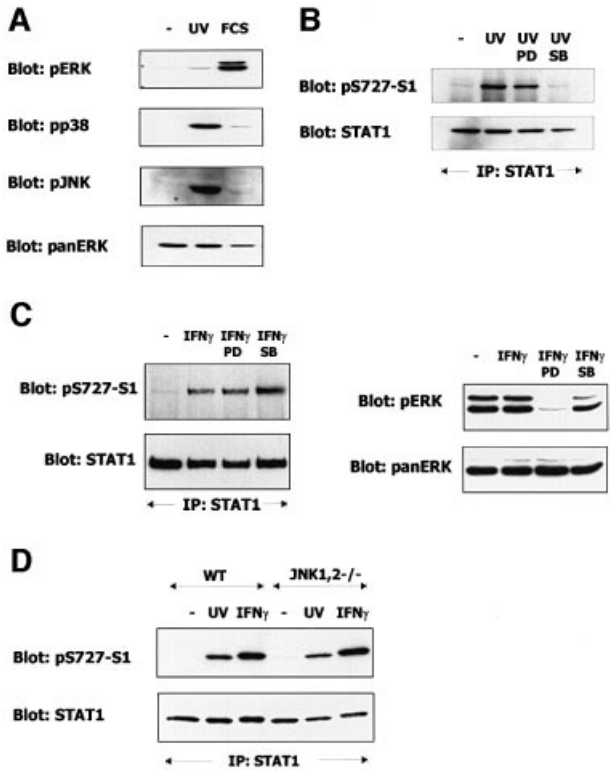

Fig. 5. Contribution of MAPK pathways to stress-induced phosphorylation of STAT1 on S727. Where indicated, 3T3 mouse fibroblasts were exposed to UV light, treated with 10% FCS, or with IFN-γ. (**A**) Activation of MAPK pathways was determined in cell lysates with phosphospecific antibodies to ERK (pERK), JNK (pJNK) or p38MAPK (pp38). The loading control was performed with a monoclonal panERK antibody. (**B**) The contributions of ERKs and p38MAPK were assessed by adding the MEK inhibitor PD98059 (PD) or the p38MAPK inhibitor SB203580 (SB) 30 min before UV irradiation. STAT1 was precipitated from the cell extracts using a STAT1 C-terminal antiserum. Western blots of the precipitates were stained with phosphospecific antiserum for S727 (upper panel) and reprobed with a STAT1 C-terminal antiserum (lower panel). (**C**) The contributions of ERKs and p38MAPK to the IFN-γ-induced S727 phosphorylation of STAT1 were assessed as in (B). Aliquots of the cell extracts used for immunoprecipitation of STAT1 were analyzed directly by western blotting with pERK and pan-ERK antibodies to assess ERK activity and confirm equal loading, respectively. (**D**) The contribution of JNKs was analyzed in fibroblasts deficient for both JNK1 and JNK2. STAT1 was precipitated from control (WT) and JNK/JNK2$^{-/-}$ fibroblasts using STAT1 C-terminal antiserum. STAT1 phosphorylation on S727 (upper panel) and loading (lower panel) were determined with phosphospecific or STAT1 C-terminal antisera.

these cells is below the detection threshold of a northern blot. By comparison, the reduction of basal IRF1 expression in STAT1-S727A cells was less pronounced (28% of wild type; Figure 4C) and a smaller reduction in inducibility was found (data not shown).

A number of IFN-γ-inducible genes not previously analyzed by northern blotting were investigated by real-time PCR (Figure 4B). In agreement with the reporter gene assay, induction of the endogenous IFP53 gene by IFN-γ was reduced by ~50% and a similar reduction was found for the p21 cyclin-dependent kinase (CDK) inhibitor gene. In contrast, the reduction of IFN-γ-inducibility found for the transporter associated with the antigen presentation 1 (TAP1) gene was even stronger than that found for the GBP1 gene. Basal expression of the TAP1 and IFP53 genes was also strongly affected by the S727A mutation (Figure 4C), whereas very little effect was noted for the p21 gene. The comparison between IFP53 and p21 suggests that effects on basal and induced expression are not necessarily coupled.

