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Stat1 combines signals derived from IFN-γ and LPS receptors during macrophage activation

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Complete activation of macrophages during immune responses results from stimulation with the activating cytokine interferon-γ (IFN-γ) and a second stimulus, usually a microbial product. Bacterial infection of macrophages, or treatment with bacterial lipopolysaccharide (LPS), resulted in rapid Stat1 phosphorylation on Ser727 (S727) independently of concomitant tyrosine phosphorylation. IFN-γ also caused rapid phosphorylation of S727. In both situations, S727 phosphorylation was reduced by pre-treatment of cells with the serine kinase inhibitor H7. When macrophages were treated sequentially or simultaneously with LPS and IFN-γ, the pool of molecules phosphorylated on both Tyr701 (Y701) and S727 was strongly increased. Consistently, Stat1-dependent transcription in response to IFN-γ was significantly enhanced if the cells were pre-treated with bacterial LPS. The relative amount of S727-phosphorylated Stat1 in the non-tyrosine phosphorylated fraction was considerably smaller than that in the tyrosine-phosphorylated fraction. No evidence was found for an effect of S727 phosphorylation on the phosphorylation of Y701 by IFN-γ. Thus, serine and tyrosine phosphorylation of Stat1 are caused independently of each other, but the serine kinase may recognize tyrosine-phosphorylated Stat1 preferentially in the course of an IFN-γ response. The data suggest Stat1 to be a convergence point for immunological stimuli in a macrophage proinflammatory response.
Keywords: IFN/LPS/serine phosphorylation/Stat/transcription

Introduction

Innate immunity provides an immediate host response against bacterial infection. The antibacterial mechanisms recruited by the innate immune system usually are triggered by the invading microorganism itself. The adaptive immune response, ultimately orchestrated by lymphocytes, arises as a consequence of antigen presentation and enhances the antibacterial response through antigen-specific mechanisms. However, cells of the adaptive immune system also provide a positive feed-back to the innate immune system through the synthesis of cytokines that either increase effector cell numbers or activate these cells for an increased antibacterial performance. This has been well documented in the case of macrophages. These respond to the initial bacterial infection with an immediate synthesis of proinflammatory mediators and contribute in many ways to natural immunity, but also to the onset of a specific immune response (Kielian and Blecha, 1995; Viriyakosol and Kirkland, 1995). Once activated Th1 cells are present, they provide the macrophage-activating factor interferon-γ (IFN-γ) which acts in concert with products of the microbe, for example the cell wall component lipopolysaccharide (LPS), to generate macrophages with a maximal capacity to ingest and kill microbes, as well as to synthesize membrane receptors and secreted molecules that function in a proinflammatory immune response (Nacy and Meltzer, 1991).

The intracellular events leading to macrophage activation have been uncovered to some extent. IFN-γ binds to a hetero-oligomeric, class II cytokine receptor and activates a Jak–Stat signaling pathway consisting of the Jak1 and Jak2 kinases as well as the transcription factor signal transducer and activator of transcription 1 (Stat1). Stat1 binds to the cytoplasmic portion of the ligand-activated IFN-γ receptor and is phosphorylated by Jaks on a single tyrosine residue (Y701). Phosphorylation on Y701 causes SH2 domain-mediated dimerization, nuclear translocation and binding of Stat1 to its cognate promoter DNA, the gamma interferon-activated site (GAS; reviewed in Darnell *et al*., 1994; Muller *et al*., 1994; Schindler, 1995; Leaman *et al*., 1996; Darnell, 1997; Decker, 1997; Decker *et al*., 1997). To act with maximal efficiency as a transcription factor, Stat1 must also be phosphorylated on S727 which lies within a potential MAPK consensus motif at the C-terminus, a putative transactivation domain (Wen *et al*., 1995). At present, the kinase-phosphorylating S727 is not clearly defined. The MAPK ERK2 was suggested to exert Stat1 serine kinase activity (David *et al*., 1995), but the extent to which this applies is currently a matter of debate (Chung *et al*., 1997; Nishiya *et al*., 1997; Zhu *et al*., 1997). Zhu and colleagues recently provided preliminary evidence for a Stat1 serine kinase distinct from ERKs (Zhu *et al*., 1997).

Early post-receptor events in LPS-induced signal transduction involve tyrosine phosphorylation, but it is unclear which tyrosine kinases are critically involved in signal transduction downstream of the CD14 or other LPS receptors (reviewed in Sweet and Hume, 1996). In addition to the tyrosine kinases, a number of serine kinases were shown to be activated by LPS, including c-Raf, the MAP kinases Erk1 and Erk2, the p38 kinase, c-Jun kinase and ceramide-activated kinase (CAK; Joseph *et al*., 1994; Reimann *et al*., 1994; Büscher *et al*., 1995; Wright and Kolesnick, 1995; Hambleton *et al*., 1996; Sanghera *et al*., 1996; Meng and Lowell, 1997). Importantly, LPS also activates IκB kinase. NF-κB, and to some extent AP1, plays a central role in the transcriptional activation of

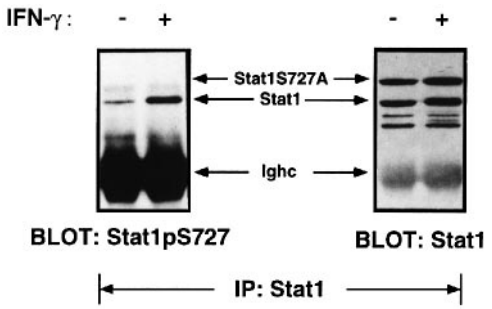


Fig. 1. Specificity of the anti-Stat1 pS727 serum. 293 cells were transfected with an S727A mutant of Stat1 which was tagged at the N-terminus with myc epitopes and thus migrated more slowly in SDS–PAGE. Half of the transfected cells were treated with IFN-γ. Extracts from IFN-γ-treated and untreated cells were subjected to immunoprecipitation with anti-Stat1α serum and the precipitates were stained with anti-pS727 serum or normal anti-Stat1α serum. The additional, lower molecular weight bands in the Stat1 blot stem from degradation products derived from transfected Stat1S727A.

numerous genes encoding immunologically important cytokines or other mediators (reviewed in Baeuerle and Henkel, 1994; Baeuerle and Baltimore, 1996). Therefore, the functional cooperation between IFN-γ and LPS can be explained in terms of each inducing a set of proinflammatory gene products. However, some genes, such as those encoding iNOS, transcription factor IRF-1 or the cell adhesion molecule ICAM-1, were also shown to be induced cooperatively by NF-κB and Stat1 (Sims *et al*., 1993; Caldenhoven *et al*., 1994; Look *et al*., 1994; Xie *et al*., 1994; Ledebur and Parks, 1995; Gao *et al*., 1997; Pine, 1997).

