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Stat1 Serine Phosphorylation Occurs Independently of Tyrosine Phosphorylation and Requires an Activated Jak2 Kinase

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Gamma interferon (IFN-γ) induces both tyrosine and serine phosphorylation of Stat1. Stat1 serine phosphorylation is required for maximal transcriptional activity of Stat1. In this report, we present evidence that Stat1 tyrosine phosphorylation is not a prerequisite for Stat1 serine phosphorylation, although an active Jak2 kinase is required for both phosphorylation events. Stat1 serine phosphorylation occurs with a more delayed time course than tyrosine phosphorylation. The occurrence of serine phosphorylation without tyrosine phosphorylation suggests that serine phosphorylation takes place in the cytoplasm. Experiments performed with cells expressing either dominant-negative or constitutively active Ras protein indicated that the Ras–mitogen-activated protein kinase pathway is probably not involved in IFN-γ-induced Stat1 serine phosphorylation. Finally, a kinase capable of correct Stat1 serine phosphorylation was detected in partially purified cytoplasmic extracts from both IFN-γ-treated and untreated cells.

Polypeptide signalling that changes gene expression often involves the STAT proteins (16). These latent cytoplasmic transcription factors become activated by phosphorylation of tyrosine residues, catalyzed either by receptor-associated Janus (Jak) kinases or by receptor tyrosine kinases. The STAT molecules then dimerize by reciprocal phosphotyrosine-SH2 interactions and enter the cell nucleus to effect transcriptional changes. It has been amply demonstrated that transcriptional activation is further amplified when the serine residues of STAT proteins are phosphorylated (22). A single serine in Stat3 and Stat1, residue 727 in both cases, appears to be the major, if not the only, serine kinase target site (21). In addition to these studies, a number of other findings suggestive of serine phosphorylation of STATs that are also tyrosine phosphorylated have been described. For example, Stat3 and Stat5 show a ligand-dependent, slower migration that is also time dependent and is inhibited by the serine kinase inhibitor H7 (2, 3, 24).

The signalling pathway(s) involved in serine phosphorylation of the STATs is not known at present, although we do show here that Jak2 is required for STAT serine phosphorylation in response to gamma interferon (IFN-γ). In this study, we explored the time course and cellular locus of serine and tyrosine phosphorylation and the potential interdependence of the two. In addition, we examined the nature of the serine phosphorylation pathways, demonstrating the lack of evidence for participation of several prominent serine kinases. However, we did detect a kinase capable of catalyzing phosphorylation of Stat1, predominantly on residue 727.

MATERIALS AND METHODS

Cell culture. U3A cells (13, 14), γ2A cells (12), and their derived cell lines were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% cosmic serum (HyClone Laboratories Inc.). NIH 3T3 cells and cells stably transfected with either dominant-negative Ras (dnRas) or constitutively active Ras (CARas) were grown in DMEM supplemented with 10% bovine calf serum (HyClone Laboratories Inc.). Cells were normally starved by culturing them in DMEM without serum before labeling them and treating them with ligands.

Phosphopeptide mapping and phosphoamino acid analyses. For phosphopeptide mapping, cells that had been starved overnight in serum-free DMEM were grown for 3 h in phosphate-free DMEM (GIBCO-BRL). [^{32}P]orthophosphate (DuPont) was then added to the medium to a final concentration of 0.5 mCi/ml (5 ml of labeling medium was used per 100-mm-diameter dish). Following 2.5 h of incubation, cell lysates from either IFN-γ-treated or untreated cells were prepared and precleared with a mouse anti-Stat3C antibody (25). Stat1α proteins were immunoprecipitated with anti-Stat1C antibody and fractionated by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS–7.5% PAGE). The ^{32}P-labeled Stat1α proteins were identified by autoradiography and excised from the gel. The proteins were then digested with trypsin (100 ng/ml; Worthington Biochemical Corporation) in 50 mM NH_4CO_3. Phosphopeptides were separated by electrophoresis at pH 3.5 followed by chromatography on thin-layer cellulose plates (Kodak) (19). For phosphoamino acid analyses, phosphopeptides were eluted from thin-layer chromatography plates with pyridine and hydrolyzed in 6 M HCl at 110°C for 90 min. Phosphoamino acids were loaded on thin-layer chromatography plates and separated by two-dimensional electrophoresis at pH 1.9 (first dimension) and pH 3.5 (second dimension) (19).

Cell extracts and immunocomplex kinase assay. Cytoplasmic, nuclear, and whole-cell extracts were prepared as previously described (22). Erk2 protein was immunoprecipitated with an anti-Erk2 antibody (Santa Cruz Biotechnology). The immunoprecipitates were resuspended in kinase buffer (20 mM Tris, 10 mM $MgCl_2$, 0.3 mM Na_3VO_4, 5 mM ATP, and 5 μCi of [^{32}P]ATP) containing MBP (Sigma) and incubated at 30°C for 30 min. The phosphorylated MBP was separated by SDS-PAGE and visualized by autoradiography.