The C-terminus determines the specific interaction of STAT1 with serine kinases

The identical position of a $PMS_{727}P$ motif in the C-terminus of STAT3 implies a similar role and regulation of S727 phosphorylation to those in STAT1. Since the PMSP sequence is a potential phosphorylation site for members of the MAPK family, we first addressed the question of if and how STAT1 and STAT3 serine phosphorylation is triggered by different stimuli and different signaling pathways. Further experiments investigated the structural basis of differential responsiveness.

UV irradiation strongly activated c-Jun kinases (JNKs) and p38MAPK. Activation of ERK1/2 was very weak by comparison (Figure 5A). To rule out contributions of the ERK pathway to stress-induced STAT1-S727 phosphorylation, we irradiated the cells with UV light in the presence of PD98059. The MEK inhibitor did not affect STAT1 phosphorylation. In marked contrast, the p38MAPK inhibitor SB203580 completely inhibited the S727 kinase (Figure 5B). In accordance with our previous results, IFN-γ-induced STAT1 phosphorylation on S727 was not affected by either PD98059 or SB203580 (Figure 5C). 3T3 fibroblasts obtained from mice deficient in both JNKs (JNK1,2$^{-/-}$ cells) displayed no impairment in UV-induced STAT1 phosphorylation on S727 (Figure 5D). We conclude that at least in the case of UV irradiation, the STAT1 C-terminus is targeted specifically by the p38MAPK pathway.

Sensitivity to MEK inhibition and other lines of evidence support the idea that growth factor receptors signal to STAT3 through the ERK pathway (Chung *et al*., 1997; Ng and Cantrell, 1997; Jain *et al*., 1998; Stephens *et al*., 1998). Virtually no phosphorylation of STAT1 on S727 was observed in response to epidermal growth factor (EGF) or serum treatment (Figure 6A), particularly if compared with UV or IFN-γ treatment. In an identical situation, S727 of STAT3 was phosphorylated marginally by IFN-γ treatment or UV irradiation, but strongly phosphorylated by treatment with EGF or fetal calf serum (FCS; Figure 6B). Phosphorylation by EGF was completely abrogated by simultaneous treatment with the MEK inhibitor (Figure 6C). Consistent with the findings of a previous report (Wright *et al*., 1999), neither STAT3 phosphorylation on S727 nor ERK activation was inhibited by PD98059 if stimulated by FCS (data not shown). Thus, STAT3, but not STAT1, responds to a growth factor-activated ERK1/2 pathway. In conclusion, the STAT1 C-terminus appears to be a good substrate for the p38MAPK pathway, but not the ERK pathway, whereas the opposite is true for STAT3.

MAPK substrates contain docking domains that specify the interaction with a particular family member (Tanoue *et al*., 2000). The portion of the STATs conferring specificity for either a MAPK or a MAPK substrate kinase (MAPKAP) has not been determined. We changed C-terminal amino acids of STAT1 (amino acids 716–750) to those of STAT3 (amino acids 706–770).

