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Maximal Activation of Transcription by Stat1 and Stat3 Requires Both Tyrosine and Serine Phosphorylation

Zilong Wen, Zhong Zhong, and James E. Darnell, Jr.
Laboratory of Molecular Cell Biology
The Rockefeller University
New York, New York 10021-6399

Summary

Stat1 and Stat3 are latent transcriptional factors activated initially through phosphorylation on single tyrosine residues induced by cytokine and growth factor occupation of cell surface receptors. Here we show that phosphorylation on a single serine (residue 727) in each protein is also required for maximal transcriptional activity. Both cytokines and growth factors are capable of inducing the serine phosphorylation of Stat1 and Stat3. These experiments show that gene activation by Stat1 and Stat3, which obligatorily require tyrosine phosphorylation to become active, also depends for maximal activation on one or more of the many serine kinases.

Introduction

The STAT proteins (signal transducers and activators of transcription) are latent transcription factors that become activated by phosphorylation on tyrosine in response to polypeptide–receptor interaction at the cell surface (Darnell et al., 1994). The activated STATs dimerize, translocate to the cell nucleus, and bind specific DNA elements. The STAT proteins were originally recognized in studies of interferon α (IFN-α) and IFN-γ transcriptional activation: Stat1 and Stat2 are phosphorylated in response to IFN-α (Schindler et al., 1992b), they heterodimerize (Schindler et al., 1992b; Qureshi et al., 1995), and together with a 48 kDa protein that is not phosphorylated (Fu et al., 1990; Veals et al., 1992), they bind to the IFN-α-specific DNA element, the IFN-stimulated response element (Levy et al., 1988). Stat1, but not Stat2, is activated by IFN-γ (Shuai et al., 1992), homodimerizes (Shuai et al., 1994), translocates to the nucleus, and binds to a different DNA element, the IFN-γ-activated site (GAS) (Decker et al., 1991; Lew et al., 1991). Stat3 (Zhong et al., 1994a, 1994b), which proved identical to the acute phase response factor (Akira et al., 1994), was the first of a series of additional STAT family members to be described and is activated by ligands that bind to receptor-tyrosine kinases such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and colony-stimulating factor 1, as well as cytokine receptors such as interleukin-6 (IL-6), which lack intrinsic tyrosine kinases but have janus kinases associated with their internal domains (Akira et al., 1994; Schindler and Darnell, 1995). Stat3 is also activated by IFN-α (Beadling et al., 1993; Boulton et al., 1995), but not by IFN-γ (Beadling et al., 1993; Akira et al., 1994; Zhong et al., 1994b). Stat1 can be activated also by EGF and PDGF, but is not activated as strongly as Stat3 by cytokines such as IL-6.

Cell lines (U3A cells) that completely lack Stat1α and Stat1β, which is missing the COOH-terminal 38 amino acids of Stat1α, are defective in response to either IFN-α or IFN-γ (Muller et al., 1993). Cells that lack Stat2 are deficient for the IFN-α response, but still respond to IFN-γ (Leung et al., 1995). In U3A cells, Stat1α or Stat1β suffices to restore the IFN-α pathway for most genes (Muller et al., 1993). However, Stat1α can restore the IFN-γ pathway, but Stat1β cannot (Muller et al., 1993), despite the fact that Stat1β is phosphorylated on tyrosine, dimerizes, enters the nucleus, and can bind DNA (Muller et al., 1993; Shuai et al., 1993a). Since the only difference between Stat1α and Stat1β is the lack of the COOH-terminal 38 amino acids in Stat1β, this focused our attention on these residues in IFN-γ-dependent transcriptional activation.

Furthermore, we had earlier encountered some parallels and some differences in drug sensitivity in the IFN-α and IFN-γ transcriptional pathways (Lew et al., 1989), which remained unexplained. Both pathways are inhibited by genistein or staurosporine, which are primarily inhibitors of tyrosine phosphorylation, in line with the obligatory requirement for tyrosine phosphorylation for STAT dimer formation and DNA binding (Schindler et al., 1992b; Shuai et al., 1993a). However, H7, which is also a serine/threonine kinase inhibitor, blocked IFN-γ-induced transcription, but had very much less effect on IFN-α-induced transcription (Lew et al., 1989). In addition, ^{32}P is incorporated into phosphoserine in Stat1α to a much greater extent than in Stat1β in response to IFN-γ (Shuai et al., 1992, 1993a). All of these results suggested that perhaps Stat1α contained a critical serine in the 38 terminal amino acids that served in gene activation. The present experiments demonstrate that Ser-727, which is lacking in Stat1β, is in fact phosphorylated, possibly through the mitogen-activated protein (MAP) kinase pathway. Furthermore, the phosphorylation of Ser-727 is induced by IFN-γ and PDGF treatments. Finally, Stat1 protein that is mutant in Ser-727 (Ser-727→Ala-727) is phosphorylated normally on tyrosine, dimerizes, and binds DNA, but in cells bearing the mutant protein, only about 20% as much IFN-γ-dependent transcription occurs. Evidence is also given that Stat3 is phosphorylated on an analogous serine residue and that full transcriptional activation by Stat3 also requires serine phosphorylation.

