The EMBO Journal vol.15 no.22 pp.6262–6268, 1996

The rapid inactivation of nuclear tyrosine phosphorylated Stat1 depends upon a protein tyrosine phosphatase

Richard L.Haspel, Marianne Salditt-Georgieff and James E.Darnell,Jr[1]

Laboratory of Molecular Cell Biology, The Rockefeller University, New York, NY 10021-6399, USA

[1]Corresponding author

After interferon-γ (IFN-γ) treatment of cells the appearance of tyrosine phosphorylated Stat1 in the nucleus was maximal within 20–30 min, remained for 2–2.5 h and activated molecules disappeared by 4 h. In the absence of continued signaling from the receptor (imposed by staurosporine treatment) previously activated Stat1 disappeared completely within 60 min, implying continuous generation and removal of active molecules during extended IFN-γ treatment. Proteasome inhibitors prolonged the time of activation of Stat1 by prolonging signaling from the receptor but not by blocking removal of already activated Stat1 molecules. By analyzing with ^{35}S labeling the distribution of total Stat1 and activated Stat1, we concluded that the Stat1 molecules promptly cycle into the nucleus as tyrosine phosphorylated molecules and later return quantitatively to the cytoplasm as non-phosphorylated molecules. Therefore, the removal of the activated Stat1 molecules from the nucleus appears not to be proteolytic but must depend on a protein tyrosine phosphatase(s).

Keywords: interferon-γ receptor/phosphatase/proteasome/signal transduction/Stat1

Introduction

Early observations on polypeptide-induced transcription charted not only the rapid rise in transcription rate in response to such ligands as IFN-α, EGF and PDGF but, within at most a few hours, a subsequent decline in transcription rate (Greenberg and Ziff, 1984; Larner *et al.*, 1984, 1986). In the ensuing years, the positive-acting transcription factors that are induced by several different polypeptides have been purified or identified and in a large number of cases have been found to be members of the STAT (signal transducers and activators of transcription) protein family (Darnell *et al.*, 1994; Schindler and Darnell, 1995). The ligand-activated forms of the STATs are phosphorylated on a single tyrosine and after homo- or heterodimerization the proteins are translocated to the nucleus where they bind specific DNA sites and direct gene activation. Using DNA binding assays it has been shown in a number of instances that STAT activation is transient with activated molecules disappearing from nuclear and cytoplasmic extracts within 1–4 h of ligand treatment (Shuai *et al.*, 1992; Silvennoinen *et al.*, 1993; Pallard *et al.*, 1995). The underlying basis for the inactivation of the STAT proteins in this cycle has not been explained. Internalization of receptors from the cell surface with either detachment of polypeptide ligand or destruction of the ligand and receptor would presumably halt signals from the receptor (Goldstein *et al.*, 1985) but it is not known how long activated receptors continue to generate activated STAT molecules. In addition to the physical dislocation of the receptor, a number of cell surface receptors have tyrosine phosphatases recruited to specific sites on their cytoplasmic domains (Feng and Pawson, 1994). Although the quantitative role and the precise substrates for these phosphatases in stopping ligand activated signaling are not known, it is clear they are important. Mutation of these phosphatases—as in the motheaten mouse (Schulz *et al.*, 1993; Tsui *et al.*, 1993)—or loss of their attachment sites (Klingmuller *et al.*, 1995) leads to abnormally increased signaling from the affected receptors. Thus, not only internalization and inactivation of receptors but also enzymatic inactivation of Jak kinases or receptor docking sites can potentially limit the activation of STAT molecules. However, cessation of signaling from a receptor would not lead to automatic removal of activated STAT molecules; these molecules must either be dephosphorylated or potentially destroyed proteolytically. The proteasome, a multi-subunit protease (Goldberg and Rock, 1992; Hershko and Ciechanover, 1992) that degrades molecules tagged with the small protein ubiquitin, is known to degrade a number of transcription factors including c-fos and c-jun (Trier *et al.*, 1994; Stancovski *et al.*, 1995). There is currently no definitive evidence for a phosphatase or a protease acting on members of the STAT family.

In the present experiments we have examined the time-course of the activation–inactivation cycle of Stat1 in cells treated with IFN-γ and show that the entire course is essentially over by ~4 h, even in the continued presence of the ligand. Using staurosporine, a kinase inhibitor, to quickly stop any further signaling from the receptor, we find that the half-life of the active STAT molecules to be no longer than 15 min. Through the use of inhibitors of proteasome action, such as the peptide analog MG132 (Palombella *et al.*, 1994; Rock *et al.*, 1994), we show that the DNA binding form of Stat1 remains present for several hours longer than normal. However, this maintenance of active Stat1 required prolonged cell surface signaling because the MG132-supported signal disappears quickly in a staurosporine chase. We then measured directly the stability of ^{35}S-labeled Stat1 during IFN-γ stimulation and found that it is quantitatively preserved through the activation–inactivation cycle. These results argue strongly against proteolysis as a major cause for removal of active STAT molecules and in favor of protein tyrosine phosphatase(s) as the mechanism of removal.