Cell fractionation and Stat1 kinase assay. NIH 3T3 cells were treated with IFN-γ for 35 min, washed twice with ice-cold phosphate-buffered saline, and pelleted by centrifugation at 2,000 × *g* for 5 min. The cell pellet was washed briefly in 4 volumes of hypotonic buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM Na_3VO_4, 0.5 mM dithiothreitol, 1 μg of leupeptin per ml, 2 μg of aprotinin per ml, and 1 μg of pepstatin per ml) before being resuspended in 3 volumes of the same buffer. After swelling on ice for 10 min, cells were broken by 10 strokes of a Dounce homogenizer. The supernatant from a 15-min spin at 20,000 × *g* was further clarified by centrifugation at 100,000 × *g* for 30 min. Proteins were applied to an anion-exchange column (Perceptive Biosystems) equilibrated in hypotonic buffer A. After the column was washed with 10 ml of buffer A, proteins were eluted with a linear gradient of NaCl (0 to 0.3 M) in buffer A at a flow rate of 10 ml/min, and 1-ml fractions were collected. Stat1 serine kinase activity in every other fraction was measured by an in vitro kinase assay using full-length Stat1 as the substrate; an aliquot of the column fraction (2 μl) was added to the kinase reaction mixture, which contained 40 mM HEPES (pH 7.9), 10 mM $MgCl_2$, 50 μM ATP, 5 μCi of [^{32}P]ATP, and 1 μg of full-length Stat1α in a final volume of 20 μl. The reaction mixture was incubated at 30°C for 1 h before termination of the reaction by addition of an equal volume of 2× SDS sample buffer. The phosphorylated Stat1 was separated by SDS-PAGE and visualized by autoradiography.

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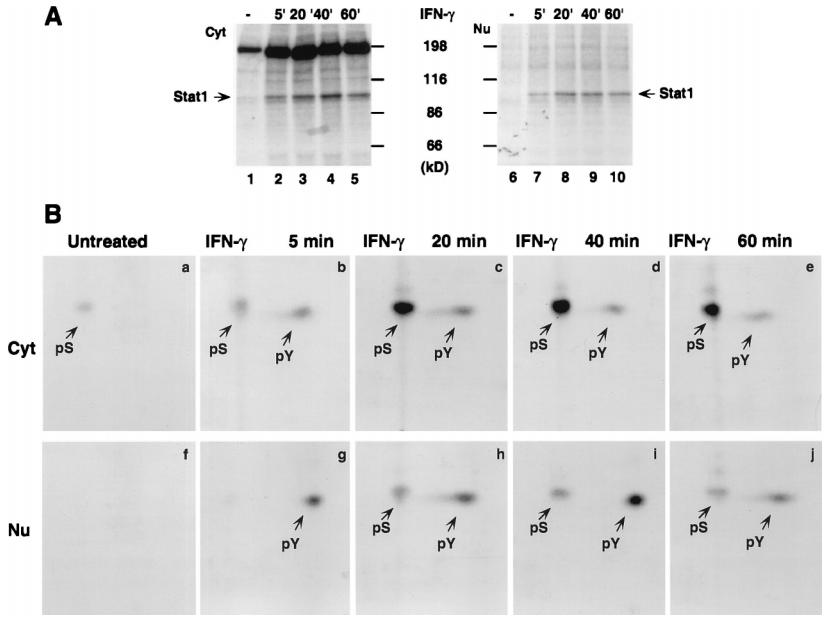

FIG. 1. Time course of Stat1 serine phosphorylation following IFN-γ treatment of NIH 3T3 cells. (A) Immunoprecipitation of ^{32}P-labeled Stat1 from untreated (lanes 1 and 6) and IFN-γ-treated (lanes 2 to 5 and 7 to 10) cytoplasmic (Cyt) extracts (lanes 1 to 5) and nuclear (Nu) extracts (lanes 6 to 10) of NIH 3T3 cells. The numbers between the panels correspond to the positions of molecular size markers (in kilodaltons). (B) Tryptic phosphopeptide maps of ^{32}P-labeled Stat1 from cytoplasmic extracts (a to e) and nuclear extracts (f to j) of untreated cells (a and f) and cells treated with IFN-γ (b to e and g to j) for different time periods.