Results

Sequence Alignment of STATs Reveals Conserved PMSP Box

Amino acid sequence comparison of the known STATs shows conserved regions scattered throughout nearly the entire length of the proteins (Fu et al., 1992; Schindler et al., 1992a; Akira et al., 1994; Hou et al., 1994; Wakao et al., 1994; Yamamoto et al., 1994; Zhong et al., 1994a, 1994b). However, the COOH-terminal region of the STATs (from residue ~710 to the end) is quite diverse (Figure 1), except for a highly conserved sequence PMSP in Stat1α,

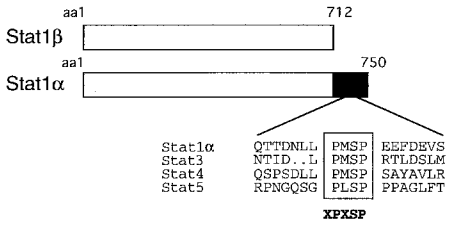

Figure 1. Alignment of Amino Acid Sequences at COOH-Terminal of STAT Proteins

Stat1β lacks the COOH-terminal 38 amino acids of Stat1α, and within this region the amino acid sequence PMSP (boxed) is highly conserved in Stat1α, Stat3, Stat4, and Stat5. Amino acids residues are shown with standard single-letter abbreviations.

Stat3, Stat4, and Stat5 (PLSP). This conserved sequence is missing from the Stat1β spliced variant encoded by the Stat1 gene (Schindler et al., 1992a; Yan et al., 1995) and is also missing in Stat2 (Fu et al., 1992) and Stat6 (Hou et al., 1994), both of which are about 100 amino acids longer than Stat1, Stat3, Stat4, and Stat5. The PMSP sequence is similar to the MAP kinase recognition consensus sites (Clark-Lewis et al., 1991; Gonzalez et al., 1991). We had earlier observed in mapping the ^{32}P-labeled tyrosine phosphopeptides induced by IFN-α in Stat1 and Stat2 that Stat2 contained very little if any labeled phosphoserine, and as mentioned above Stat1α did contain phosphoserine that was lacking in Stat1β after IFN-γ treatment.

Ser-727 of Stat1α Is the Major Serine Kinase Target In Vivo

Therefore, we first determined directly whether the Ser-727 residue of Stat1 could be phosphorylated in vivo. U3A cells (Muller et al., 1993) that lack Stat1 protein were permanently transfected with expression vectors for wild-type Stat1α or mutant Stat1αs (Ser-727→Ala-727). Individual clones of cells expressing Stat1α or Stat1αs to comparable levels were used in comparisons in the remainder of this work. Cells expressing either wild-type Stat1α or Stat1αs were exposed to [^{32}P]orthophosphate for 2.5 hr and treated with IFN-γ for 20 min. (As a control, the wild-type cells were also labeled without IFN-γ treatment.) Protein extracts were prepared from both nucleus and cytoplasm, exposed to anti-Stat1C, an antiserum against the COOH-terminus of Stat1, and the 91 kDa ^{32}P-labeled band was selected after SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 2A). The labeled Stat1 samples were digested with trypsin, applied to thin-layer cellulose plates, and separated by a two-dimensional analysis (Figure 2B; Walaas and Nairn, 1989). Autoradiographs of the separated samples revealed an IFN-γ-induced phosphopeptide (labeled 1 in Figure 2Bb) that migrated similarly to the earlier described phosphotyrosine-containing peptide, GIYTEK (Shuai et al., 1992, 1993a). This phosphopeptide was not present in the sample from cells expressing wild-type protein that were not treated with IFN-γ (Figures 2Ba and 2Bd). A second prominently labeled peptide (labeled 2 in Figure 2B; actually a double spot in some samples, possibly owing to either incomplete trypsin digestion or perhaps oxidation of the methionine residue in the peptide) contained phosphoserine (Figures 2Ba, 2Bb, 2Bd, and 2Be; Figures 2C and 2D). This phosphoserine-containing peptide was present in either nuclear or cytoplasmic samples of IFN-γ-treated or untreated U3A-derived cells containing the wild-type protein, but was absent from cells containing the mutant protein Stat1αs (Figures 2Bc and 2Bf). Thus, a single serine to alanine mutation at residue 727 apparently removed from Stat1 the major target site for serine phosphorylation. Note that in these samples of U3A-derived cells (Figure 2) the serine phosphorylation occurred whether or not the cells were treated with IFN-γ. There was somewhat more phosphoserine than phosphotyrosine, suggesting that more Stat1α molecules were phosphorylated on serine than on tyrosine, since there is apparently a single residue of each amino acid phosphorylated, at least in these Stat1α-complemented U3A cells.