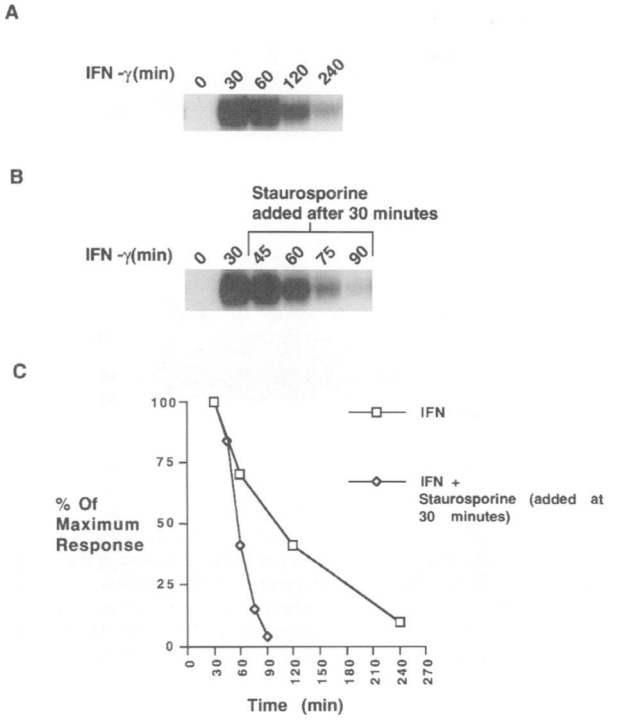

Fig. 1. Effect of staurosporine treatment on pre-induced Stat1 DNA-binding activity. (**A**) EMSA analysis with an M67 probe. Nuclear extracts were prepared from Bud-8 normal human fibroblasts treated with IFN-γ for the indicated times. (**B**) Same as (A) except after 30 min of IFN-γ treatment, staurosporine (500 nM) was added to the cells and nuclear extracts prepared every 15 min over the next 60 min. (**C**) Plot of the PhosphorImager analysis of Stat1 DNA-binding complexes at various times after IFN-γ and IFN-γ plus staurosporine treatment.

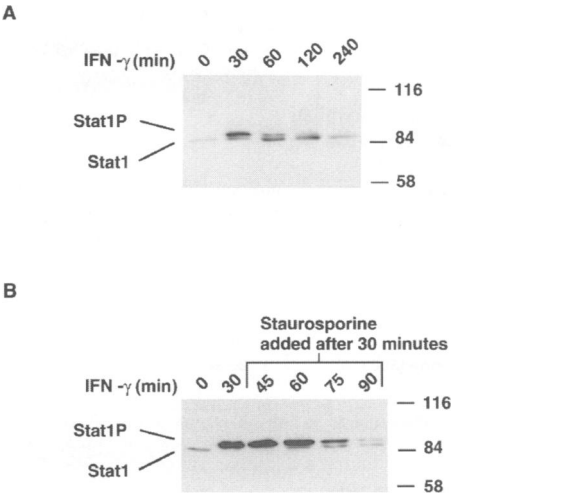

Fig. 2. Western blots of Stat1 in IFN-γ treated cells with and without staurosporine. (**A**) Western blot analysis. Nuclear extracts were prepared from Bud-8 fibroblasts treated with IFN-γ for the indicated times. These extracts were then subjected to SDS–PAGE and blotted with anti-Stat1 C-terminal antiserum. (**B**) Same as (A) except after 30 min of IFN-γ treatment, staurosporine (500 nM) was added to the cells and nuclear extracts prepared every 15 min for the next 60 min.

Results

The cycle of activation and inactivation of Stat1

To determine the time-course of inactivation of Stat1 after IFN-γ treatment, human fibroblasts (Bud-8 cells) were treated and nuclear extracts prepared at time points up to 4 h. Activated Stat1 was detected by a DNA binding assay (electrophoretic mobility shift assay, EMSA). The peak of the induced Stat1 DNA binding activity occurred at ~30 min (Figure 1A, lane 2; also, data not shown) and over the next few hours, there was a gradual decay with only 5–10% of the DNA binding activity remaining by 4 h (Figure 1A, lane 5 and Figure 1C).

The decay in DNA binding activity must be due first to attenuation of signaling from cell surface receptors but must also include removal of the activated Stat1. In order to distinguish between these two steps, the protein kinase inhibitor staurosporine (Schindler *et al.*, 1992) was used in a variation of a pulse–chase experiment. Cells were treated with IFN-γ for 30 min, and then signaling from the receptor was halted by adding the kinase inhibitor. In several experiments the amount of nuclear DNA binding activity was about the same for the 15 min after staurosporine treatment. However, after an additional 15 min or so, the DNA binding activity decreased by at least one-half and the signal disappeared almost completely within 1 h of staurosporine treatment (Figure 1B and C). The disappearance of active Stat1 after staurosporine includes the time required for the drug to block signaling and the time for the cytoplasmic pool of phosphorylated Stat1 to enter the nucleus. Assuming that the staurosporine only blocked further signaling and did not induce premature decay of activated Stat1 molecules, the half-life of a nuclear phosphorylated Stat1 molecule is ≤15 min.

We next determined whether the decay in DNA binding activity was synchronous with the disappearance of phosphorylated Stat1. Using Stat1 C-terminal antiserum that precipitates the full-length Stat1 molecule, extracts from untreated cells show a single reactive band at 91 kDa (Figure 2A and B, lane 1) but extracts from cells treated for 30 min with IFN-γ show two closely spaced bands. The second, slower-moving band is tyrosine phosphorylated Stat1 (Figure 2A and B, lane 2; Shuai *et al.*, 1992; Wen *et al.*, 1995). With constant IFN-γ treatment, the upper band gradually disappeared over 4 h (Figure 2A). After staurosporine treatment, the upper band disappeared almost completely within 60 min (Figure 2B), showing that the decay of DNA binding activity (Figure 1) paralleled the loss of tyrosine phosphorylated Stat1 (Figure 2).