RESULTS

Time course of tyrosine and serine phosphorylations of Stat1. As a first step in unraveling the biochemical pathway that leads to serine phosphorylation of the STATs, we examined the relative time of induction by IFN-γ of Stat1 tyrosine and serine phosphorylation in NIH 3T3 cells. Before being exposed for 3 h to [^{32}P]orthophosphate, cells were starved overnight in serum-free medium to lower the initial level of serine phosphorylation. The $^{32}PO_4$-labeled cells were then either left untreated or treated with IFN-γ, and samples were examined at various intervals. Cytoplasmic and nuclear extracts were prepared, and Stat1α was immunoprecipitated with an antibody produced against the COOH terminus of Stat1 (anti-Stat1C) and fractionated by SDS-PAGE (Fig. 1A). The radiolabeled Stat1α bands were excised from the gel and digested with trypsin prior to two-dimensional peptide separation by chromatography and electrophoresis. Similar to earlier results (22), two predominant phosphopeptides were observed, one containing phosphotyrosine (pY) and one containing phosphoserine (pS). Tyrosine phosphorylation was clearly detectable in both the cytoplasm and nucleus within 5 min of IFN-γ treatment, indicating a rapid transport of Stat1 dimers to the nucleus. In contrast, induction of serine phosphorylation occurred later, being clearly increased in the cytoplasm (Fig. 1B, panel c) or present in the nucleus only after 20 min of IFN-γ treatment. Note also that in the continuous presence of $^{32}PO_4$ the amount of labeled serine recovered from Stat1α after 20 min reached a higher level in the cytoplasmic fraction than in the nuclear fraction (Fig. 1B; compare panels c to e with panels h to j), suggesting that phosphorylation of serine can definitely occur on non-tyrosine-labeled molecules in the cytoplasm of cells. In addition, the total amount of pS (nucleus plus cytoplasm) was considerably higher than the total amount of pY by 20 min of IFN-γ treatment.

Independence of tyrosine and serine phosphorylations. As another means of assessing the possible interconnection of serine and tyrosine phosphorylations, we determined whether the mutation of the tyrosine at position 701 of Stat1 (the single tyrosine residue that is phosphorylated) to phenylalanine (Y701F mutation) would interfere with the phosphorylation of serine (Fig. 2). U3A cells (12), which lack endogenous Stat1 expression, were stably transfected with either wild-type Stat1α or the Stat1α Y701F mutant. The two cell lines were treated with IFN-γ and subjected to phosphopeptide analysis as described for Fig. 1. IFN-γ was still capable of inducing serine phosphorylation of the Y701F mutant of Stat1 (Fig. 2B). Note that pY was absent in the Y701F cell line (Fig. 2B), confirming that only this single tyrosine is phosphorylated. Therefore, as suggested by the excess of serine compared to tyrosine in the experiment of Fig. 1, tyrosine phosphorylation is not an obligatory step for IFN-γ-induced serine phosphorylation. By studying the S727A mutation, we had earlier concluded that tyrosine phosphorylation, dimerization, and DNA binding do not require phosphorylation of serine 727 (22). Therefore, not only do serine and tyrosine phosphorylations follow distinctly different time courses; they also appear to be independent of each other.

The classical Ras-MAPK pathway is not responsible for IFN-γ-induced serine phosphorylation. Because the sequence surrounding the serine residue, PMSP, matches the mitogen-activated protein kinase (MAPK) consensus sequence (6, 8), and because MAPK could phosphorylate a synthetic Stat1 peptide containing PMSP in vitro (22), it was originally suggested that MAPK could be responsible for phosphorylating the serine residue. We therefore tested whether MAPK was activated by IFN-γ treatment. NIH 3T3 cells were treated with IFN-γ for different lengths of time, and the level of MAPK activation was assessed by both an immunocomplex kinase assay (Fig. 3A) and a tyrosine phosphorylation assay (Fig. 3B). Only a weak and transient activation of MAPK (Erk2) activity was observed in IFN-γ-treated cells, and the low level of activation did not parallel the time course of Stat1 serine phosphorylation in response to IFN-γ treatment.