In the present study, we investigated whether bacteria and their products directly influence the output of the Jak–Stat signal transduction pathway. Our results suggest that bacterial infections augment Stat1-mediated transcription by activating a kinase capable of phosphorylating S727.

Results

An antiserum specifically recognizing Stat1 phosphorylated on Ser727

We immunized rabbits with a peptide corresponding to part of the Stat1 C-terminus and including phosphorylated S727. The resulting serum (hereafter designated anti-pS727) was tested by enzyme-linked immunosorbent assay (ELISA) and in Western blots of gels in which immunoprecipitates of cellular Stat1 were compared with recombinant (unphosphorylated) Stat1 C-terminus or Stat1S727A mutant C-terminus. These experiments revealed an ~1000-fold preference of the antibodies for the phosphorylated C-terminus of Stat1 (data not shown). The specificity of anti-pS727 was demonstrated further in an experiment shown in Figure 1. 293 kidney fibroblasts were transfected with a plasmid expressing high levels of myc epitope-tagged Stat1S727A. The transfected cells expressed approximately equal amounts of endogenous wtStat1 and exogenous Stat1S727A. Following stimulation with IFN-γ and immunoprecipitation of cellular extracts, an increased signal of wtStat1 was observed in Western blots with anti-pS727. In contrast, Stat1S727A did not react with the antibody in either unstimulated or IFN-γ-treated cells. The experiment thus indicates that some wtStat1 phosphorylated on S727 is present in 293 cells which, in accordance with published results (Wen *et al*., 1995), is strongly increased by treatment with IFN-γ. The lack of reactivity with our antiserum of the S727A mutant under all conditions suggested the suitability of our antibodies to reveal the activity of the Stat1 S727 kinase and thus to study its regulation by stimuli of cell-surface receptors. To obtain further evidence for this assumption, we tested a number of stimuli previously implicated in causing Stat1 phosphorylation on S727 (Figure 2A; Wen *et al*., 1995). U937 promonocytes grown in the presence of 10% fetal calf serum (FCS) produced a Stat1 signal with anti-pS727. Starvation from serum for 20 h completely abolished Stat1 reactivity with anti-pS727, and a brief stimulation with 10% re-established it. Both types of IFN and phorbol ester caused a Stat1 signal in Western blots stained with anti-pS727. These results show beyond doubt that anti-pS727 correctly reveals the regulation of the Stat1 S727 kinase in intact cells.

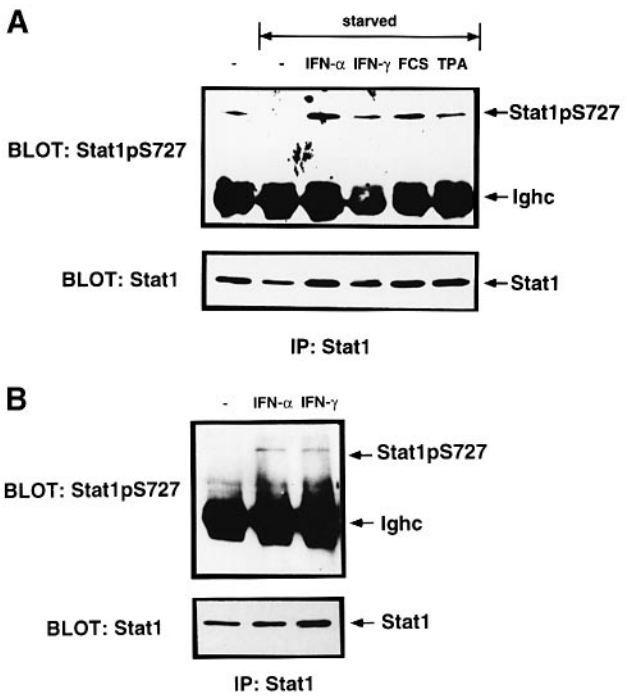


Fig. 2. Induction of Stat1 S727 phosphorylation in U937 cells and human PBMC. (**A**) Cells from a proliferating culture (lane 1) or after overnight starvation were stimulated for 20 min with the indicated agents. (**B**) Human PBMC were prepared under serum-free conditions and stimulated with IFNs for 20 min. Determination of Stat1 S727 phosphorylation was with anti-pS727 as described for Figure 1.

The presence of factors stimulating S727 kinase activity in serum raised the question of whether such factors are present in blood plasma, thus causing constitutive S727 phosphorylation in blood leukocytes. Peripheral blood mononuclear cells (PBMC) isolated from whole blood demonstrated a low constitutive S727 phosphorylation of Stat1 which could be stimulated significantly by both IFN-α and IFN-γ (Figure 2B). This result confirms the importance of regulating S727 phosphorylation *in vivo*.

Bacterial infection and LPS activate the Stat1 S727 kinase

The experiment shown in Figure 2A demonstrates rapid phosphorylation of Stat1 S727 in response to the phorbol

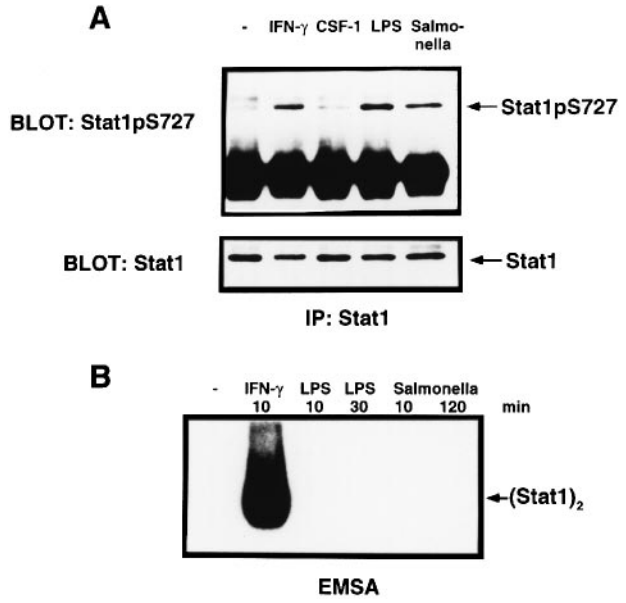


Fig. 3. The effect of bacteria, LPS or CSF-1 on Stat1 S727 phosphorylation in mouse macrophages. (**A**) Bac1.2F5 macrophages were either infected with *S.typhimurium* or treated with LPS for 20 min. Stimulation with human recombinant CSF-1 was for 10 min. Determination of Stat1 S727 phosphorylation was with anti-pS727 as described for Figure 1. (**B**) Extracts from Bac1.2F5 cells after LPS treatment or *Salmonella* infection were assayed for tyrosine phosphorylation of Stats in the EMSA with a β-casein GAS sequence as a labeled probe.