Proteasome inhibitors prolong IFN-γ signaling

The disappearance of the phosphorylated Stat1 protein from the nucleus might be due either to proteolytic destruction and/or dephosphorylation of that protein. The proteasome is involved in the destruction of many cellular proteins (Hershko and Ciechanover, 1992) and we sought to determine whether this pathway played a role in IFN-γ signaling. Bud-8 cells were pretreated for 1 h with either MG132 or lactacystin, compounds that are potent but structurally unrelated inhibitors of proteasome action (Fenteany *et al.*, 1995; Palombella *et al.*, 1994; Rock *et al.*, 1994). The pretreated cells or control cells were then treated with IFN-γ (Figure 3) and nuclear extracts prepared. After 30 min, all nuclear extracts had strong DNA-binding activity with the activity in the extracts from cells pretreated with the proteasome inhibitors being slightly stronger (Figure 3, compare lanes 2, 3 and 4). In extracts from cells without pretreatment with proteasome

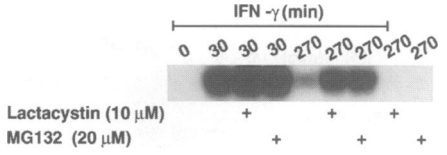

Fig. 3. Proteasome inhibitors prolong IFN-γ signaling. EMSA analysis with an M67 probe. Bud-8 fibroblasts were pretreated with lactacystin (10 μM) or MG132 (20 μM) or an equivalent volume of DMSO (carrier used to dissolve compounds) for 1 h. IFN-γ was then added (except in lanes 8 and 9) and nuclear extracts prepared at the indicated time points.

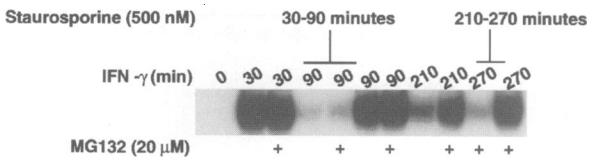

Fig. 4. MG132 does not prolong signaling in the presence of staurosporine. EMSA analysis with an M67 probe. Bud-8 fibroblasts were pretreated with MG132 (20 μM) or DMSO for 1 h. IFN-γ was then added to the cells and nuclear extracts prepared at the indicated time points. Where noted, staurosporine (500 nM) was added to the cells for 60 min.

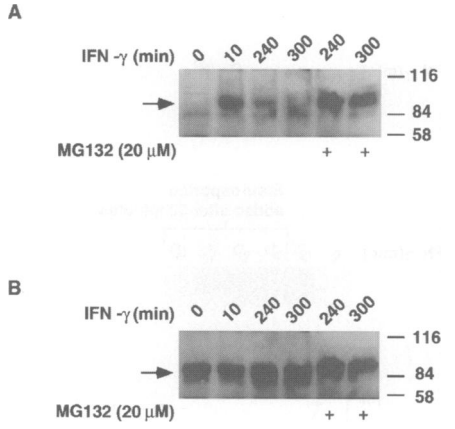

Fig. 5. MG132 prolongs phosphorylation of the IFN-γ receptor α chain. (**A**) Protein immunoblot of precipitated α chain of IFN-γ receptor with anti-phosphotyrosine antibody. WI-38 cells were pretreated with MG132 (20 μM) or DMSO for 1 h. IFN-γ was then added to the cells and whole-cell extracts were generated at the indicated time points. The IFN-γ receptor α chain was then precipitated, PAGE and transfer to filters was carried out and filters were then blotted with anti-phosphotyrosine antibody. (**B**) The blot in (A) was stripped and reprobed with the antibody to the IFN-γ receptor α chain.

inhibitor, the great majority of the DNA binding activity had disappeared after 4.5 h (lane 5). However, the nuclear extracts from the cells treated with proteasome inhibitor still had at least half as much DNA binding activity as at 30 min (Figure 3, compare lanes 3 and 4 with lanes 6 and 7). When cells were treated with only the proteasome inhibitors for 4.5 h, there was no detectable Stat1 DNA-binding activity (lanes 8 and 9). Thus, the proteasome inhibitors MG132 and lactacystin do not independently generate a signal but prolong the presence of activated Stat1.

The effect of the proteasome inhibitor MG132 is upstream from the Stat1 molecule

We next sought to determine at what point in the IFN-γ signaling cascade the proteasome inhibitors exerted their effect. Staurosporine was once again used to block signaling at the receptor after a pool of phosphorylated Stat1 had been accumulated. If the proteasome acts to destroy the Stat1 molecule, then proteasome inhibitor pretreatment should still prolong the activated Stat1 signal, even in the absence of constantly generated active Stat1. Bud-8 cells were pretreated with MG132 or carrier and IFN-γ was added for 30 min or for 3.5 h. At each time point cell samples were treated with staurosporine. Even in the presence of MG132, whether early or late in the IFN-γ treatment course, the DNA-binding activity still disappeared 1 h after addition of the staurosporine (Figure 4, lanes 5 and 10). We concluded that the proteasome inhibitor does not spare the DNA-binding protein from removal but must exert its effect upstream by sustaining the signal that causes activation of Stat1 at the cell surface.

MG132 prolongs activation of the IFN-γ cell surface receptor

The human IFN-γ receptor is composed of both α and β chains (Farrar *et al.*, 1992; Hemmi *et al.*, 1994; Soh *et al.*, 1994). The activation of Stat1 after IFN-γ treatment is preceded by ligand-dependent activation of JAK kinases associated with the α and β chains (reviewed in Darnell *et al.*, 1994; Ihle, 1995) and subsequent phosphorylation of a number of tyrosines on the α chains (Greenlund *et al.*, 1994). We tested activation of the α chain of the receptor by treating cells with IFN-γ, making cell extracts, precipitating with an antiserum to the α chain, separating on SDS–PAGE and using antiphosphotyrosine antibody to determine the phosphorylation state of the receptor. For these experiments, WI-38 cells were used which are identical to Bud-8 fibroblasts in their responses to IFN-γ, staurosporine and MG132 (data not shown).