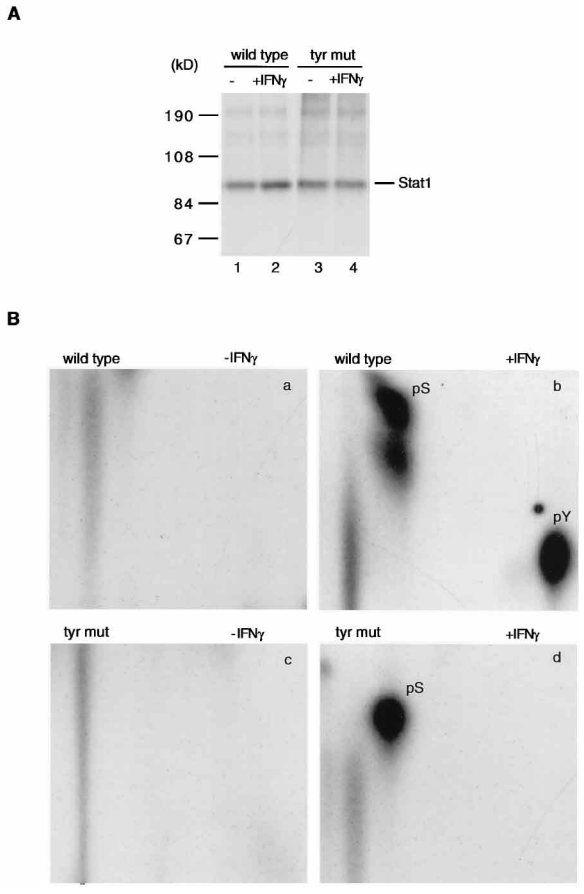

FIG. 2. Tyrosine phosphorylation is not required for serine phosphorylation of Stat1. (A) Immunoprecipitation of ^{32}P-labeled Stat1 from untreated (−) (lanes 1 and 3) or IFN-γ-treated (+IFNγ) (lanes 2 and 4) U3A Stat1α wild-type (lanes 1 and 2) and U3A Stat1α Y701F tyrosine mutant (tyr mut) (lanes 3 and 4) cells. The numbers on the left correspond to the positions of molecular size markers (in kilodaltons). (B) Tryptic phosphopeptide maps of ^{32}P-labeled wild-type Stat1α (a and b) and tyrosine mutant Stat1α Y701F (c and d) from untreated (a and c) and IFN-γ-treated (b and d) cells.

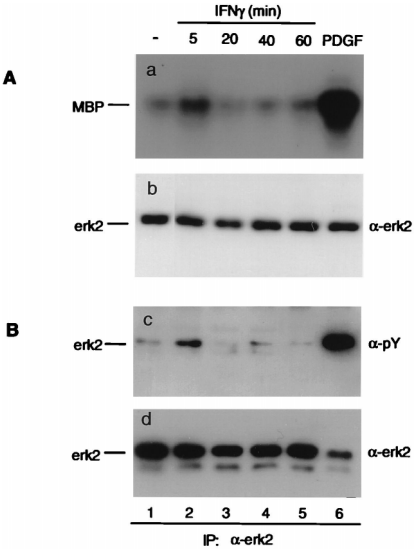

FIG. 3. Erk2 is weakly and transiently activated by IFN-γ treatment. (A) Panel a: MBP kinase assay of immunoprecipitated Erk2 from untreated cells (lane 1), PDGF-treated cells (lane 6), and cells treated with IFN-γ for different durations (lane 2 to 5); panel b: immunostaining of immunoprecipitated Erk2 used for the MBP kinase assay. (B) Panel c: immunostaining with an antiphosphotyrosine antibody (α-pY) of immunoprecipitated Erk2 from untreated cells (lane 1), platelet-derived growth factor (PDGF)-treated cells (lane 6), and cells treated with IFN-γ for different durations (lane 2 to 5); panel d: immunoblotting of immunoprecipitated (IP) Erk2 used for the phosphotyrosine assay.

A second group of experiments aimed at determining the role of MAPK in Stat phosphorylation was carried out based on the well-established role that the G-protein Ras plays in MAPK activation by growth factors (Fig. 4). Both CARas, which permanently activates MAPK, and a dexamethasone-inducible dnRas which prevents MAPK activation are available. Introduction of the dnRas (4) and induction of this protein by dexamethasone in NIH 3T3 cells did not prevent activation of Stat1 by IFN-γ, as evidenced by the labeling of the pY-containing peptide (Fig. 4B, panel d). In contrast, dexamethasone induction of the dnRas did completely suppress MAPK (Erk2) activation. There was a small (approximately twofold) induction of Erk2 by IFN-γ that was totally suppressed by the dnRas (compare samples +dex and −dex in Fig. 4C). Nevertheless, in these cells the IFN-γ-induced serine phosphorylation of Stat1 was unaffected by the presence of the dnRas protein (Fig. 4B, panels b and d).

In separate stable transfection experiments, CARas was introduced into 3T3 cells (7); this markedly elevated the level of active MAPK (Erk2) (Fig. 5C). However, the basal level of serine phosphorylation was not significantly affected by the expression of CARas (Fig. 5B, panels a and c). Moreover, IFN-γ treatment resulted in a similar significant induction of serine phosphorylation of Stat1 in cells with or without CARas (Fig. 5B, compare panels b and d). Taken together, the results of all of these experiments (Fig. 3 to 5) make it very unlikely that the Ras-activated MAPK catalyzes IFN-γ-induced serine phosphorylation.