The site of in vivo serine phosphorylation was confirmed as residue 727 by synthesizing a 29 residue long peptide matching the human Stat1α sequence from residue 712 to 740 (Figure 2D). This peptide was treated with purified Xenopus MAP kinase in the presence of [γ-^{32}P]ATP. The resulting labeled peptide was subjected to two-dimensional separation and eluted from the thin-layer chromatography (TLC) plate. The purified ^{32}P-labeled peptide was then digested with trypsin, and the synthetic and authentic [^{32}P]phosphoserine–labeled tryptic peptides were compared by two-dimensional analysis (Figure 2D). The two labeled peptides migrated very similarly (each sample was analyzed in a different chromatography tank, leading to the slight differences in migration) and when mixed yielded a single serine-containing spot (Figure 2Dc), the conventional method of demonstrating phosphopeptide identity (Walaas and Nairn, 1989). The experiment also established that the Stat1 peptide was a substrate for the MAP kinase, which was suspected to be possible because the sequence of the potential phosphorylation site, PMSP, matched the known MAP kinase recognition site (Clark-Lewis et al., 1991; Gonzalez et al., 1991). This, of course, does not prove the nature of the responsible kinase inside cells.

Induction of Serine Phosphorylation

We sought to determine a possible inductive signal for the serine phosphorylation, first by depriving Stat1α-complemented U3A cells of serum and testing whether without IFN-γ treatment such cells lacked Stat1α Ser-727 phosphorylation. Figure 3 shows that serum starvation (for 14 hr) did not block the labeling of the serine-containing phosphopeptide, which was labeled about half as well as in cells treated with IFN-γ (compare Figures 3Ba and 3Bb). We then became aware that HT1080 cells, the parental cell line of U3A cells, have a well-documented, mutant constitutively activated N-*ras* allele that might cause Ser-727 to be permanently phosphorylated (Hall et al., 1983; Brown et al., 1984).

We therefore shifted to NIH 3T3 cells, diploid mouse fibroblasts with normal Ras protein(s). These cells were deprived of serum for 14 hr, and samples were then la-