As shown in Figure 5A, the receptor was not phosphorylated until IFN-γ treatment, and was maximally phosphorylated within 10 min (lanes 1 and 2; also data not shown). By 4 h, very little phosphorylated receptor remained (lane 3) even though all lanes contained approximately equivalent amounts of the α chain of the IFN-γ receptor (Figure 5B). In the presence of MG132, the receptor remained heavily phosphorylated even 5 h after the beginning of IFN-γ treatment (Figure 5A, lane 6). Thus, prolonged signaling from the receptor in the presence of MG132 could account for the sustained activation of Stat1 in the presence of the proteasome inhibitors.

Vanadate prolongs the Stat1 signal in the presence of staurosporine

At this point we could invoke a possible role for proteolysis in decreasing receptor signaling. However, the disappearance of the phosphorylated nuclear Stat1 appeared not to be due to proteolysis but to disappearance of DNA binding activity associated with loss of phosphotyrosine (Figures 1 and 2). To attempt to show that the removal of active Stat1 required a tyrosine phosphatase we used Na vanadate, a compound well known to be a phosphatase inhibitor (Gordon, 1991). Bud-8 cells were treated with IFN-γ to establish a pool of active molecules and were then treated with staurosporine or staurosporine plus Na vanadate. As expected from the experiments in Figure 1, treatment of

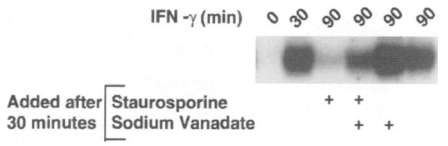

Fig. 6. Vanadate prolongs IFN-γ signaling even in the presence of staurosporine. EMSA analysis with an M67 probe. Bud-8 fibroblasts were treated with IFN-γ for 30 min. Staurosporine or carrier (DMSO) was then added to the cells. In lanes 4 and 5 vanadate (1 mM) was also added and the incubation continued for an additional 60 min. Nuclear extracts were generated at the indicated time points.

the IFN-γ-induced cells with staurosporine for a further 60 min caused almost complete disappearance of the Stat1 signal (Figure 6, lane 3). If vanadate was added together with the staurosporine, the disappearance of active Stat1 was partially prevented (lane 4). Thus, we conclude that at least part of the removal of Stat1 can be demonstrated to be due to phosphatase action. Two other samples were included in this experiment. As expected, IFN-γ-treated cells by 90 min had somewhat less active Stat1 than at 30 min (lane 6). This decline did not occur in the presence of vanadate (lane 5). While these results support the possibility that a phosphatase(s) removed the activated Stat1, there are complications in their interpretation. First, vanadate may not act instantly or completely to block phosphatases; had it done so there would be no decline of active Stat1 in the presence of both staurosporine and vanadate (Figure 6, lane 4). Second, Na vanadate by itself can lead to accumulation of active Stat1 (Igarashi *et al.*, 1993), complicating the nature of a continuing signal in the presence of vanadate. It is nevertheless clear that vanadate does affect the removal of activated Stat1, even in the presence of staurosporine; this is consistent with a role for a phosphatase in the deactivation process.

Direct evidence for cytoplasmic–nuclear cycling of Stat1 involving a tyrosine phosphatase

To test directly the fate of Stat1 molecules after activation, a quantitative recovery experiment with ^{35}S-labeled cells was carried out. Cells were labeled for 2.5 h with [^{35}S]-methionine, washed and medium containing non-radioactive methionine was added. IFN-γ was then used to activate Stat1 and samples were taken at frequent intervals thereafter. Anti-Stat1 antiserum was used to precipitate the protein from nuclear and cytoplasmic extracts; subsequently gel electrophoresis, autoradiography and quantitation of both the non-phosphorylated and the more slowly migrating, tyrosine phosphorylated form was carried out. The experimental results were clear-cut (Figure 7A–D). Before treatment, the large majority of ^{35}S-labeled Stat1 was cytoplasmic; after treatment, the ^{35}S-labeled, phosphorylated Stat1 was evident at a maximal level in the cytoplasm by 10 min, at which time there was already a strong phosphorylated Stat1 signal in the nucleus. (The X-ray film exposures in Figure 7A and B are not equal, but quantitation of data from two experiments is presented in Figure 7C and D.) By 30–45 min the amount of phosphorylated Stat1 had increased to a maximum in the nucleus and equalled ~20–25% of the total labeled Stat1 in the cell. The nuclear phosphorylated Stat1 started to slowly decline at this point, remaining at about one-third of peak levels at 3 h. Coincident with the fall in nuclear