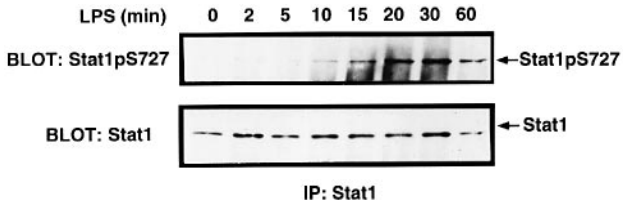


Fig. 4. The kinetics of Stat1 S727 phosphorylation by LPS. Bac1.2F5 macrophages were stimulated with LPS for the indicated periods. Determination of Stat1 S727 phosphorylation in cellular extracts was with anti-pS727 as described for Figure 1.

ester TPA. Since TPA does not cause Stat1 phosphorylation on tyrosine (Eilers *et al.*, 1994), the drug exploits the possibility of independently regulating Y701 and S727 phosphorylation. We investigated the possibility that this might also occur in response to (patho-)physiological stimuli that cooperate with IFN-γ in causing macrophage activation. Macrophages were infected with *Salmonella typhimurium* for 20 min. Alternatively, macrophages were treated with LPS. In either case, a strong stimulation of Stat1 phosphorylation on S727 was observed which was similar, or even superior, to that caused by IFN-γ (Figure 3A). By contrast, no evidence for tyrosine-phosphorylated Stats was observed in the EMSA upon brief treatment of macrophages with LPS or after brief infection with *S.typhimurium*, suggesting that LPS did not activate a Jak–Stat pathway (Figure 3B). The kinetics of S727 kinase activation by LPS are shown in Figure 4. Importantly, LPS rapidly activated S727 kinase, thus ruling out the possibility of activation by indirect mechanisms (see also Figure 9C). The kinetics of LPS- and IFN-γ-induced S727 phosphorylation were found to be similarly rapid

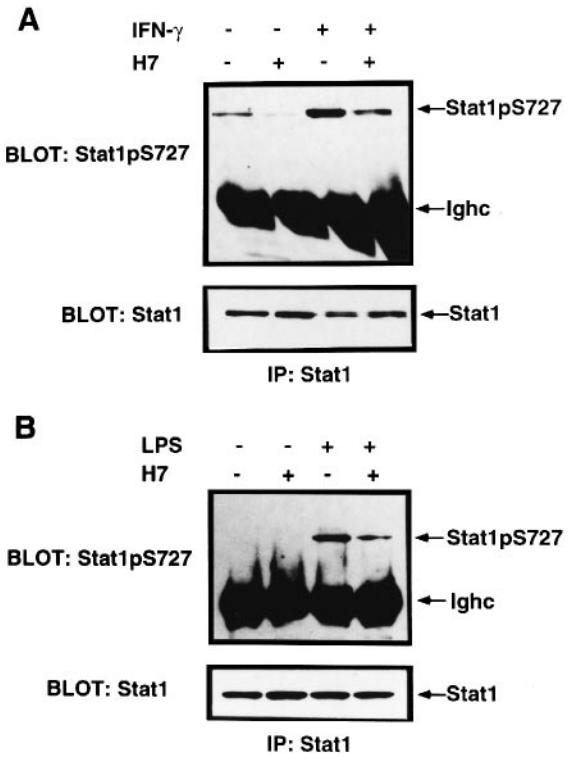


Fig. 5. The effect of serine kinase inhibitor H7 on Stat1 S727 phosphorylation. (**A**) Bac1.2F5 macrophages were pre-treated with 50 μM H7 for 10 min and subsequently stimulated with IFN-γ for 25 min. (**B**) Macrophages were pre-treated similarly with H7 and subsequently stimulated with LPS for 25 min. Determination of Stat1 S727 phosphorylation was with anti-pS727 as described for Figure 1.