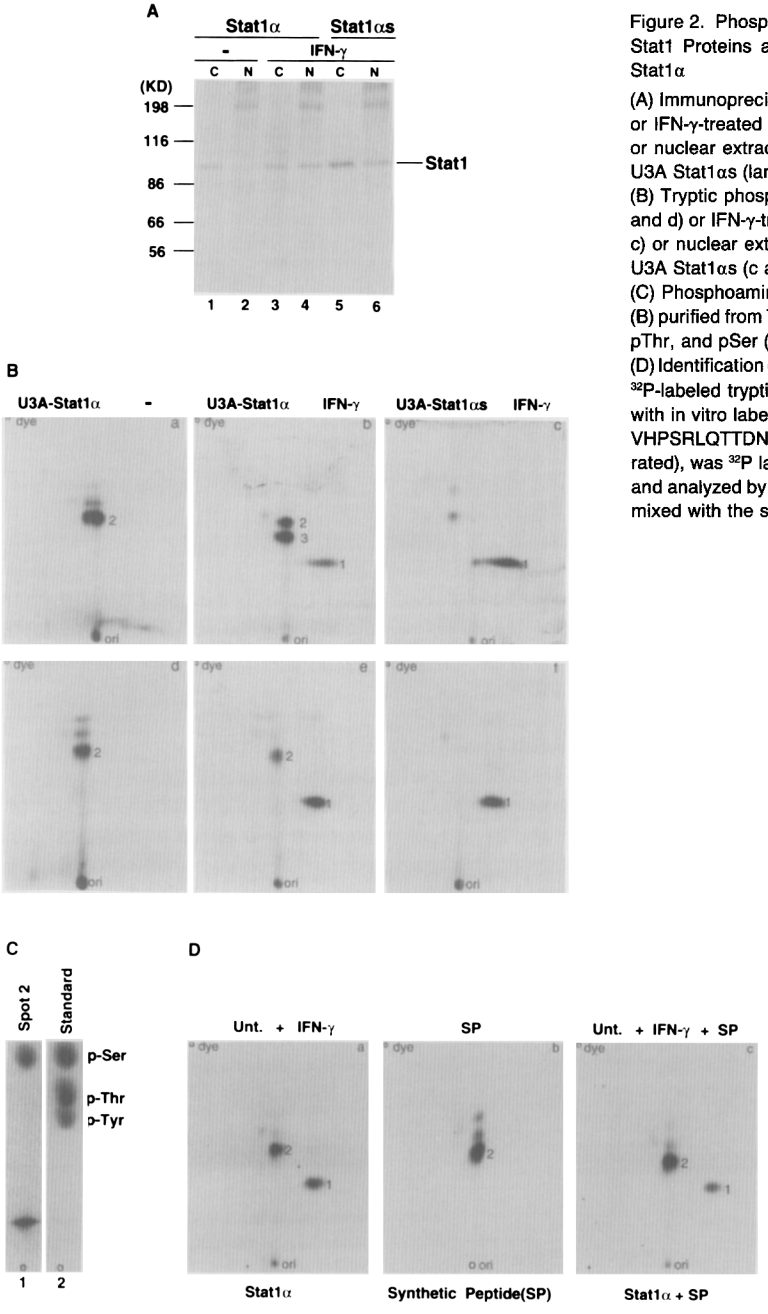

Figure 2. Phosphopeptide Mapping of Trypsin Digests of ^{32}P-Labeled Stat1 Proteins and Identification of the Phosphoserine Residue in Stat1α

(A) Immunoprecipitation of ^{32}P-labeled Stat1 of untreated (lanes 1–2) or IFN-γ-treated (lanes 3–6) cytoplasmic extracts (lanes 1, 3, and 5) or nuclear extracts (lanes 2, 4, and 6) of U3A Stat1α (lanes 1–4) or U3A Stat1αs (lanes 5 and 6) cells.

(B) Tryptic phosphopeptide map of ^{32}P-labeled Stat1 of untreated (a and d) or IFN-γ-treated (b, c, e, and f) cytoplasmic extracts (a, b, and c) or nuclear extracts (d, e, and f) of U3A Stat1α (a, b, d, and e) or U3A Stat1αs (c and f).

(C) Phosphoamino acid analysis of the spot 2 phosphopeptide from (B) purified from TLC plates (left) compared with the migration of pTyr, pThr, and pSer (right).

(D) Identification of the phosphoserine residue 727 in Stat1α. Authentic ^{32}P-labeled tryptic phosphopeptides (a) from Stat1α were compared with in vitro labeled synthetic phosphopeptide (b); synthetic peptide, VHPSRLQTTDNLLPMSPEEFDEVSRIVGS (Bio-Sythesis, Incorporated), was ^{32}P labeled by MAP kinase in vitro, digested with trypsin, and analyzed by two-dimensional peptide mapping either alone (b) or mixed with the synthetic peptide (c).

beled with [^{32}P]O_4 for 2.5 hr and treated for 20 min with IFN-γ or PDGF or left untreated. A clear induction by IFN-γ was seen in both phosphopeptide 1, the tyrosine-containing peptide, and in phosphopeptide 2, the Ser-727-containing peptide. After treatment with PDGF, a similar smaller degree of induction in tyrosine phosphorylation and a robust induction of serine phosphorylation were also observed (Figures 3Bd and 3Be). Two additional phosphopeptides that gave weaker signals were detected in the phosphoproteins precipitated by anti-Stat1α from the PDGF-induced cells. Since PDGF also activates Stat3 in these cells and Stat1–Stat3 heterodimers form (Zhong et al., 1994b), these two phosphopeptides could be due to phosphorylation of Stat3 or other coprecipitating phosphorylated proteins.

Tyrosine Phosphorylation and DNA Binding of Stat1αs

We next tested the functional importance of phosphorylation of Stat1 on Ser-727 using the mutant construct in which alanine was substituted for serine at residue 727. We first examined the phosphorylation of tyrosine and the ability of the Stat1αs mutant to bind DNA compared with that of wild-type protein. Cells expressing either wild-type Stat1α or Stat1αs protein were treated with IFN-γ for 20 min, and protein extracts were prepared. Electrophoretic