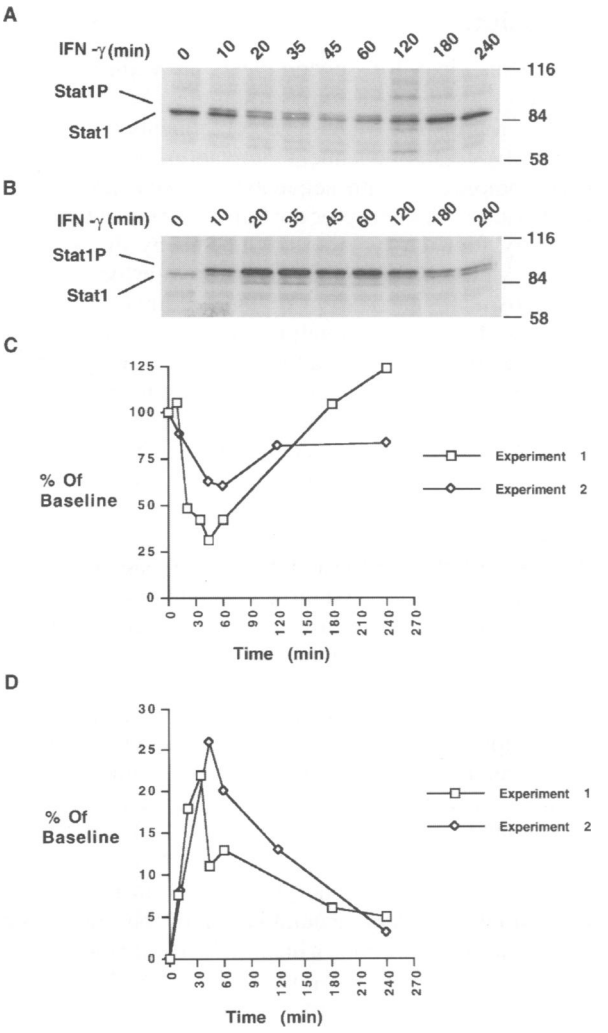

Fig. 7. The Stat1 activation/inactivation cycle analyzed by ^{35}S labeling. (**A**) ^{35}S-Labeling followed by immunoprecipitation of Stat1. Bud-8 fibroblasts were labeled with ^{35}S for 2.5 h as described in Materials and methods. Label was removed, cells washed in normal medium and IFN-γ was added. At the indicated time points, cytoplasmic and nuclear extracts were prepared. The cytoplasmic extracts were then subjected to SDS–PAGE and autoradiography. (**B**) Same as (A) except SDS–PAGE and autoradiography of nuclear extracts [~10-fold longer exposure than in (A)]. (**C**) Plot of the PhosphorImager analysis of the Stat1 bands (both phosphorylated and unphosphorylated) shown in (A) (Experiment 1) and a separate experiment (Experiment 2). Data are expressed as percent of baseline (the value in the 'no treatment' lane of the cytoplasmic extracts). (**D**) Plot of the PhosphorImager analysis of the phosphorylated Stat1 band shown in (B) (Experiment 1) and a separate experiment (Experiment 2). Data are expressed as percent of baseline (the value in the 'no treatment' lane of the cytoplasmic extracts).

phosphorylated Stat1 there was a rise in non-phosphorylated cytoplasmic Stat1 to initial levels. It was perhaps significant that the total recovery of the ^{35}S radiolabel was higher at the beginning and end of these experiments than at intermediate times after IFN-γ treatment, when the maximal nuclear phosphorylated Stat1 was evident. It is possible therefore that the nuclear phosphoprotein may not be quantitatively extracted. If this were true then even more Stat1 molecules could be present in the nucleus as phosphorylated Stat1 in the period from ~30 to 120 min after IFN-γ treatment.

Discussion

It has been clear for several years now that cytokine activation of the STAT family of molecules is in many instances a transient event. The components of the cycle include: (i) ligand-dependent activation of a receptor–kinase complex through sequential tyrosine phosphorylation of kinases and receptor docking sites; (ii) cytoplasmic phosphorylation on tyrosine of the Stat by the activated receptor–kinase complex; (iii) nuclear translocation; and (iv) removal or inactivation of the accumulated nuclear molecules. For Stat1 activation by IFN-γ it was known that activated nuclear molecules appeared within ~15 min and were gone within several hours (Shuai *et al.*, 1992). The length of time the receptors remained active in continuing to generate activated Stat1 was not known and the mechanism for removal of active nuclear molecules was not clear. The present results help to clarify these questions.

Because of the indeterminate time of signaling from activated receptors, we first turned our attention to stopping signaling and observing how long activated Stat1 molecules persisted. Staurosporine, a kinase inhibitor, showed that the inactivation of previously tyrosine phosphorylated, dimeric Stat1—the DNA-binding form of the molecule—is very rapid. The half-life of a phosphorylated nuclear Stat1 molecule appears to be at most 15 min. We also examined, during the early phases of activation, the time to accumulate maximal activated Stat1. By observing either DNA binding activity, or particularly the accumulation of ^{35}S-labeled phosphorylated Stat1 in the nucleus, the maximum nuclear accumulation of phosphorylated Stat1 occurred by 30 min, which includes time to saturate receptors, kinases and transport mechanisms. At this point of maximal accumulation of nuclear Stat1 there must be a balance of arrival and removal of activated Stat1 molecules. Thus, during the early stages of the IFN-γ treatment cycle, when signaling is still presumably maximal, the half-time to reach a maximum of activated molecules in the nucleus accords well with the half-time to remove the activated molecules. These results suggest quite strongly that activated molecules go through the cycle of activation and removal in ≤30 min. A most important point is that by 30 min of IFN-γ treatment, ~25% of the total Stat1 in the cell was activated and translocated to the nucleus. (As noted, this value could be higher because all nuclear molecules may not be recovered in preparing nuclear extracts.) Thereafter, the level of activated nuclear Stat1 remains high for at least another ~90 min and detectably elevated for up to 4 h. With a cycle that has a half-time of ~15 min and an elevated level of nuclear phosphorylated Stat1 that remains for 2 h, signaling must continue through several cycles. The majority of Stat1 molecules in the cell must be activated, cycle through the nucleus and reappear in the cytoplasm in a dephosphorylated state. In spite of this conclusion, the total amount of ^{35}S-labeled Stat1 (within the limits of experimental error) was the same at the end of the IFN-γ cycle as at the beginning. Kim and Maniatis have detected phosphorylated Stat1 associated with ubiquitin, indicating some protein turnover. However, in their experiments it was not possible to estimate the fraction of Stat1 molecules bound to ubiquitin (Kim and Maniatis, 1996). Our results on quantitative (or near-quantitative) recovery of labeled protein through an IFN-γ treatment cycle argue very strongly against removal of any major fraction of nuclear Stat1 activity by proteolysis. We therefore conclude that dephosphorylation occurs on Stat1 Tyr701, either in the nucleus with prompt egress of dephosphorylated protein to the cytoplasm, or almost immediate dephosphorylation upon re-emergence of a molecule back into the cytoplasm. Since a number of other activated, DNA-binding forms of STATs also undergo a rapid cycle of activation and inactivation (Silvennoinen *et al.*, 1993; Pallard *et al.*, 1995), the suggested regulation by a phosphatase could be general for this pathway. The only phosphatases that at present are known to affect the JAK–STAT pathway are molecules that are associated with the cytoplasmic domain of the cell surface receptor (Yi *et al.*, 1993; Klingmuller *et al.*, 1995) and these molecules would not seem to be correctly located to be candidates for dephosphorylation of Stat1 after its nuclear transit. Other tyrosine phosphatases that exist in the cell nucleus are known, but in the absence of enzyme-specific inhibitors or genetic removal of particular enzymes it is not possible to ascertain which enzyme(s) is involved.