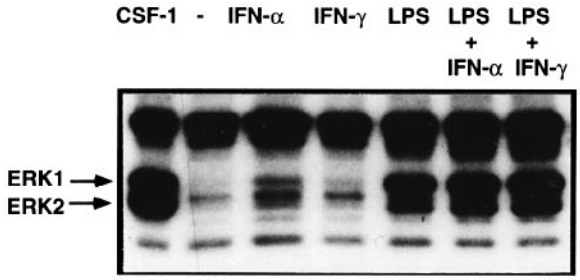


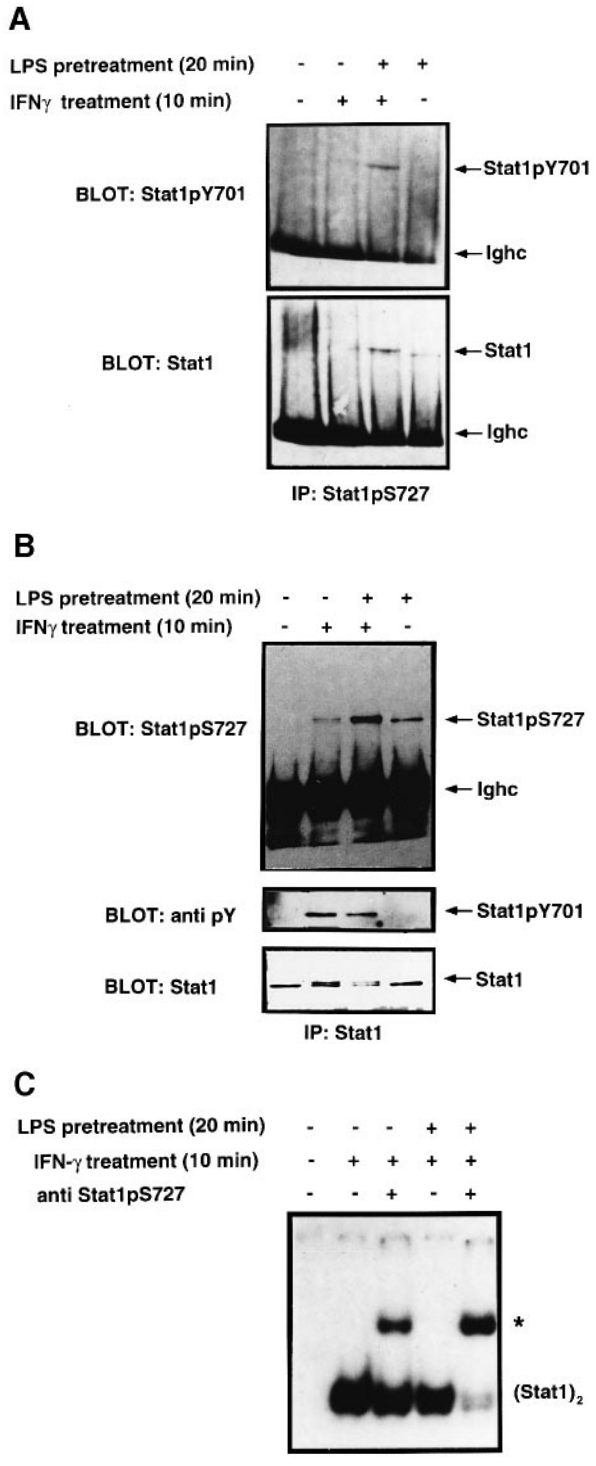
Fig. 6. Activation of MAP kinases ERK1 and ERK2 by IFNs and LPS. Bac1.2F5 cells were stimulated with CSF-1 for 10 min, or with IFN-α, IFN-γ or LPS for 20 min. Simultaneous treatment with IFNs and LPS was for 25 min. Following stimulation, cellular extracts were prepared and ERK activation was measured by in-gel phosphorylation of co-polymerized myelin basic protein.

(Figures 4 and 9C). Consistent with previous data (Zhu *et al.*, 1997), maximal levels of tyrosine phosphorylation in response to IFN-γ were reached earlier than maximal levels of S727 (data not shown).

LPS- and IFN-γ-induced activation of the Stat1 S727 kinase were sensitive to the serine kinase inhibitor H7 (Figure 5). Moreover, H7 also suppressed basal S727 phosphorylation caused by serum factors (Figure 5A). In six independent experiments, the inhibition of IFN-γ-mediated S727 phosphorylation ranged between 50 and 80%. On average, the drug was less efficient in inhibiting kinase activation by LPS (between 20 and 70% inhibition in five independent experiments). It is unclear at present whether incomplete inhibition by H7, particularly of LPS-

induced S727 phosphorylation, indicates the presence of H7-sensitive and -insensitive pathways targeting this Stat1 phosphorylation site.

We tested whether the MAP kinases ERK1 and ERK2 might be a common downstream target of LPS and IFN-γ to cause S727 phosphorylation in macrophages. As a positive control, colony-stimulating factor 1 (CSF-1), which is known to cause robust MAPK activation in Bac1.2F5 macrophages, was used. Our results (Figure 6) demonstrate strong activation of both ERKs by CSF-1 and LPS. By contrast, IFN-γ caused very little ERK2 activation and no activation of ERK1. IFN-α activated ERKs, but to a fairly small extent compared with CSF-1 or LPS. Combined treatment with IFNs and LPS slightly increased activation by LPS alone, but this effect was not very pronounced. Thus, MAPK activity can be uncoupled from S727 kinase because IFN-γ causes robust S727 phosphorylation whereas CSF-1 causes no detectable S727 kinase activation (Figure 3). To assess further the possibility of Stat1 phosphorylation by ERKs, for example in the case of LPS, we performed in-gel kinase assays or ERK2 immune complex kinase assays using recombinant Stat1 C-terminus as a substrate. These experiments revealed no detectable ability of CSF-1 or *Salmonella*-activated ERKs to phosphorylate S727 (data not shown).



LPS pre-treatment increases the transcriptional response to IFN-γ

To be functionally meaningful, LPS must cooperate with IFN-γ to increase the pool of Stat1 molecules phosphorylated on both Y701 and S727, and this pool of molecules must cause an increased target gene transcription rate. We first tested the effect of LPS pre-treatment on IFN-γ activation of Stat1. Macrophage extracts after treatment with IFN-γ alone, or after LPS pre-treatment and subsequent IFN-γ stimulation, were immunoprecipitated with either anti-pS727 antibodies or with antibodies to the Stat1 C-terminus. Western blots of these precipitates were developed with either pS727 serum (in the case of Stat1 immunoprecipitation only), with anti-phosphotyrosine monoclonal antibody or with Stat1 antibodies. This experiment (Figure 7A) showed that the pS727-specific antibodies precipitate much more tyrosine-phosphorylated Stat1 from LPS-pre-treated cells pulsed for a brief period (10 min) with IFN-γ. In fact, precipitation of tyrosine-phosphorylated Stat1 with anti-pS727 serum from cells treated for 10 min with IFN-γ alone was below the level of detection. The control Stat1 antibody precipitated equal amounts of tyrosine-phosphorylated protein from IFN-γ-treated and pre-LPS/IFN-γ-treated cells (note that the antibody to the Stat1 C-terminus precipitates more efficiently compared with the pS727 antiserum; therefore signal intensities in Figure 7A and B cannot be compared directly). This result indicates a larger amount of tyrosine-phosphorylated molecules in the S727-phosphorylated Stat1 pool after LPS pre-treatment and allows the conclu-

Fig. 7. The effect of LPS pre-treatment on the S727 phosphorylation status of tyrosine-phosphorylated Stat1 isolated from IFN-γ- or LPS/IFN-γ-treated macrophages. Bac1.2F5 macrophages were pre-treated with LPS for 20 min, and untreated or pre-treated cells were stimulated with IFN-γ and LPS for 10 min. Pre-treated control cells were stimulated further with LPS alone for another 10 min. Extracts were subjected to immunoprecipitation with either anti-pS727 serum (**A**) or serum against the Stat1 C-terminus (**B**) as indicated. Western blots of the immunoprecipitates were developed with anti-pS727 antiserum [only Stat1Ab immunoprecipitate in (B)], anti-phosphotyrosine or anti-Stat1 antibodies. Note that the antibody to the Stat1 C-terminus precipitates more efficiently compared with the pS727 antiserum; therefore, signal intensities in (A) and (B) cannot be compared directly. Levels of Stat1 precipitated by the anti-pS727 serum from cells treated for 10 min with IFN-γ alone were below the level of detection. (**C**) Extracts derived from macrophages after 10 min of treatment with IFN-γ alone, or with IFN-γ for 10 min after pre-treatment with LPS for 20 min, were subjected to EMSA with a β-casein probe. Where indicated, supershift analysis was performed with anti-pS727 antibodies. The supershifted Stat1 band is marked by an asterisk.