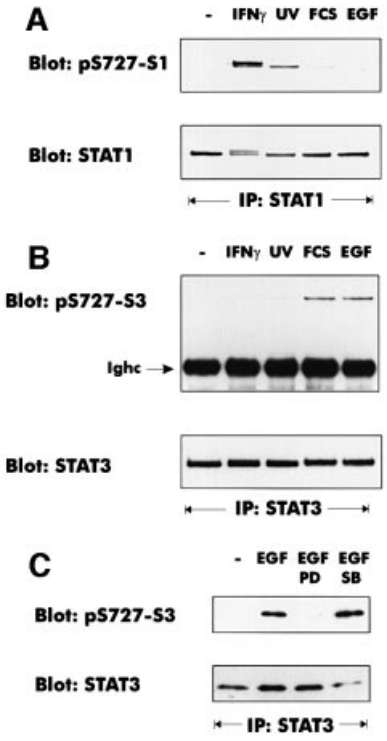

Fig. 6. Different signaling pathways cause S727 phosphorylation of STAT1 and STAT3. Where indicated, mouse 3T3 fibroblasts were treated with IFN-γ for 30 min, irradiated with UV light, or stimulated with 10% FCS (30 min) or EGF (100 ng/ml, 30 min). (**A**) STAT1 phosphorylation on S727 was analyzed with phosphospecific antiserum. Loading was controlled by reprobing with a monoclonal antibody to the STAT1 N-terminus. (**B**) STAT3 was precipitated from cellular lysates with an antiserum to the C-terminus. Phosphorylation of S727 was determined with a phosphoserine 727-specific antiserum (Ighc indicates the immunoglobulin heavy chain). The loading control was performed by reprobing the blot with the STAT3 C-terminal antiserum. (**C**) The pathway-specific inhibitors PD98059 (PD) and SB203580 (SB) were added 30 min prior to EGF treatment. STAT3 phosphorylation on S727 was determined as described for (B).

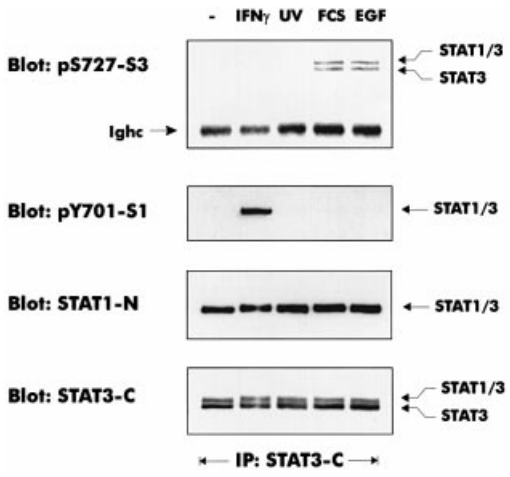

Fig. 7. The STAT3 C-terminus determines the link to MAPK pathways. Fibroblasts from STAT1-deficient mice were reconstituted with a STAT1–STAT3 chimera containing STAT3 C-terminal amino acids. Treatment with UV, IFN-γ, FCS or EGF was carried out as described for Figure 6. Endogenous STAT3 and the STAT1–STAT3 chimera were precipitated from cellular lysates using antiserum against the STAT3 C-terminus. Phosphorylation of both proteins on S727 was determined using an antiserum to the phosphoserine 727 of STAT3 (pS727-S3). The position of the immunoglobulin heavy chain is marked by Ighc. Tyrosine phosphorylation of STAT1–STAT3 was determined using an antiserum directed to phosphotyrosine 701 of STAT1 (indicated as pY701-S1). A monoclonal STAT1 N-terminal antibody (indicated as STAT1-N) was used to visualize the STAT1–STAT3 chimeric protein. A STAT3 C-terminal antiserum (indicated as STAT3-C) was employed to detect both the STAT1–STAT3 chimera and the endogenous STAT3.

If the exchanged portion of the molecule contained a specificity element, the chimeric STAT molecule should acquire the substrate properties of STAT3.

STAT1-deficient fibroblasts were stably transfected with the STAT1–STAT3 chimera and clonal lines were selected. Phosphorylation of the chimeric molecule that displayed a slower SDS–PAGE mobility than the endogenous STAT3 protein was analyzed by immunoprecipitation using a STAT3 C-terminal antibody that precipitated both the STAT1–STAT3 chimera and the endogenous STAT3. Tyrosine phosphorylation of STAT1–STAT3 occurred in response to IFN-γ, confirming the structural integrity of the chimera and its ability to interact with the IFN-γ receptor (Figure 7). The IFN-γ-induced expression of the endogenous IFP53 gene was affected similarly to that in the case of the STAT1-S727A mutation (Figure 4B).

Following UV irradiation, treatment with IFN-γ, FCS or EGF, S727 phosphorylation of the chimera now followed the pattern of endogenous STAT3 (Figure 7). Thus the specificity for connecting to MAPK pathways appears to reside in the C-terminus of the STATs.