MAPK belongs to a large family of proline-directed serine kinases which includes JNK and P38 kinase (23). To test whether these kinases might be involved in the IFN-γ-induced serine phosphorylation in 3T3 cells, we measured the activities of these two kinases in both untreated and IFN-γ-treated cells. In no case were we able to observe any induction of these kinase activities by IFN-γ (26). Therefore, most likely the MAPK family is not involved in Stat1 serine phosphorylation. Further, we performed phosphopeptide mapping experiments with cells treated with either wortmannin (1), which inhibits PI3 kinase, or bisindolylmaleimide (18), which specifically blocks a broad spectrum of protein kinase C enzymes. None of these agents inhibited the IFN-γ-induced phosphorylation of the serine at position 727 (26).

Requirement of Jak2 for serine phosphorylation of Stat1. The activity of Jak1 and Jak2 kinases is required for tyrosine phosphorylation of Stat1 following IFN-γ treatment (11, 20). In cells that lack or are mutated in either Jak1 or Jak2, the Stat1 tyrosine is not phosphorylated upon IFN-γ treatment (20). It is likely that a serine kinase cascade is initiated by the activated Jak kinases or is activated by some other kinase that is associated with the ligand-activated IFN-γ receptor chains. To distinguish between these possibilities, we analyzed IFN-γ-induced phosphopeptides of Stat1 in γ2A cells (12), which lack Jak2 activity, and γ2A cells complemented with wild-type Jak2 (Fig. 6). No IFN-γ-dependent induction of Stat1 serine or tyrosine phosphorylation was observed in γ2A cells (Fig. 6B, panels c and d), whereas the Jak2-complemented cells (wild type [Fig. 6B, panels a and b]) exhibited substantial IFN-γ-dependent increases in both serine and tyrosine phosphoryla-

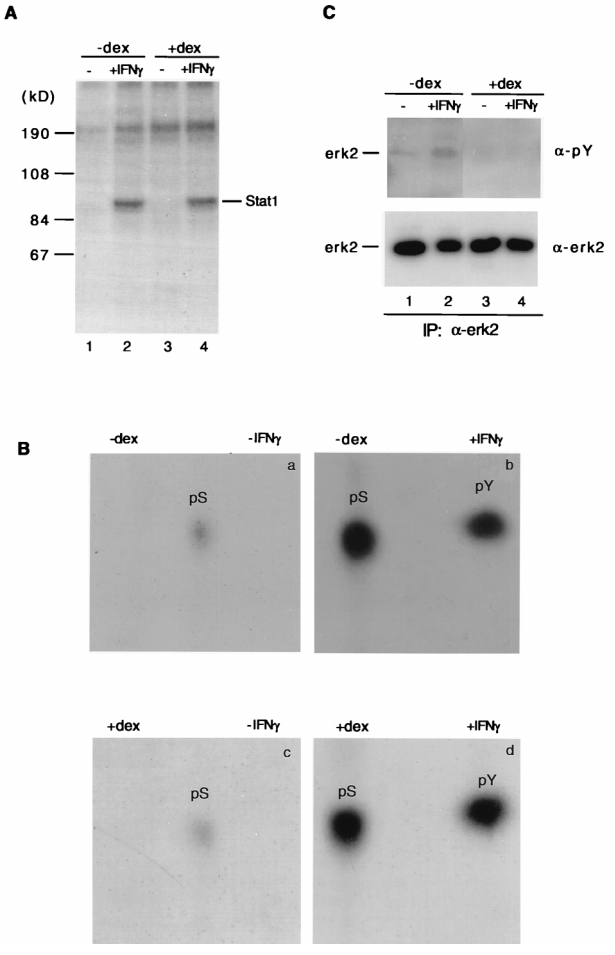

FIG. 4. dnRas does not interfere with IFN-γ-induced Stat1 serine phosphorylation. (A) Immunoprecipitation of ^{32}P-labeled Stat1 from untreated (−) (lanes 1 and 3) and IFN-γ-treated (+IFNγ) (lanes 2 and 4) dnRas NIH 3T3 cells grown in the absence (−dex) (lanes 1 and 2) or the presence (+dex) (lanes 3 and 4) of dexamethasone. The numbers on the left correspond to the positions of molecular size markers (in kilodaltons). (B) Tryptic phosphopeptide maps of ^{32}P-labeled Stat1 of untreated (a and c) and IFN-γ-treated (b and d) cells grown in the presence (c and d) or absence (b) of dexamethasone. (C) Tyrosine phosphorylation of immunoprecipitated (IP) Erk2 from untreated (lanes 1 and 3) and IFN-γ-treated (lanes 2 and 4) dnRas NIH 3T3 cells grown in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of dexamethasone. The top panel is blotted with antiphosphotyrosine antibody (4G10), and the bottom panel is blotted with anti-Erk2 antibody.