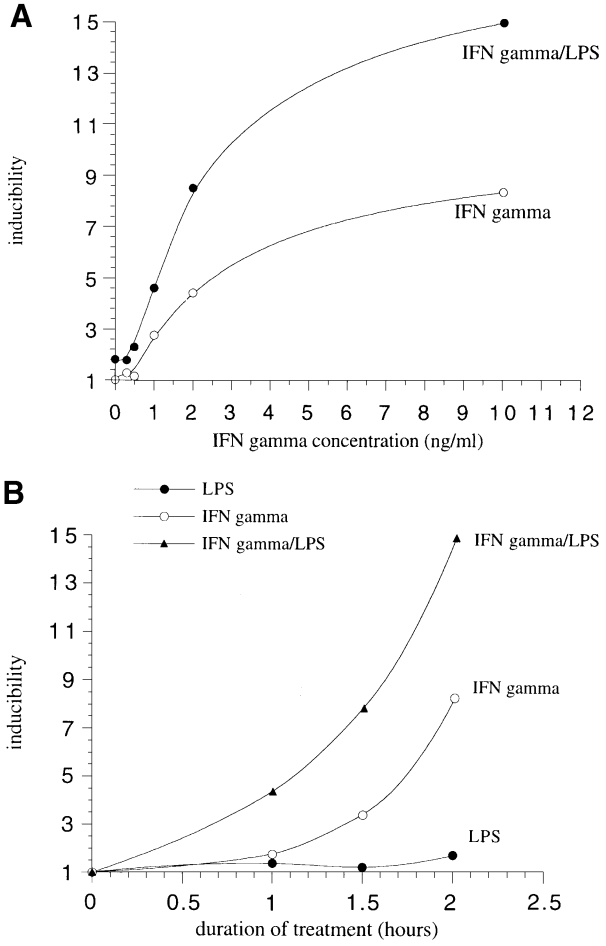


Fig. 8. LPS pre-treatment enhances Stat1-dependent transcription in Bac1.2F5 cells stably transfected with a Stat-dependent luciferase reporter gene (for details, see Materials and methods). (**A**) Cells were pre-treated with LPS for 30 min. These cells, as well as non-pre-treated controls, were stimulated with different doses of IFN-γ for 2 h, followed by preparation of extracts and determination of luciferase activity. LPS-induced transcription due to indirect effects was 1.8-fold. (**B**) LPS-pre-treated or untreated cells were stimulated with 10 ng/ml of IFN-γ for the indicated periods, followed by determination of luciferase activity.

sion that LPS treatment before IFN-γ stimulation causes a larger pool of Stat1 molecules to be phosphorylated on both Y701 and S727. In further support of this conclusion, the Stat1 band seen in EMSA experiments with extracts from macrophages pre-treated with LPS and subsequently stimulated with IFN-γ contained more material reacting with anti-pS727 serum than the band derived from extracts after stimulation with IFN-γ alone (Figure 7B).

To test whether the elevated number of Y701/S727-phosphorylated Stat1 molecules caused a corresponding increase in transcription, we prepared stable transfectants of the Bac1.2F5 macrophage line with a luciferase reporter gene regulated by a synthetic promoter containing two GAS elements. Pre-treatment of these cells with LPS caused a strong increase in the transcriptional response to IFN-γ over a range of cytokine doses (Figure 8A). Therefore, the LPS effect on Stat1 S727 phosphorylation corresponds to increased transcriptional competence. LPS did not lower profoundly the IFN-γ dose necessary to produce a transcriptional response under the conditions employed in our experiments. This suggests that tyrosine phosphorylation is rate limiting and that S727 phosphorylation does not lower the K_m of Jaks for their Stat1 substrate to a degree detectable in this experimental set-up.

The IFN-γ dose–response curve in Figure 8A was obtained after 30 min of pre-treatment with LPS, followed by 2 h of treatment with IFN-γ. Within this period, an ~2-fold stimulation of reporter gene expression by LPS alone was noted. LPS stimulates cytokine release from macrophages. Therefore, Stat activation by indirect means might contribute to the transcriptional effect of LPS reported in Figure 8A. In contrast to this assumption, the activity of LPS alone seemed insufficiently large to explain our transcriptional data because combined LPS/IFN-γ treatment by far exceeded the added effects of IFN-γ alone and LPS alone (e.g. 15-fold versus 7 + 2-fold for stimulation with 10 ng/ml of IFN-γ). To rule out that we were measuring Stat1-independent, indirect effects on transcription rather than the effect of Stat1 S727 phosphorylation, we determined the kinetics of the responses to LPS or IFN-γ alone, or to pre-LPS/IFN-γ treatment (Figure 8B). The combined treatment caused a significant expression of the reporter gene already after 60 min. An effect of IFN-γ alone was noted after 90 min, and this was doubled by LPS pre-treatment. A 2-fold transcriptional induction by LPS alone did not occur before 2 h of treatment, i.e. the period chosen for the experiment in Figure 8A. Therefore, synergistic enhancement of Stat-dependent transcription by LPS and IFN-γ occurs in the absence of any indirect effect caused by LPS alone and can be attributed entirely to Stat1, the only IFN-γ-activated Stat in these cells.