Discussion

STAT serine phosphorylation is a layer of signaling specificity superimposed on the intrinsic specificity provided by the JAK–STAT paradigm. Here we provide evidence that the implications of STAT1 phosphorylation at S727 for cellular cytokine responses are determined at several distinct stages. First, cytokines such as IFN-γ that cause tyrosine phosphorylation can stimulate a p38MAPK-independent mode of serine phosphorylation, which requires phosphotyrosine binding to SH2 domains. Secondly, when S727 phosphorylation involves MAPK, the connection to individual MAPK family members is determined by C-terminal amino acids, which, at least in the case of STAT3, behave like an independent specificity module. Thirdly, while the effect of S727A mutation is generally to reduce transcription factor activity, striking differences were observed in the magnitude of reduction at individual target genes. Therefore, a specific effect on gene expression is generated by the dependence of individual STAT1 target genes on a phosphorylated S727.

Y701 and S727 phosphorylation are linked in IFN-γ signaling

The strict dependence of IFN-γ-mediated S727 phosphorylation on tyrosine phosphorylation is in line with the previously reported need for JAK2 or the PYK2 PTK (Zhu *et al*., 1997; Takaoka *et al*., 1999). In further agreement with our results, tyrosine phosphorylation of STAT1 precedes serine phosphorylation in the IFN-γ response of NIH 3T3 fibroblasts (Zhu *et al*., 1997). SH2 domain-based interactions involving STAT1 might be necessary for

serine phosphorylation at different steps during signaling. The IFN-γ-responsive serine kinase could be part of the receptor complex and phosphorylate STAT1 only after it is bound to the IFNGR1 chain or to one of the JAKs, or the serine kinase may recognize only dimerized STATs. Binding of STAT1 to an unidentified molecular platform might also be necessary or, more simply, its recruitment by an SH2 domain-containing serine kinase. Partial purification of STAT1-S727 kinase produced an enzyme requiring the entire STAT1 for phosphorylation (Zhu *et al*., 1997). This mode of substrate recognition appears to contrast with that of the major MAPK.

In obvious contrast to our results, S727 phosphorylation of STAT1 by IFN-γ was found to be independent of Y701 phosphorylation in U3A cells. U3A is a human fibrosarcoma line selected for deficiency in IFN signaling after mutagenesis with a frameshift mutagen (Pellegrini *et al*., 1989; Muller *et al*., 1993). Thus, there are three major differences from the fibroblasts used in our studies: they are transformed, have been mutagenized and originate from human, not mouse. Wen *et al*. (1995) reported transfected STAT1α to be constitutively phosphorylated on S727 in U3A cells, even after serum deprivation. Perhaps a mutation in the U3A DNA deregulates a signaling pathway (p38MAPK?), causing phosphorylation of STAT1 on S727 in the absence of tyrosine phosphorylation. The activity of the deregulated kinase might obscure the need for phosphotyrosine-based interactions, as they would occur if phosphorylation involved the proper IFN-γ-stimulated serine kinase. According to our results, the requirement for the JAK2 kinase may derive entirely from the need for tyrosine-phosphorylated STAT1. The role of JAK2 was established in a JAK2-deficient sibling cell line of U3A, γ2A. If our reasoning were correct, the deregulated kinase activity of U3A would not be found in γ2A cells.

The human and mouse origins could also provide an explanation for the differences observed between U3A cells and mouse fibroblasts. In support of this assumption, species differences were noted recently in the activation of STAT4 by type I IFN (Farrar *et al*., 2000). Moreover, the role of the TYK2 kinase in IFN-α signaling is indispensable in the case of human TYK2-deficient cells, but ancillary in cells from TYK2-deficient mice (Velazquez *et al*., 1992; Karaghiosoff *et al*., 2000). Both findings point out major differences in the deployment of JAKs and STATs in mice and men.

Finally, one must bear in mind that the scenario depicted above does not include the PYK2 PTK or PKR. An involvement of these kinases stresses the possibility that the IFN-γ-stimulated signaling network, hence a potential necessity for SH2 domains, extends beyond the JAK–STAT pathway.