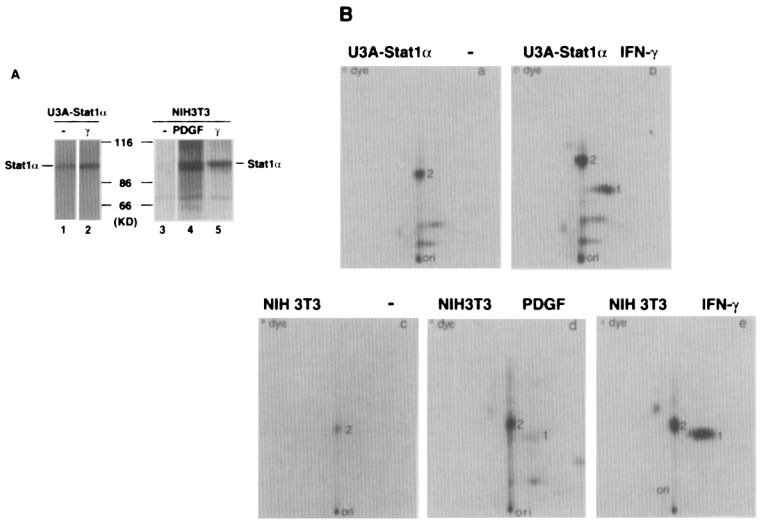

Figure 3. Inducible and Constitutive Phosphorylation of Ser-727 in Stat1α in Two Cell Lines

(A) Immunoprecipitation of ^{32}P-labeled Stat1α of untreated (lanes 1 and 3) or IFN-γ-treated (lanes 2 and 5) and PDGF-treated (lane 4) serum-starved U3A cells bearing Stat1α (lanes 1 and 2) or NIH 3T3 cells (lanes 3, 4, and 5).

(B) Tryptic phosphopeptide map of Stat1α in serum-starved U3A Stat1α cells (a and b) and NIH 3T3 cells (c, d, and e) treated with IFN-γ (b and e) or PDGF (d) for 20 min or left untreated (a and c).

mobility shift assays (EMSAs) (Fried and Crothers, 1981) with nuclear extracts of cells treated for 20 min with IFN-γ showed equal induction of DNA-binding activity using the ^{32}P-labeled interferon-regulatory factor 1 (IRF-1) GAS oligonucleotide (Sims et al., 1993) as a probe (Figure 4A). In fact, both wild-type and mutant proteins bound equally, but to varying extents (data not shown) using three different labeled DNA probes: binding with M67 SIE was greater than with IRF-1 GAS, which was greater than with Ly6E GAS (Wagner et al., 1990; Khan et al., 1993; Sims et al., 1993). The gel shift bands were specific because the Stat1C antiserum produced a supershift, while the preimmune serum had no effect (Figure 4A, lanes 3, 4, 7, and 8). In addition, both wild-type and mutant proteins were equally phosphorylated on tyrosine, as tested by anti-phosphotyrosine antibody reaction with Stat1 immunoprecipitates separated on polyacrylamide gels (Figures 4B and 4C).

Requirement for Ser-727 in Stat1α Transcriptional Induction

Having demonstrated that serine phosphorylation of residue 727 in Stat1 occurs in vivo and that mutant protein lacking this residue could be phosphorylated on tyrosine and bind DNA, we tested for the effect on IFN-γ-induced transcription. Two different experiments indicated that the Ser-727 was required for maximal IFN-γ-induced transcription. First, U3A cells were transfected with either wild-

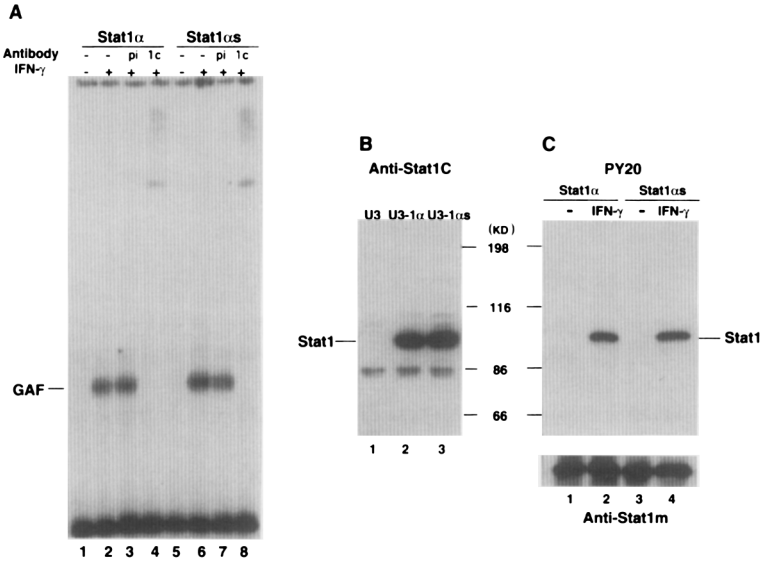

Figure 4. DNA Binding and Tyrosine Phosphorylation of Ser-727→Ala-727 Mutant of Stat1α in U3A-Complemented Cells

(A) Gel mobility shift analysis was performed with ^{32}P-labeled IRF-1 GAS probe. Nuclear extracts were prepared from U3A Stat1α cells (lanes 1–4) and U3A Stat1αs (the Ser-727→Ala-727 mutant, lanes 5–8) cells untreated (lanes 1 and 5) or treated with IFN-γ (lanes 2–4 and 6–8). Preimmune serum (pi, lanes 3 and 7) or anti-Stat1C serum (1C, lanes 4 and 8) was added in the reaction.