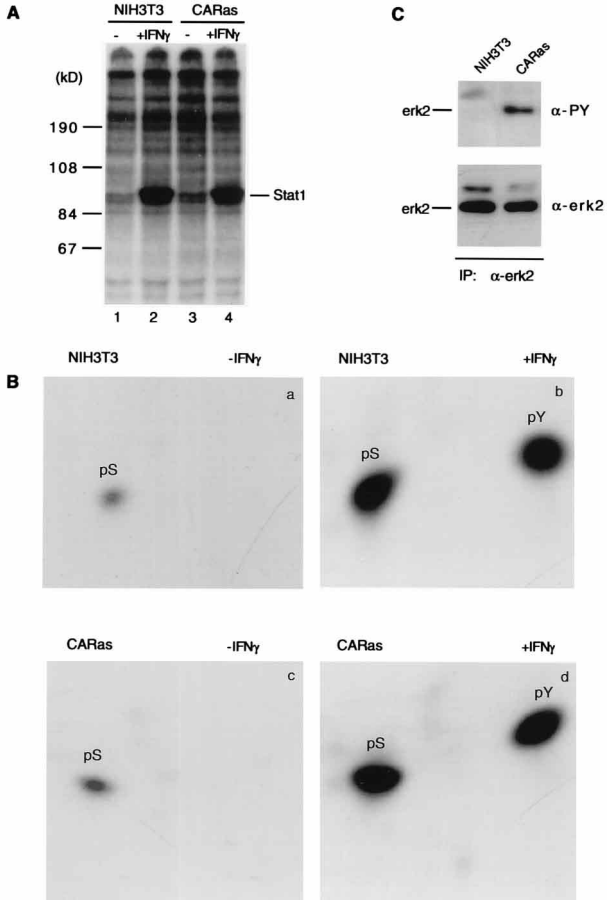

FIG. 5. CARas does not cause an increased level of Stat1 serine phosphorylation. (A) Immunoprecipitation of ^{32}P-labeled Stat1 from untreated (−) (lanes 1 and 3) and IFN-γ-treated (+IFNγ) (lanes 2 and 4) NIH 3T3 cells (lanes 1 and 2) or CARas-transformed 3T3 cells (lanes 3 and 4). Numbers on the left correspond to the positions of molecular size markers (in kilodaltons). (B) Tryptic phosphopeptide maps of ^{32}P-labeled Stat1 of untreated (a and c) and IFN-γ-treated (b and d) NIH 3T3 cells (a and b) or CARas-transformed 3T3 cells (c and d). (C) Tyrosine phosphorylation of immunoprecipitated (IP) Erk2 from NIH 3T3 cells or 3T3 cells transformed with CARas.

tion. Therefore, a functional Jak2 kinase is required for IFN-γ-induced serine phosphorylation of Stat1. Since Jak2 kinase has only been demonstrated to exhibit tyrosine kinase activity (15), it is most likely not the direct kinase that phosphorylates serine 727 of Stat1. We also attempted to assess the role of Jak1 in induced serine phosphorylation, but several independent Jak1$^-$ cell lines had high levels of constitutive Stat1 serine phosphorylation, and we were unable to test for an IFN-γ-induced event.

Detection of a serine kinase that phosphorylates Stat1. Since we had not uncovered a known serine kinase as the enzyme likely to be responsible for phosphorylation of Stat1, we attempted, by an in vitro kinase assay, to identify a serine kinase activity that does phosphorylate Stat1. After trying several different substrates, we found that a full-length Stat1 protein expressed in and purified from insect cells produced the most reliable results. Moreover, it is the full-length non-tyrosine-phosphorylated Stat1 that seems to be the correct in vivo substrate for IFN-induced serine phosphorylation. Since the majority of the IFN-γ-induced phosphoserine in Stat1 was observed in the cytoplasm, we searched for kinase activity there. Crude cytoplasmic extracts from IFN-γ-treated 3T3 cells produced no specific phosphorylation on the 91-kDa Stat protein substrate but yielded a high background with many different protein species (data not shown). Separation of the cytoplasmic extract on an anion-exchange column (Fig. 7A) and testing of eluted fractions as sources of enzyme for phosphorylation of Stat1 did reveal a serine kinase activity for the added Stat1α. This result was observable even without antibody precipitation of the Stat1 substrate (Fig. 7A). However, the IFN-γ-treated and untreated cell kinase activities separated by this column assay were identical (Fig. 7A, compare panels a and b). When in vitro $^{32}PO_4$-labeled Stat1 phosphoprotein was hydrolyzed to amino acids and examined, only labeled pS was observed (Fig. 7B). To determine whether the same kinase activity phosphorylated serine 727 of Stat1, the in vitro-phosphorylated Stat1 was excised from an SDS-PAGE gel, digested with trypsin, and separated by two-dimensional chromatography and electrophoresis. In this experiment, several