Y701 and S727 phosphorylation occur independently, but one of the kinases preferentially uses Stat1 phosphorylated by the other

Using cells containing Stat1 mutated either on S727 or Y701, the Darnell group demonstrated that neither phosphorylation event is a necessary pre-condition for the other (Zhu *et al*., 1997). Our result with LPS confirms the independence of the phosphorylation events in macrophages containing wtStat1. Despite the ability of the Stat1 kinases to use a substrate phosphorylated neither on Y701 nor S727, the phosphorylation of one residue might generate a molecule which is in some way discriminated by the second kinase. To test this, we treated macrophages with IFN-γ and determined the distribution of S727-phosphorylated Stat1 between the Y701-phosphorylated and non-tyrosine-phosphorylated Stat1 pools. Extracts of IFN-γ-treated cells were subjected to immunoprecipitation and the precipitates were analyzed in Western blots. These were stained with anti-pS727, anti-phosphotyrosine antibody and, subsequently, with normal Stat1 antiserum (Figure 9A). Under optimal conditions, the Y701-phosphorylated and non-tyrosine-phosphorylated Stat1 bands stained in a ratio of ~9:1 with anti-pS727, indicating that much more S727-phosphorylated Stat1 is in the tyrosine-phosphorylated pool of molecules. On the other hand, staining with control Stat1 antibody revealed that only ~50% of Stat1 molecules were phosphorylated on tyrosine. Therefore, a larger proportion of Y701-phosphorylated

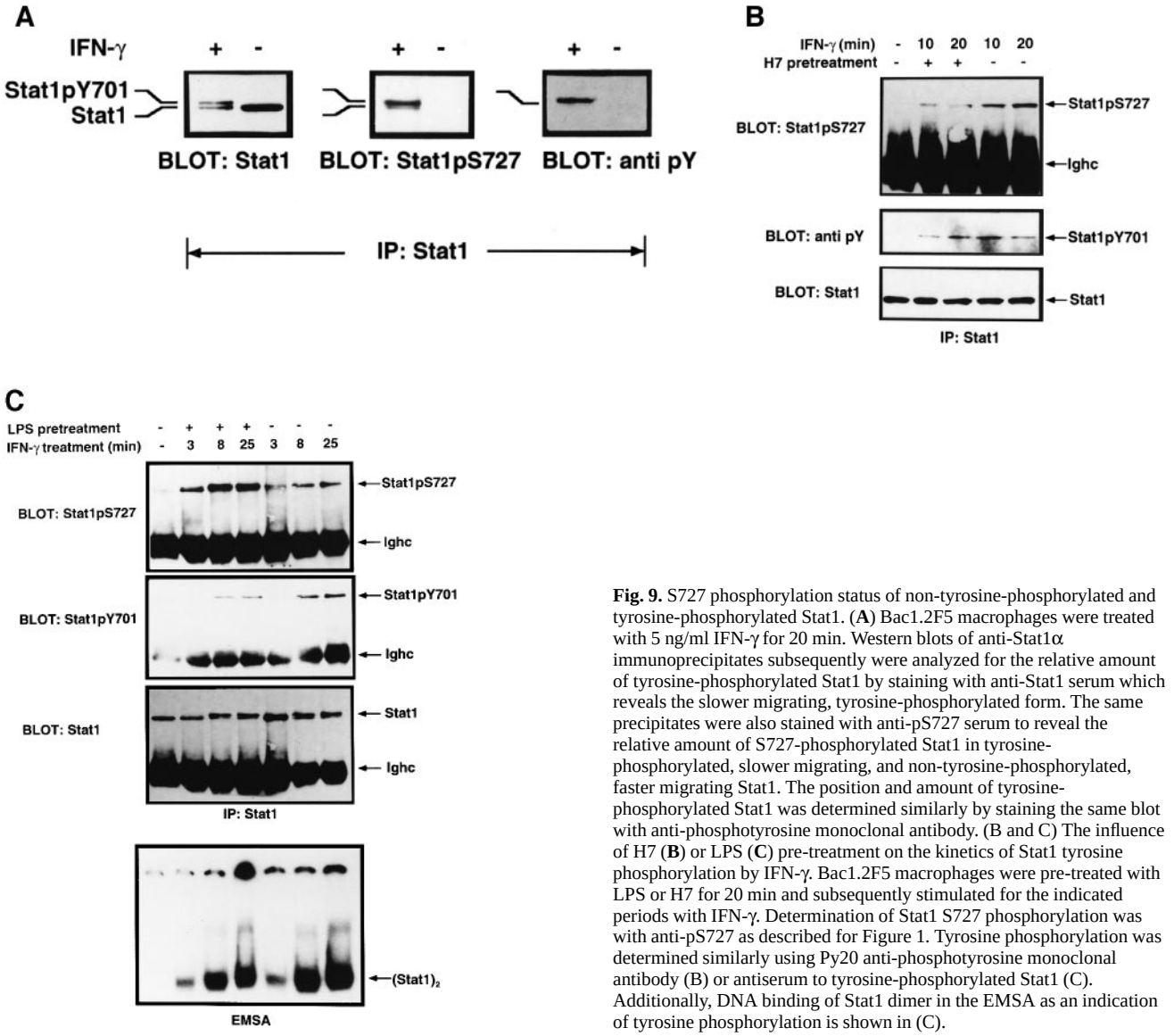


Fig. 9. S727 phosphorylation status of non-tyrosine-phosphorylated and tyrosine-phosphorylated Stat1. (**A**) Bac1.2F5 macrophages were treated with 5 ng/ml IFN-γ for 20 min. Western blots of anti-Stat1α immunoprecipitates subsequently were analyzed for the relative amount of tyrosine-phosphorylated Stat1 by staining with anti-Stat1 serum which reveals the slower migrating, tyrosine-phosphorylated form. The same precipitates were also stained with anti-pS727 serum to reveal the relative amount of S727-phosphorylated Stat1 in tyrosine-phosphorylated, slower migrating, and non-tyrosine-phosphorylated, faster migrating Stat1. The position and amount of tyrosine-phosphorylated Stat1 was determined similarly by staining the same blot with anti-phosphotyrosine monoclonal antibody. (B and C) The influence of H7 (**B**) or LPS (**C**) pre-treatment on the kinetics of Stat1 tyrosine phosphorylation by IFN-γ. Bac1.2F5 macrophages were pre-treated with LPS or H7 for 20 min and subsequently stimulated for the indicated periods with IFN-γ. Determination of Stat1 S727 phosphorylation was with anti-pS727 as described for Figure 1. Tyrosine phosphorylation was determined similarly using Py20 anti-phosphotyrosine monoclonal antibody (B) or antiserum to tyrosine-phosphorylated Stat1 (C). Additionally, DNA binding of Stat1 dimer in the EMSA as an indication of tyrosine phosphorylation is shown in (C).

molecules are phosphorylated on S727 compared with the non-tyrosine-phosphorylated Stat1. This result suggests that either the S727 kinase prefers molecules already phosphorylated on Y701, or that the Jak kinase prefers Stat1 phosphorylated on S727. Two further experiments were performed to test whether serine phosphorylation affects the rate of tyrosine phosphorylation. The cells were pre-treated with either H7 (Figure 9B) or LPS (Figure 9C) before addition of IFN-γ. In neither case could we observe a significant effect of S727 phosphorylation on maximal levels of IFN-γ-stimulated Y701 phosphorylation (determined by Western blot with anti-phosphotyrosine). Judging from maximal levels of tyrosine phosphorylation, the results shown in Figure 9 are in line with those of Figure 8A, showing that pre-treatment with LPS and thus phosphorylation of S727 did not lower the minimal dose of IFN-γ necessary to cause a transcriptional response. In cases of a better recognition of S727-phosphorylated Stat1 by Jaks, one would expect an increased sensitivity of the transcriptional response to IFN-γ.