STAT1 serine phosphorylation—the MAPK connection

Signaling through MAPK pathways downstream of G-proteins generally proceeds without SH2 domain-based protein interactions (Whitmarsh and Davis, 1998; Burack and Shaw, 2000). During stress responses that do not activate JAKs, but strongly activate MAPK, STAT1 is linked to p38MAPK. However, when IFN induces tyrosine phosphorylation, S727 phosphorylation is not linked to the classical MAPK pathways, even though these pathways may be active, as recently observed for IFN-α (Uddin *et al*., 1999, 2000).

A role for p38MAPK was postulated recently for STAT1 serine phosphorylation in IFN-γ-treated HeLa cells (Goh *et al*., 1999). However, our studies and those performed in other laboratories show that most cell types do not employ p38MAPK either in type I or type II IFN-mediated S727 phosphorylation (Kovarik *et al*., 1999; Uddin *et al*., 2000). Using JNK-deficient cells, we now provide conclusive evidence that JNKs are not required for stress- or IFN-γ-induced STAT1 phosphorylation on S727. Likewise, and despite some previous support for their potential engagement (David *et al*., 1995; Wen *et al*., 1995; Takaoka *et al*., 1999), a major role for ERKs 1 and 2 in STAT1 phosphorylation can be ruled out. First, MEK inhibition had no influence on stress-induced S727 phosphorylation. Secondly, Zhu *et al*. (1997) demonstrated that forced ERK activation or disruption of the ERK pathway does not affect the phosphorylation of STAT1. Thirdly, IFN-γ has very limited potential to stimulate ERK, JNK or p38MAPK activation (Kovarik *et al*., 1998, 1999). The low level of ERK activation by IFN-γ is not sufficient for cross-talk to STATs because phosphorylation of STAT3, a good ERK substrate *in vitro* and *in vivo*, was barely detectable upon stimulation with IFN-γ. More importantly, transplanting the STAT3 C-terminus to STAT1 generated a connection to ERKs in growth factor responses, but not in the IFN-γ response. In conclusion, p38MAPK is the only member of the family with a clear link to STAT1, and this link appears to be disconnected once Y701 is phosphorylated.

The striking preferences of the STAT1 and STAT3 C-termini for different MAPK family members are in agreement with current ideas about the cell biology of individual STATs and MAPK family members. STAT1 is linked to the p38MAPK pathway, hence a classical inflammation or stress response, which is likely to occur during TH1-dominated immune responses. In contrast, STAT3 is connected to growth factor responses through the ERK pathway. Surely this simple concept will not apply to all STAT-mediated cellular responses. However, as a rule, the C-terminal amino acids may support the specific biological function of STATs through their influence on serine phosphorylation.

Promoter-specific requirement for STAT1 serine phosphorylation

The results obtained with STAT1-S727A cells confirm that S727 phosphorylation stimulates the function of the transactivating domain. Fairly little is known about the mechanism of the stimulatory activity. Two proteins were recently reported to associate preferentially with S727-phosphorylated STAT1. MCM5, a protein important for DNA replication, causes a fluctuation of STAT1 transcriptional activity during the cell cycle (Zhang *et al*., 1998). The tumor suppressor BRCA1 enhanced transcription of the $p21^{WAF}$ gene in response to IFN-γ, but had no effect on the induced expression of the IRF1 gene (Ouchi *et al*., 2000).

Northern blotting and real-time PCR experiments revealed that the STAT1-S727A mutation differentially affects the investigated STAT1 target genes. Inducibility

by IFN-γ was significantly affected (~50% reduction) in the case of the IRF1, IFP53 and p21 genes. These genes are bona fide targets specifically for the STAT1 dimer (Strehlow *et al*., 1993; Pine *et al*., 1994; Chin *et al*., 1996). An 80% reduction in IRF1 gene expression had been noted under similar conditions in U3A cells expressing STAT1-S727A (Wen *et al*., 1995).