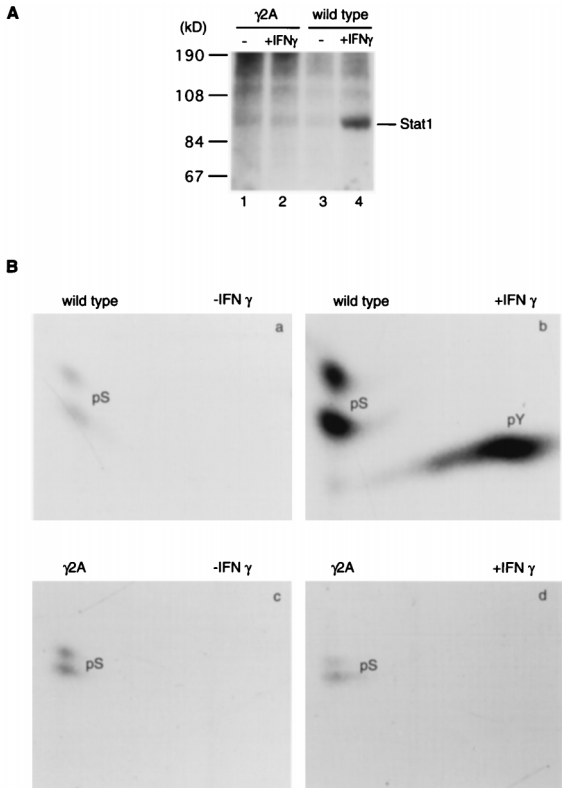

FIG. 6. Jak2 kinase is required for serine phosphorylation of Stat1. (A) Immunoprecipitation of ^{32}P-labeled Stat1 from untreated (−) (lanes 1 and 3) and IFN-γ-treated (+IFNγ) (lanes 2 and 4) cells. The cells used were γ2A cells (Jak2 mutant) (lanes 1 and 2) and Jak2-complemented γ2A cells (lanes 3 and 4). The numbers on the left correspond to the positions of molecular size markers (in kilodaltons). (B) Tryptic phosphopeptide maps of ^{32}P-labeled Stat1 from untreated (a and c) and IFN-γ-treated (b and d) γ2A cells (c and d) cells or Jak2-complemented γ2A cells (a and b).

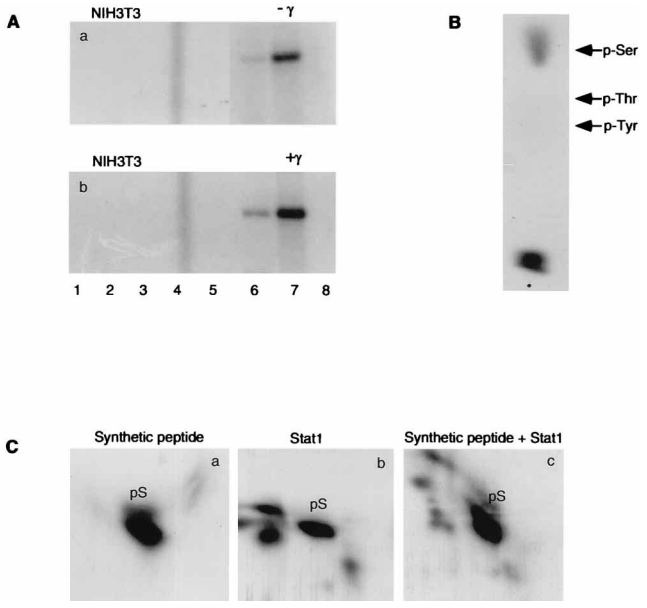

FIG. 7. Identification in NIH 3T3 cells of a kinase activity that phosphorylates Stat1 on serine 727. (A) In vitro kinase assay of Stat1 phosphorylation with fractions (1 to 8) purified from untreated (−γ) (a) or IFN-γ-treated (+γ) (b) NIH 3T3 cells. The Stat1 kinase activity is eluted at a concentration of approximately 0.1 M NaCl (fractions 6 and 7). (B) Phosphoamino acid analysis of the major phosphopeptide derived from the Stat1 protein phosphorylated in vitro by the active fraction. p-Ser, phosphoserine; p-Thr, phosphothreonine; p-Tyr, phosphotyrosine. (C) The tryptic phosphopeptide map of in vitro-phosphorylated Stat1 (b) was compared with that of an in vitro-labeled synthetic phosphopeptide (a). The synthetic peptide, VHPSRLQTTDNLLPMSPEEFDEVSRIVGS, was ^{32}P labeled by MAPK in vitro, digested with trypsin, and analyzed by two-dimensional peptide mapping either alone (a) or in a mixture with the synthetic peptide (c).

phosphopeptides were observed in the digested kinase product from in vivo-labeled protein, but one prominent phosphopeptide accounted for about half of the incorporated ^{32}P: the serine 727 peptide. To confirm that the synthetic phosphopeptide was identical to the natural phosphopeptide that encompasses serine 727, we mixed the phosphopeptides from trypsin-digested, in vitro-phosphorylated Stat1 with a phosphorylated synthetic peptide that covered amino acids 719 to 740 in Stat1 and was also trypsin treated. The major activities in the two samples comigrated, indicating the identity of the most prominent peptide (Fig. 7C, panel c). Therefore, Stat1 is phosphorylated on the correct serine residue by the serine kinase(s) in the fraction separated by the anion-exchange column. It may be that some other serines are also phosphorylated by the partially purified enzyme preparation, but the major peptide appears to contain serine 727.