Discussion

Stat1 plays an indispensable and non-redundant role in immune responses (Durbin *et al*., 1996; Meraz *et al*., 1996). Probably the most important outcome of our studies is that independent regulation by phosphorylation of the two sites involved in turning Stat1 from a latent into an activated form, Y701 and S727, is not only mechanistically possible, but is in fact employed as a means of priming the Stat1 molecule for higher transcriptional competence in a situation of microbial infection. Therefore, the Stat1 protein is a physical entity combining input from the infectious agent *per se*, as well as from the immune response directed against the infectious agent, both of which cooperate in bringing about maximal activation of a macrophage. Stat1 can thus be regarded as a physical link between the innate and adaptive immune response.

The precise role of S727 phosphorylation in the mechanism of Stat1 action is unclear. Studies with the S727A mutant showed that phospho-S727 enhances transcription

factor activity, and it is clear from the data provided here that LPS-induced S727 phosphorylation increases the Stat1-dependent transcriptional response. A likely explanation for this effect is the phosphorylation-dependent association of a transcriptional Stat1 cooperation partner or coactivator. CBP/p300 proteins, coactivators recently implicated in Stat transcriptional responses, appear to bind to Stat1 independently of S727 phosphorylation (Zhang *et al*., 1996). Therefore, the relevant target of the phosphorylated C-terminus currently is elusive.

The occurrence of regulated serine phosphorylation recently has been recognized for most Stats (Boulton *et al*., 1995; Eilers *et al*., 1995; Lutticken *et al*., 1995; Wen *et al*., 1995; Zhang *et al*., 1995; Beadling *et al*., 1996; Cho *et al*., 1996; Kirken *et al*., 1997a,b). However, only in the case of Stat1 and Stat3 has a target site for proline-directed serine kinase activity been defined precisely (Wen *et al*., 1995; Wen and Darnell, 1997). The data provided in our study, as well as recent experimental evidence published by other laboratories (Chung *et al*., 1997; Nishiya *et al*., 1997; Zhu *et al*., 1997), while not ruling out specific situations in which Erks may act as Stat1 kinases, disprove the notion that the Stat1 C-terminus might generally couple ERK activity to Stat activation. Firstly, ERK activation by ligands that activate Stats does not correspond to S727 phosphorylation in a number of situations, and this includes CSF-1 or IFN-γ stimulation of macrophages as shown in our report. Secondly, in accordance with a previously published report, we find that the Stat1 C-terminus is not phosphorylated readily by ERKs *in situ* or *in vitro* (Chung *et al*., 1997). Finally, Darnell and co-workers recently have presented preliminary evidence for a Stat1 serine kinase distinct from ERKs with the ability to phosphorylate S727 (Zhu *et al*., 1997). In the case of Stat3, the situation may be different, and evidence for both ERK-dependent and -independent phosphorylation of Stat3 S727 has been published (Chung *et al*., 1997). Thus, the question remains open as to what extent Stat1 and Stat3 S727 kinases are identical. For both Stat1 and Stat3, it is clear, however, that either distinct serine kinases exist or that there is more than one signal transduction pathway that targets them. In the case of Stat3, this was shown by Cantrell's group using the kinase inhibitors H7 and PD98059 and stimulation of serine phosphorylation either by CD3 activation or by stimulation with interleukin-2 (IL-2) (Ng and Cantrell, 1997). Stat1 S727 may be targeted similarly by H7-sensitive and -insensitive signaling pathways, as suggested by the incomplete inhibition of S727 phosphorylation, especially in the case of LPS treatment. For IFN-γ-stimulated S727 kinase activation, a requirement for Jak2 has been demonstrated. We and others (Deng *et al*., 1996) have not observed stimulation of a Jak–Stat signaling pathway in LPS-treated macrophages, and would therefore conclude that Jaks are not needed for LPS-induced S727 phosphorylation. However, Tsukada and colleagues recently reported activation of a tyrosine-phosphorylated, Stat-like transcription factor by LPS and IL-1 which does not bind to GAS elements (Tsukada *et al*., 1996). Therefore, the potential role of Jaks in LPS signal transduction still needs to be clarified.

The experiment shown in Figure 9A suggests that the phosphorylation events on Y701 or S727 influence each other. Unlike what was recently suggested for Stat3 (Chung *et al*., 1997), we find no evidence for a negative interference of serine with tyrosine phosphorylation. The data clearly indicate a relatively larger proportion of S727 phosphorylated in the tyrosine-phosphorylated fraction of molecules. Our further experiments (Figure 9B and C) argue against a positive effect of phospho-S727 on Y701 phosphorylation, at least in the sense that the maximal levels of tyrosine phosphorylation that can be caused by IFN-γ are the same with or without priming Stat1 on S727. Therefore, we are led to conclude that the S727 kinase may phosphorylate tyrosine-phosphorylated Stat1 preferentially. The Y701 phosphogroup might increase the affinity of association, or the S727 kinase might preferentially recognize Stat1 dimers. Alternatively, the altered subcellular localization of tyrosine-phosphorylated Stat1 might increase the accessibility for the serine kinase.

In conclusion, our study provides evidence for the importance of Stat serine phosphorylation during the immune response to bacterial infections. Issues connected to signal transduction and the function of the S727 kinase will be a further subject of future studies.