The inducibility of the GBP1 and TAP1 genes by IFN-γ was reduced dramatically to <15% of that found with STAT1-WT. The murine GBP1 promoter contains both a GAS and an ISRE, and the IFN-γ-induced GBP1 gene expression essentially depends on the association of IRF1 with the ISRE (Briken *et al*., 1995). Similarly, the bidirectional promoter rendering the neighboring TAP1 and LMP2 genes responsive to IFN-γ contains binding sites for both STAT1 and IRF1 (Wright *et al*., 1995; White *et al*., 1996; Cramer *et al*., 2000). The comparatively weak reduction of IRF1 mRNA expression caused by the STAT1-S727A mutation argues against an indirect effect of the mutation on the GBP1 and TAP1 promoters through IRF1. While this cannot be ruled out formally, we favor as one possible explanation that serine phosphorylation might be particularly important when the STAT1 dimer needs to interact with IRF1 for an efficient transcriptional response.

The GAS of the human GBP1 promoter binds a STAT1 dimer associated with the IRF9 (p48) protein (Seegert *et al*., 1994). The same STAT1–IRF9 complex has also been found to provide for the IFN-γ responses of other genes (Bluyssen *et al*., 1995; Majumder *et al*., 1998). Therefore, a second possibility would be that STAT1-S727 phosphorylation is particularly important for inducibility in the context of a STAT1–IRF9 complex.

Surprisingly, the absence of STAT1-S727 phosphorylation affected the basal expression of the GBP1, TAP1 and IFP53 genes and to a lesser extent the IRF1 gene. Again, published results offer at least two potential explanations for this finding. It has been shown that the absence of STAT1 or STAT2 reduces basal expression of IFN-inducible genes (Lee *et al*., 1999; Park *et al*., 2000). This effect can be explained at least in part by the autocrine secretion of type I IFN, because a similar reduction in IFN-inducible gene expression is also seen in the absence of the type I IFN receptor. The permanent expression of genes with GAS or ISRE promoter sequences due to autocrine IFN might depend on phosphorylated S727. An alternative explanation for some IFN-inducible genes was suggested recently by Chatterjee-Kishore *et al*. (2000). This report showed a contribution of non-tyrosine-phosphorylated STAT1 to the constitutive expression of the LMP2 gene, regulated by the bidirectional TAP1/LMP2 promoter. An interaction with IRF1 was important for this constitutive activity of STAT1, thus providing a potential explanation for the strong effect of the S727A mutation on genes containing GAS elements and IRF1-binding sites. In this respect, our analysis would suggest that as in the case of induced GBP1 or TAP1 induction, the 'constitutive' STAT1–IRF1 interaction requires serine phosphorylation. This is in agreement with an earlier report by Stark's group (Kumar *et al*., 1997).

The differential requirement for S727 phosphorylation might also be explained at the level of association with gene-specific coactivators or chromatin components. The BRCA1 study would be in agreement with this assumption. Thus, while our results support a role for phospho-S727 in promoting the selective transcription of subgroups of STAT1 target genes, it must be the goal of future studies to provide a mechanistic explanation for this finding.

Materials and methods

Cells, cytokines and drugs

3T3 fibroblasts from wild-type mice, STAT1-deficient mice (Durbin *et al*., 1996), or from JNK1/JNK2$^{-/-}$ embryos (a kind gift from Erwin Wagner, Institute of Molecular Pathology, Vienna) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. Prior to treatment, cells were starved for 16 h in DMEM without FCS. IFN-γ (kindly provided by G.Adolf, Boehringer Ingelheim, Vienna) was used at a concentration of 5 ng/ml for the periods indicated in the figure legends. UV irradiation was with UVC (254 nm, 40 J/m^2). Cells were processed for further experimentation 20–30 min after irradiation. EGF was purchased from Calbiochem and used at a concentration of 100 ng/ml. The p38MAPK inhibitor SB203580, kindly provided by Ken Murray (Smith-Kline Beecham), was used at 5 μM. The MEK inhibitor PD98059 was purchased from Calbiochem and used at 50 μM. Both drugs were added 30 min before further treatment.