Finally, we note that other investigators have attempted to use vanadate inhibition of protein tyrosine phosphatases to argue that a nuclear tyrosine dephosphorylation occurs (David *et al.*, 1993; Haque *et al.*, 1995). While this may be a correct conclusion, these earlier experiments are not persuasive. They rely basically on the ability of vanadate to support for a longer time a declining level of IFN-γ-induced phosphorylated Stat1 signal. However, these previous experiments cannot discriminate between a continuing IFN-γ-induced cell surface signal that also is destroyed by tyrosine phosphatase, and a nuclear event that removes the nuclear phosphorylated Stat1. We did show (Figure 6) that, with no further incoming signal (staurosporine treatment), vanadate did partially block the Stat1 decrease. Thus, the Na vanadate experiments are compatible with a nuclear phosphatase, but do not prove its existence.

Even though our major conclusion is that the removal of nuclear-activated Stat1 must require phosphatase action, the experiments with proteosome inhibitors do indicate an important role for proteolysis in the overall receptor–JAK–STAT activation and deactivation pathway. The proteosome inhibitor, MG132 (and lactacystin, a chemically different proteosome inhibitor), supports the presence of nuclear phosphorylated Stat1 long after it would have disappeared in an untreated cell. However, the MG132-supported signal could still be eliminated in ≤1 h by staurosporine, implying that continued signaling from the cell surface was required to maintain the level of active Stat1 in the presence of MG132. This continued signaling in the presence of MG132 obviously could originate at the receptor–ligand complex. We therefore tested for phosphotyrosine on the receptor and found that MG132 prevented loss of tyrosine phosphorylation from the receptor as it normally occurs without proteosome inhibitor. We conclude that internalization of the receptor with consequent proteolysis of either ligand or receptor or both normally stops the IFN-γ signaling. This conclusion matches that for several other receptors where proteolytic destruction by proteosomes has been suggested (Cenciarelli *et al.*, 1992; Mori *et al.*, 1992, 1995).

Materials and methods

Cell culture, antibodies and inhibitors

Bud-8 normal human fibroblasts were grown in DMEM supplemented with 10% fetal calf serum (Hyclone Laboratories Incorporated) and non-essential amino acids. WI-38 cells were grown in DMEM supplemented with 10% calf serum (Hyclone). Anti-Stat1 C-terminal sera were raised in rabbits as previously described (Shuai *et al.*, 1992). Anti-IFN-γ receptor α chain monoclonal antibody (GIR-94) was the kind gift of Robert Schreiber. Anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology. IFN-γ (a gift from AMGEN) was used at 5 ng/ml. Staurosporine (Sigma) was dissolved in dimethylsulfoxide (DMSO) and used at a final concentration of 500 nM. MG132 and lactacystin (the kind gifts of Alfred Goldberg and Julian Adams, MyoGenics) were dissolved in DMSO and used at final concentrations of 20 μM and 10 μM respectively.

Cell extracts, immunoprecipitations and SDS–PAGE

When generating cytoplasmic and nuclear extracts, cells were first lysed at 4°C by gently pipetting after 5 min in hypotonic buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM Na_3VO_4, 1 mM EDTA, 10% glycerol, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin and 1 mM DTT) with 0.2% NP-40. After centrifugation at 4°C (13 000 r.p.m. in microfuge) for 10 s, supernatants were collected as cytoplasmic extracts. Nuclear extracts were prepared by resuspension of the crude nuclei in high-salt buffer (hypotonic buffer with 20% glycerol and 420 mM NaCl) at 4°C for 30 min and the supernatants were collected after centrifugation at 4°C (13 000 r.p.m.) for 5 min. Whole-cell lysates were prepared by lysing cells in whole-cell extract buffer (50 mM Tris, 280 mM NaCl, 0.5% NP-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM DTT and 0.1 mM Na_3VO_4) for 10 min at 4°C. Supernatants were collected after centrifugation at 4°C (13 000 r.p.m.) for 10 min.

Immunoprecipitations were carried out by adding the precipitating antibody (5 μg of GIR-94 or 3 μl of anti-Stat1 C-terminal antiserum) to each extract and incubating for 1–2 h at 4°C followed by incubation at 4°C with protein G–Sepharose for 2 h (in the case of GIR-94) or protein A–Sepharose overnight (in the case of anti-Stat1). Samples were then washed three times with whole-cell extract buffer and then twice with PBS. 25 μl of 2× Laemmli running buffer was added to each sample. Samples were then heated at 80°C for 4 min and subjected to SDS–PAGE on a 7% gel for the IFN-γ receptor α chain or a 6% gel for Stat1. For Western blots of Stat1, 8 μg of each nuclear extract was mixed with 2× Laemmli running buffer, heated to 80°C for 4 min and subjected to SDS–PAGE on a 6% gel.