(B) Western blot analysis. Whole-cell lysate from U3A cells (lane 1), U3A cells complemented with wild-type Stat1α (lane 2), and U3A cells complemented with mutant Stat1αs (lane 3) was subjected to SDS–PAGE and blotted with anti-Stat1 COOH-terminal serum (anti-Stat1C).

(C) Protein immunoblot of anti-Stat1C precipitates with anti-phosphotyrosine antibody. Whole-cell lysate from U3A Stat1α cells (lanes 1 and 2) or U3A Stat1αs cells (lanes 3 and 4) either treated with IFN-γ (lanes 2 and 4) or untreated (lanes 1 and 3) were precipitated with anti-Stat1C serum and probed with anti-phosphotyrosine antibody (top). The blot was then stripped and reprobed with anti-Stat1 middle serum (anti-Stat1m, bottom).

type Stat1α or the mutant Stat1αs plus a reporter gene (luciferase) construct with three GAS sites from the promoter of the IFN-γ-inducible Ly6E gene (Khan et al., 1993; Shuai et al., 1993a). After 16 hr of transfection, the cells were either treated with IFN-γ or left untreated, and extracts were assayed for luciferase activity 6 hr later. As a control, a transfection of Stat1β was also carried out. Stat1β lacks the terminal 38 amino acids of Stat1α, including the Ser-727 residue, and is known not to drive IFN-γ-induced transcription (Muller et al., 1993; Shuai et al., 1993a). Stat1β, however, is phosphorylated on tyrosine, dimerizes, enters the cell nucleus in vivo, and can bind DNA in in vitro tests (Shuai et al., 1993a). The wild-type Stat1α produced a 25- to 30-fold higher luciferase signal after IFN-γ induction, whereas transfection of the Stat1β gave almost no increased signal from the reporter construct. Stat1αs gave about a 4- to 5-fold increase, consistent with the conclusion that a large fraction but not all of the IFN-γ transcriptional response required not only the phosphotyrosine (Shuai et al., 1993a), but phosphoserine on residue 727 (Figure 5A).

A second experiment tested the response of an endogenous gene that is transcriptionally induced by IFN-γ treatment. Permanently tranfected U3A-derived cell lines containing wild-type Stat1α or mutant Stat1αs were treated with IFN-γ for 3 hr, and poly(A)$^+$ RNA was extracted and subjected to Northern blot analysis for mRNA from the IRF-1 gene (Maruyama et al., 1989), a gene that is transcriptionally induced by IFN-γ (Pine et al., 1990; Pine et al., 1994). There was about a 12-fold increase in IRF-1 mRNA in cells containing wild-type Stat1α, whereas cells with Stat1αs were induced about 3-fold (Figures 5B and 5C), consistent with the transfectional analysis in Figure 5A.

Stat3 Also Requires Serine Phosphorylation for Maximal Transcriptional Activation

Stat3 is highly similar to Stat1 in amino acid sequence and is activated by an array of ligands, some of which also activate Stat1 and some of which do not or do so weakly. We wished to test whether Stat3 would also require serine phosphorylation to activate transcription. Although Stat3 is 770 amino acids long, 20 amino acids longer than Stat1α, the PMSP box is conserved in Stat3 in an analogous position in the COOH-terminus of the protein (see Figure 1). Furthermore, we knew from the experiments of Boulton et al. (1995) and the recent report of Zhang et al. (1995) that a ligand- and time-dependent retardation of Stat3 in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) had been observed after treatment with cells by several ligands. Moreover, in these experiments the serine kinase inhibitor H7 blocked the change to slower mobility, which indicated that the mobility shift might be due to serine phosphorylation, since both the slow and fast forms were tyrosine phosphorylated. We therefore mutated Stat3 at Ser-727 and introduced it into COS (monkey) cells, which have a low amount of endogenous Stat3. We had earlier shown using these cells that the overexpressed Stat3 in COS cells can be activated by EGF (Zhong et al., 1994b). Stat3 was precipitated from EGF-treated COS