DISCUSSION

The first conclusion that stems from the present study is that tyrosine and serine phosphorylations in Stat1 are induced independently, and the site of phosphorylation for both serine and tyrosine is most likely the cytoplasm. The phosphorylation of tyrosine in advance of serine, and the ability of the Y701F mutant to be inducibly phosphorylated on serine 727 and the S727A mutant to be inducibly phosphorylated on Y707 (22), argue for independent inducible enzymatic events. Also, the serine phosphorylation of the Y701F mutant, which does not dimerize and enter the nucleus, shows that cytoplasmic serine phosphorylation can occur. The fact that there is greater total serine phosphorylation than tyrosine phosphorylation also argues for cytoplasmic serine phosphorylation of monomeric Stat1. Tyrosine phosphorylation almost certainly occurs while the STAT molecule is associated by its SH2 domain with a receptor docking site (9); serine phosphorylation clearly could occur while the protein is free in the cytoplasm. Most STAT molecules in cell extracts behave as single 91-kDa molecules (17), but it is still possible that a subset is bound to a specific site, perhaps even the receptor itself. Binding of Stat2 to the IFN-α receptor has been described previously (10). Finally, however, since it is clear that [^{32}P]pY-labeled Stat1 appears in the nucleus before serine 727 phosphorylation occurs, it is not possible to rule out the occurrence of serine phosphorylation in the nucleus for those molecules that enter the nucleus without prior serine phosphorylation.

The nature of the serine kinase responsible for Stat1 phosphorylation remains unknown. While the phosphorylation event itself is clearly induced by IFN-γ, there was no evidence that either a classical MAPK (Erk1 or -2) or either of two related kinases, JNK or p38, was induced. dnRas protein, which obliterated all evidence of MAPK, failed to affect IFN-γ-induced Stat1 serine phosphorylation. Also, CARas protein, which results in constitutively active MAPK, did not result in substantial Stat1 serine phosphorylation compared to IFN-γ induction of serine phosphorylation. This, of course, does not prove that a similar kinase is not the active kinase for STAT serine phosphorylation. Agents that block PI3 kinase and pro-

tein kinase also failed to affect IFN-γ-induced serine phosphorylation. These unsuccessful attempts to implicate known kinases led us to examine cell extracts for an enzyme that would catalyze the correct phosphorylation of Stat1. Such an activity was identified in partially purified cytoplasmic extracts, but no heightened activity of this kinase in IFN-γ-treated cells was observed. Nevertheless, it is possible that this enzyme is involved in IFN-γ-induced serine phosphorylation, and this possibility deserves further study. For example, no activity that efficiently phosphorylated Stat1 on serine was evidenced in crude extracts from IFN-γ-treated or untreated cells, but the background of serine phosphorylation was extremely high, as might be expected. However, after column chromatography, an activity with the ability to phosphorylate serine 727 was uncovered. It is clearly possible that the conditions of isolation unmasked a serine kinase that was inactive in vivo in untreated cells but which had already been activated in treated cells. Since this enzyme has the correct substrate specificity (for serine 727 only or mainly), it should be purified and examined with the aim of understanding specific kinase function. An additional reason for continuing to search for a possibly new and specific kinase that activates Stat1 is the knowledge that Stat3 (and perhaps other STATS) is also phosphorylated on a single serine (also residue 727) (21, 22), and reports on gel mobility differences consistent with serine phosphorylation also exist for Stat5A, Stat5B, and Stat4 (2, 5). It is clearly important that we learn whether all of these proteins are phosphorylated inducibly by the same enzyme (or family of enzymes).

Finally, while the pathway by which the serine kinase(s) responsible for STAT serine phosphorylation is activated is not clear, these experiments do establish that Jak2 is required for IFN-γ induction of serine phosphorylation. The role of the Jak kinases associated with cytokine receptors appears to be the provision of an active tyrosine kinase after transactivation of the two receptor-associated Jak molecules. These activated kinases then phosphorylate receptor tyrosine residues, which can attach adapter molecules just as has been studied in great detail for receptor tyrosine kinases. Of course, the best-studied instances of such pathways involve Ras and the MAPKs, which apparently are not used in the case of IFN-γ-induced serine phosphorylation. However, parallel pathways involving other kinases are obvious candidates and provide another attractive experimental goal.

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