Western blotting

Following SDS–PAGE, proteins were transferred to nitrocellulose and blocked for 2 h at room temperature with 5% BSA in TBST (TBS plus 0.05% Tween) if blotting with anti-phosphotyrosine antibody (4G10) or blocked with 5% milk in TBST if blotting with anti-Stat1. 4G10 antibody was added at 1:4000 for overnight incubation at 4°C while anti-Stat1 C-terminal antiserum was added at 1:2500 for 2 h at room temperature. Membranes were then washed with TBST and incubated with a 1:4000 dilution of horseradish peroxidase conjugated goat anti-mouse (for 4G10) or goat anti-rabbit (for anti-Stat1) for 45 min (Jackson ImmunoResearch), washed with TBST and subsequently developed using Renaissance chemiluminescence reagent (NEN-Dupont). Where indicated, membranes were stripped with a solution containing 2% SDS, 62.5 mM Tris, pH 6.8 and 0.7% β-mercaptoethanol for 30 min at 50°C, washed extensively with TBST and blocked with 5% milk in TBST overnight at 4°C. Membranes were then reprobed with GIR-94 antibody (1 μg/ml) for 1 h at room temperature, washed with TBST and incubated with a 1:4000 dilution of horseradish peroxidase conjugated goat anti-mouse and subsequently developed using Renaissance chemiluminescence reagent.

Electrophoretic mobility shift assay

Nuclear extracts (see above) were used for electrophoretic mobility shift assay (EMSA). EMSA was carried out on 4% 29:1 acrylamide–bisacrylamide gels as described (Fried and Crothers, 1981) using M67 SIE (Wagner *et al.*, 1990) oligonucleotide as probe.

Pulse–chase experiments

Bud-8 fibroblasts were cultured in methionine-free DMEM containing 1% fetal bovine serum and non-essential amino acids. 100 μCi/ml of [^{35}S]methionine was added to each 10 cm plate for 2.5 h. Label was removed and fresh medium was added with or without IFN-γ. At the indicated times, nuclear and cytoplasmic extracts were generated and immunoprecipitation and SDS–PAGE was carried out as described above. Gels were then soaked in fixative (25% isopropanol, 10% acetic acid) for 35 min followed by soaking in Amplify (Amersham, Inc.) for 45 min. Gels were dried at 80°C for 1 h and then exposed to film.

Quantitation using the PhosphorImager

The intensities of radioactive bands in dried gels were quantitated using a PhosphorImager (Molecular Dynamics, Inc.). The band of interest in each lane was boxed and quantitated and an identical area above each band boxed as background and subtracted. For EMSAs, the value in the 'no treatment' lane was also subtracted from all the other values and data were expressed as a percentage of the maximum value (30 min of IFN-γ treatment). For ^{35}S-labeling experiments, data were expressed as a percentage of the baseline (the value in the 'no treatment' lane of the cytoplasmic extracts).

Acknowledgements

We thank Dr Alfred Goldberg and Dr Julian Adams for generous gifts of proteasome inhibitors and Dr Robert D. Schreiber for monoclonal antibodies directed against IFN-γ receptor chains. We also thank Zhong Zhong, Curt Horvath, Jacqueline Bromberg, Xuejun Zhu and Zilong Wen for their helpful advice and Lois Cousseau for preparing the manuscript. This work was supported by NIH grants (AI32489 and AI324420) to J.E.D. R.H. is supported by MSTP grant GM07739.

References

Cenciarelli,C., Hou,D., Hsu,K.-C., Rellahan,B.L., Wiest,D.L., Smith, H.T., Fried,V.A. and Weissman,A.M. (1992) Activation-induced ubiquitination of the T cell antigen receptor. *Science*, **257**, 795–797.

Darnell,J.E.,Jr, Kerr,I.M. and Stark,G.M. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*, **264**, 1415–1421.

David,M., Grimley,P.M., Finbloom,D.S. and Larner,A.C. (1993) A nuclear tyrosine phosphatase downregulates interferon-induced gene expression. *Mol. Cell. Biol.*, **13**, 7515–7521.

Farrar,M.A., Campbell,J.D. and Schreiber,R.D. (1992) Identification of a functionally important sequence in the C terminus of the interferon-γ receptor. *Proc. Natl Acad. Sci. USA*, **89**, 11706–11710.

Feng,G.-S. and Pawson,T. (1994) Phosphotyrosine phosphatases with SH2 domains: regulators of signal transduction. *Trends Genet.*, **10**, 54–58.

Fenteany,G., Standaert,R.F., Lane,W.S., Choi,S., Corey,E.J. and Schreiber,S.L. (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science*, **268**, 726–731.

Fried,M. and Crothers,D.M. (1981) Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.*, **9**, 6505–6525.

Goldberg,A.L. and Rock,K.L. (1992) Proteolysis, proteasomes and antigen presentation. *Nature*, **357**, 375–379.

Goldstein,J.L., Brown,M.S., Anderson,R.G.W., Russel,D.W. and Schneider,W.J. (1985) Receptor mediated endocytosis. *Annu. Rev. Cell Biol.*, **1**, 1–39.

Gordon,J.A. (1991) Use of vanadate as a protein-phosphotyrosine phosphatase inhibitor. *Methods Enzymol.*, **201**, 477–482.

Greenberg,M.E. and Ziff,E.B. (1984) Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogenes. *Nature*, **311**, 433–438.

Greenlund,A.L., Farrar,M.A., Viviano,B.L. and Schreiber,R.D. (1994) Ligand-induced IFN-γ receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). *EMBO J.*, **13**, 1591–1600.