Materials and methods

Cells, cytokines and drugs

U937 promonocytes were cultured in RPMI medium containing 10% FCS. Bac1.2F5 cells, or Bac1.2F5-derived clones carrying a Stat-dependent reporter plasmid (see below), represent an early stage of mature macrophages and grow in a CSF-1-dependent manner (Morgan *et al*., 1987). They were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and 20% L-cell conditioned medium as a source of CSF-1. For stimulation with CSF-1, 10 000 U/ml of human recombinant CSF-1 (a kind gift from J.Schreurs, Chiron Corp., Emeryville, CA) were used. Primary mouse bone macrophages were obtained from femurs as described (Baccarini *et al*., 1985). Human PBMC were obtained from whole blood of healthy donors (P.K. and T.D.) by Ficoll gradient centrifugation. The cells contained ~20% monocytes. Recombinant human IFN-α and IFN-γ were kind gifts of P.von Wussow (Hannover Medical School, Hannover, Germany). Murine recombinant IFN-γ was kindly provided by G.Adolf (Bender, Vienna). Murine recombinant IFN-α was purchased from Hycult biotechnology (Uden, The Netherlands). For stimulation of cells, IFN-α was used at a concentration of 1000 U/ml; IFN-γ was used at a concentration of 5 ng/ml, unless otherwise indicated. LPS (Sigma, St Louis, MO) was either from *Salmonella* or *Escherichia coli* and was used at a concentration of 1 μg/ml, unless stated otherwise. The phorbol ester TPA (Sigma, St Louis, MO) was used at a concentration of 50 nM. The serine kinase inhibitor H7 (Calbiochem, La Jolla, CA) was used at 50 μM.

Plasmids

A Stat-dependent reporter plasmid (IFP53GAS2-Luc) was constructed by inserting two copies of an oligonucleotide comprising the IFP53-GAS sequence (Strehlow *et al*., 1993) into vector pADneo2Bgluci (a kind gift of C.Stratowa, Bender and Co., Vienna). This sets the GAS sequences in front of a β-globin minimal promoter and a luciferase reporter gene. Additionally, the vector contains a Rous sarcoma virus (RSV) promoter-driven *neo* gene for selection.

Antibodies

Rabbit antiserum to phospho-S727-Stat1 (anti-pS727) was obtained by immunization with keyhole limpet hemocyanin (KLH)-coupled phosphopeptide with the sequence DNLLPMpSPEEFDE (synthesized by Research Genetics, Huntsville, AL). The resulting serum was used in Western blots at a dilution of 1:5000, in immunoprecipitation at a dilution of 1:50 and in 'supershift' EMSA experiments at 1:125. Rabbit antiserum to the Stat1α C-terminus was obtained by immunizing with a GST fusion protein comprising the 39 C-terminal amino acids of Stat1α (plasmid kindly provided by Chris Schindler, Columbia University, NY). The serum was used for immunoprecipitation at a dilution of 1:100 and in Western blots at a dilution of 1:5000. Rabbit antiserum specific for

the tyrosine-phosphorylated Stat1 was a kind gift of David Frank and Michael Greenberg (Harvard Medical School). The antiserum was used for Western blots at a dilution of 1:500. Mouse monoclonal antibody Py20, directed against phosphotyrosine, was purchased from Transduction Laboratories (Lexington, KY) and used for Western blots at 2 μg/ml.

Cellular extracts

Methods to generate nuclear or whole-cell extracts for EMSA have been described (Eilers *et al*., 1994 and references therein). For immunoprecipitation and Western blot analysis, the cells were lysed in lysis buffer containing 10 mM Tris–HCl (pH 7.05), 50 mM NaCl, 30 mM $NaPP_i$, 50 mM NaF, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium vanadate, pepstatin 1 μg/ml, leupeptin 0.5 μg/ml, aprotinin 3 μg/ml. Extracts were cleared by centrifugation at 15 000 r.p.m., 4°C, 8 min.

Electrophoretic mobility shift assay (EMSA)

We recently have described this assay using an oligonucleotide corresponding to the β-casein GAS sequence as a probe (Gouilleux *et al*., 1995; Woldman *et al*., 1997).

Immunoprecipitation and Western blot analysis

Cellular extracts were normalized on the basis of protein determinations. Normalized volumes of extracts were incubated for 2–4 h at 4°C with specific antibodies. Immune complexes were collected following incubation (2–12 h at 4°C) with protein A–Sepharose beads. Immunoprecipitates were washed four times with lysis buffer. The beads were eluted by boiling in Laemmli sample buffer. Proteins were resolved by 10% SDS–PAGE and electrotransferred to nitrocellulose. Membranes were blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline including 0.1% Tween-20 (PBST) and incubated with the appropriate concentration of specific antibody, diluted in PBST + 2% BSA. After washing, the blots were incubated with horseradish peroxidase-conjugated second antibody and developed using an ECL system (Amersham, UK) according to the manufacturer's instructions.

In-gel assay for MAP kinase activation

Denatured protein samples (15 μg/lane) were fractionated over a 10% SDS–acrylamide gel containing 0.2 mg/ml co-polymerized substrate (myelin basic protein, Sigma). After the run, the gel was washed twice for 10 min in 50 mM HEPES, pH 7.4, 5 mM 2-mercaptoethanol and 20% isopropanol. The gel was then washed twice for 10 min in 50 mM HEPES, pH 7.4, 5 mM 2-mercaptoethanol and denatured twice for 30 min in 50 mM HEPES, pH 7.4, 5 mM 2-mercaptoethanol containing 6 M guanidine–HCl. The gel was renatured with four changes of 50 mM HEPES, pH 7.4, 5 mM 2-mercaptoethanol, 0.04% Tween-20 for 16 h total at 4°C. At 30 min before the assay, the gel was equilibrated in 25 mM HEPES, pH 7.4, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 90 mM Na_3VO_4 at 30°C. The assay was performed by incubating the gel in 25 mM HEPES, pH 7.4, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 90 mM Na_3VO_4 containing 10 μCi of [γ-^{32}P]ATP at 30°C for 60 min. The reaction was terminated by four washes in 5% trichloroacetic acid (TCA),10 mM Na pyrophosphate at room temperature for 4 h.

Transient and stable transfection

293 human kidney embryonic fibroblasts were transfected with Superfect reagent (Quiagen) according to the manufacturer's instructions. Expression of protein from the transfected gene was determined 24 h after transfection. Stable transfectants of Bac1.2F5 cells were generated by electroporating log-phase cells with IFP53GAS2-Luc plasmid. After 2–3 weeks of selection in G418, individual clones were isolated, expanded and tested for IFN-γ-inducible luciferase activity. Several clones were analyzed and gave very similar or identical results with respect to Stat-dependent luciferase induction. One of these (C11) was used for the experiments shown in this study.

Luciferase assays

Extraction of cells and determination of luciferase activity were performed according to standard protocols (Sambrook *et al*., 1989).

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