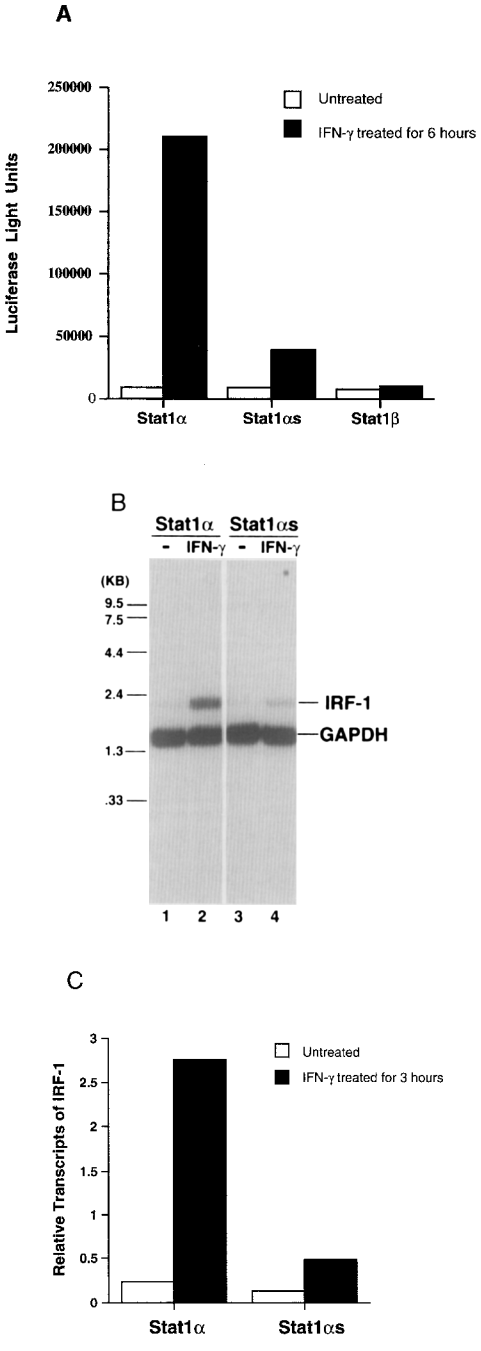

Figure 5. Transcriptional Activation Analysis of Stat1αs

(A) Luciferase assay. Transfection into U3A cells of a reporter gene (luciferase) plus either Stat1α, Stat1αs, or Stat1β expression constructs for 16 hr was followed by a 6 hr IFN-γ treatment; extracts were prepared, and luciferase assays were carried out. Luciferase light units are plotted on the Y axis (open bars, untreated cells; closed bars, IFN-γ-treated cells).

(B) Stimulation of IRF-1 mRNA accumulation by IFN-γ in U3A Stat1α and U3A Stat1αs cells. Northern blot analysis of IRF-1 transcript. Poly(A)$^+$ RNA were collected from U3A Stat1α cells (lanes 1 and 2) or U3A Stat1αs cells (lanes 3 and 4) untreated (lanes 1 and 3) or treated with IFN-γ for 3 hr (lanes 2 and 4). The blot was probed with IRF-1 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA.

(C) Quantitation of IRF-1 transcripts in (B) by PhosphorImager normalized to GAPDH. Open bars, untreated samples; closed bars, IFN-γ-treated samples (7 ng/ml for 3 hr).

cells, the precipitates were subjected to electrophoresis, and Stat3 was detected by anti-tyrosine antibody; it was clear with wild-type protein that two tyrosine-phosphorylated forms (the higher form, Stat3-H and the lower form, Stat3-L) were seen (Figure 6A, lane 1), indicating possible serine phosphorylation of the slower moving band. In the wild-type protein, the great majority was in the slow moving band. However, the mutation of Stat3, Ser-727→Ala-727, completely blocked the formation of the slower migrating band (Figure 6A, lane 2). The tyrosine phosphorylation of the mutant protein was equal to that of the wild-type protein, and the ability to bind DNA after EGF induction was also unchanged by the Ser-727 mutation, as tested by EMSA using ^{32}P-labeled M67 SIE (Wagner et al., 1990) (Figure 6). The binding of both wild type and mutant were identical using two other labeled probes, IRF-1 GAS (Sims et al., 1993) or Ly6E GAS (Khan et al., 1993; data not shown). These latter results on DNA binding are in accord with the experiments of Boulton et al. (1995), which showed Stat3 DNA binding to be equal in H7-treated and untreated cells. However, Zhang et al. (1995) report that Stat3 in a B-cell line activated by IL-6 binding was at least partially sensitive to H7 inhibition. We do not know the basis for this difference at present.

We next tested the transcriptional inductive capability of the wild-type Stat3 compared with the Ser→Ala-727 mutant in an acute tranfection experiment. We have recently shown that U3A cells have a low endogenous Stat3 activation by IFN-α, but a robust induction of newly introduced Stat3 (Horvath et al., 1995), and we therefore used that system in this test. The wild-type protein induced about 20-fold a luciferase target gene driven by Stat3-binding sites, whereas the Stat3 mutant Ser→Ala-727 protein only induced the reporter gene about 8-fold. (Two additional experiments gave very similar results.) Thus, a phosphoserine at 727 in Stat3, like the phosphoserine at residue 727 of Stat1α, is required for maximal transcriptional activation.