Plasmids

An expression vector was created by first replacing the cytomegalovirus (CMV) promoter of RcCMV (Invitrogen) with the eIF1α promoter of pEFCX, a pEFBos derivative (Mizushima and Nagata, 1990). Parts of the resulting plasmid, pEF/Rc, were combined with a portion of pcDNA3.1Zeo (Invitrogen) to derive an expression plasmid, pEF-Zeo, containing the eIF1α promoter and a zeocin resistance gene. STAT1-WT cDNA was kindly provided by Chris Schindler (Columbia University, New York), S727A- and Y701-mutated cDNAs were provided by Jim Darnell (Rockefeller Univerity, New York) and R602K-mutated cDNA was obtained from John Krolewski (UC Irvine, Irvine, CA). A chimeric STAT1–STAT3 cDNA was made by substituting the DNA encoding the STAT1 C-terminus beyond amino acid 716 with a PCR-generated fragment encoding STAT3 amino acids 708–770. Correct recombination was confirmed by DNA sequencing. All cDNAs were inserted into pEF-Zeo. The IFP-Luc plasmid containing the IFP53 promoter (Strehlow *et al*., 1993) fused to the luciferase gene was generated by inserting a 192 bp promoter fragment including the GAS into pGL2basic (Promega).

Cell transfection and selection of transfected clones

A total of 10^7 STAT1-deficient fibroblasts were transfected using 2 μg of plasmid DNA and Effectene reagent according to the manufacturer's instructions (Qiagen, Hilden, Germany). Transfected cells were selected in 100 μg/ml zeocin (Invitrogen, Carlsbad, CA). Bulk cultures of zeocin-resistant cells were cloned by limiting dilution in microtiter wells. Individual cell clones were analyzed for STAT1 expression by immunofluorescence with a monoclonal antibody recognizing the STAT1 N-terminus, and by western blotting.

Antibodies

Antisera to the STAT1 C-terminus and to phospho-S727-STAT1 have been described recently (Kovarik *et al*., 1998). Rabbit antiserum to Y701-phosphorylated STAT1 was purchased from New England Biolabs (Beverly, MA) and used at a dilution of 1:1000. A monoclonal antibody recognizing the STAT1 N-terminus was purchased from Transduction Laboratories (Lexington, KY). Antibodies against the STAT3 C-terminus or the S727-phosphorylated STAT3 C-terminus were obtained, respectively, from Santa Cruz and New England Biolabs. Phosphospecific antibodies against ERKs, p38MAPK and JNKs were bought from New England Biolabs and used for western blotting at dilutions of 1:1000. Monoclonal antibodies recognizing ERKs (panERK) were purchased from Transduction Laboratories (Lexington, KY) and used at a dilution of 1:2000 in western blotting.

Immunoprecipitation and western blotting

A protocol for these procedures has been described recently (Kovarik *et al*., 1998). Antibodies were used as indicated above or in the figure legends.

Northern blotting

Total RNA was isolated from fibroblasts using the RNeasy reagent kit and the manufacturer's instructions (Quiagen, Hilden, Germany). A 15 µg aliquot of total RNA was separated on agarose gels and blotted to membranes using standard procedures. The blots were probed with cDNA, labeled with ^{32}P by the random-prime method (Roche, Vienna, Austria). The cDNAs used for labeling encoded the following mouse genes: IRF1 (Miyamoto *et al*., 1988), GBP1 (Cheng *et al*., 1991), SOCS1/JAB and SOCS3/CIS3 (Suzuki *et al*., 1998).

Quantitation of gene expression using real-time PCR

The cDNAs used in the real-time PCR assays were reverse transcribed from 5 µg of total RNA using the Oligo$(dT)_{12–18}$ Primer and the SuperScript Preamplification System for first strand cDNA synthesis (Life Technologies) according to the manufacturer's recommendations. Real-time PCR was performed on an ABI PRISMTM7700 Sequence Detection System (Applied Biosystems). Detailed information about real-time PCR conditions, primers and quantification (Heid *et al*., 1996) is provided as Supplementary data.

Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

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