Haque,S.J., Flati,V., Deb,A. and Williams,B.R.G. (1995) Roles of protein-tyrosine phosphatases in Stat1a-mediated cell signaling. *J. Biol. Chem.*, **270**, 25709–25714.

Hemmi,S., Böhni,R., Stark,G., DiMarco,F. and Aguet,M. (1994) A novel member of the interferon receptor family complements functionality of murine interferon γ receptor in human cells. *Cell*, **76**, 803–810.

Hershko,A. and Ciechanover,A. (1992) The ubiquitin system for protein degradation. *Annu. Rev. Biochem.*, **61**, 761–807.

Igarashi,K., David,M., Larner,A.C. and Finbloom,D.S. (1993) *In vitro* activation of a transcription factor by gamma interferon requires a

membrane-associated tyrosine kinase and is mimicked by vanadate. *Mol. Cell. Biol.*, **13**, 3984–3989.

Ihle,J.N. (1995) Cytokine receptor signalling. *Nature*, **377**, 591–594.

Kim,T.K. and Maniatis,T. (1996) Regulation of Interferon-γ-Activated STAT1 by the Ubiquitin-Proteasome Pathway. *Science*, **273**, 1717–1719.

Klingmuller,U., Lorenz,U., Cantley,L.C., Neel,B.G. and Lodish,H. (1995) Specific recruitment of SH-PTP-1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell*, **80**, 729–738.

Larner,A.C., Jonak,G., Cheng,Y.-S., Korant,B., Knight,E.,Jr and Darnell,J.E.,Jr (1984) Transcriptional induction of two genes in human cells by β interferon. *Proc. Natl Acad. Sci. USA*, **81**, 6733–6737.

Larner,A.C., Chaudhuri,A. and Darnell,J.E.,Jr (1986) Transcriptional induction by interferon. *J. Biol. Chem.*, **261**, 453–459.

Mori,S., Heldin,C.-H. and Claesson-Welsh,L. (1992) Ligand-induced polyubiquitination of the platelet-derived growth factor β-receptor. *J. Biol. Chem.*, **267**, 6429–6434.

Mori,S., Tanaka,K., Omura,S. and Saito,Y. (1995) Degradation process of ligand-stimulated platelet-derived growth factor β-receptor involves ubiquitin-proteasome proteolytic pathway. *J. Biol. Chem.*, **270**, 29447–29452.

Pallard,C., Gouilleux,F., Benit,L., Cocault,L., Souyri,M., Levy,D., Groner,B., Gisselbrecht,S. and Dunsanter-Fourt,I. (1995) Thrombopoietin activates a STAT5-like factor in hematopoietic cells. *EMBO J.*, **14**, 2847–2856.

Palombella,V.J., Rando,O.J., Goldberg,A.L. and Maniatis,T. (1994) The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell*, **78**, 773–785.

Rock,K.L., Gramm,C., Rothstein,L., Clark,K., Stein,R., Dick,L., Hwang,D. and Goldberg,A.F. (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell*, **78**, 761–771.

Schindler,C. and Darnell,J.E.,Jr (1995) Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu. Rev. Biochem.*, **64**, 621–651.

Schindler,C., Shuai,K., Prezioso,V.R. and Darnell,J.E.,Jr (1992) Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science*, **257**, 809–815.

Schulz,L.D., Schweitzewr,P.A., Rajan,T.V., Yi,T., Ihle,J.N., Matthews, R.J., Thomas,M.L. and Beier,D.R. (1993) Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. *Cell*, **73**, 1445–1454.

Shuai,K., Schindler,C., Prezioso,V.R. and Darnell,J.E.,Jr (1992) Activation of transcription by IFN-γ: tyrosine phosphorylation of a 91 kDa DNA binding protein. *Science*, **259**, 1808–1812.

Silvennoinen,O., Schindler,C., Schlessinger,J. and Levy,D.E. (1993) Ras-independent growth factor signaling by transcription factor tyrosine phosphorylation. *Science*, **261**, 1736–1739.

Soh,J. *et al.* (1994) Identification and sequence of an accessory factor required for activation of the human Interferon γ receptor. *Cell*, **76**, 793–802.

Stancovski,I., Gpmem,H., Orian,A., Schwartz,A.L. and Ciechanover,A. (1995) Degradation of the proto-oncogene product c-Fos by the ubiquitin proteolytic system *in vivo* and *in vitro*: identification and characterization of the conjugating enzymes. *Mol. Cell. Biol.*, **15**, 7106–7116.

Trier,M., Staszewski,L.M. and Bohmann,D. (1994) Ubiquitin-dependent c-Jun degradation *in vivo* is mediated by the δ domain. *Cell*, **78**, 787–798.

Tsui,H.W., Siminovitch,K.A., DeSouza,L. and Tsui,F.W.L. (1993) Motheaten and viable motheaten mice have mutations in the hematopoietic cell phosphatase gene. *Nature Genet.*, **4**, 124–129.

Wagner,B.J., Hayes,T.E., Hoban,C.J. and Cochran,B.H. (1990) The SIF binding element confers sis/PDGF inducibility onto the c-fos promoter. *EMBO J.*, **9**, 4477–4484.

Wen,Z., Zhong,Z. and Darnell,J.E.,Jr (1995). Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell*, **82**, 241–250.

Yi,T., Mui,A.L.F., Krystal,G. and Ihle,J.N. (1993) Hematopoietic cell phosphatase associates with the interleukin-3 (IL-3) receptor β chain and down-regulates IL-3-induced tyrosine phosphorylation and mitogenesis. *Mol. Cell. Biol.*, **13**, 7577–7586.

Received on July 4, 1996; revised on August 19, 1996