Discussion

In this report we note that a number of the STAT proteins contain a highly conserved potential serine kinase site in the COOH-terminal residues (Figure 1). In both Stat1 and Stat3, evidence for in vivo phosphorylation of this serine is provided, and it was demonstrated that this phosphoserine was required for maximal transcriptional activation. In the case of serine phosphorylation of Stat1 of residue 727, we found in serum-starved NIH 3T3 cells that both PDGF and IFN-γ, the most potent inducer of tyrosine phosphorylation of Stat1, also induced Stat1 serine phosphorylation. PDFG is known to activate MAP kinase (Hunter et al., 1985), and it has also been claimed that IFN-γ may be capable of activating the MAP kinase pathway in monocytes (Lui et al., 1994). In addition, we found that in U3A cells, which descend from a cell line (HT1080) with a dominantly activated N-*ras* allele (Hall et al., 1983; Brown et al., 1984), the Stat1α is constitutively phosphorylated on Ser-727 even after deprivation of serum. In such cells, IFN-γ yielded only about a 2-fold increase in serine phosphorylation (Figures 3Ba and 3Bb). Furthermore, EGF, IL-6, leukemia inhibitory factor, and ciliary neurotrophic factor induced the apparent serine phosphorylation of Stat3 (detected by Boulton et al. [1995]), as in lane 1 of Figure 6A, as a slower migrating band in wild-type Stat3. These ligands are also known to activate MAP kinase (Ahn and Krebs, 1990; Ahn et al., 1990; Boulton et al., 1994). Finally, the phosphorylation of Ser-727 was carried out in vitro by purified activated MAP kinase. Thus, it seems possible that MAP kinase may be capable of catalyzing phosphorylation of the STAT proteins, although we cannot be certain at present which serine kinase(s) might be active in this reaction.

We note that not all STAT-activated transcriptional activation apparently requires the phosphorylation of Ser-727. The IFN-α activation of transcription that occurs through the assembly of the multiprotein complex interferon-stim-

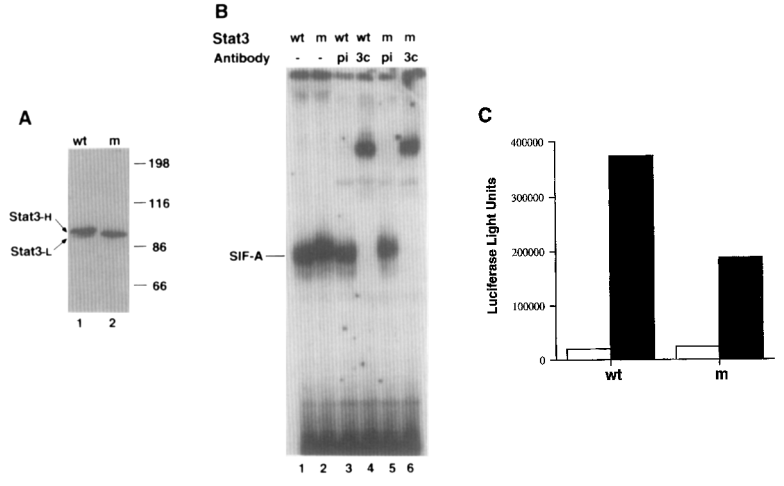

Figure 6. DNA Binding, Tyrosine Phosphorylation, and Transcription Activity of Stat3 Mutant Ser-727→Ala-727

(A) Tyrosine phosphorylation of the wild-type (wt) and Ser-727→Ala-727 mutant (m) of Stat3. Cell lysates from EGF-treated COS cells that were transfected either with wild-type (lane 1) or Ser-727→Ala-727 mutant (lane 2) were precipitated with anti-Stat3 COOH-terminal serum and probed with anti-phosphotyrosine antibody (PY20).

(B) Gel mobility shift assay was performed with ^{32}P-labeled M67 SIE probe. Nuclear extracts were prepared from EGF-treated COS cells that were transfected with either wild-type Stat3 (wt, lanes 1, 3, and 4) or Ser-727→Ala-727 mutant (m, lanes 2, 5, and 6) of Stat3. Preimmune serum (pi, lanes 3 and 5) or anti-Stat3 COOH-terminal serum (3c, lanes 4 and 6) was included in the reaction.

(C) Transcriptional activation analysis. U3A cells transfected with a 4× IRF-1 GAS-luciferase (4× IRF-1 GAS-LUC; Sims et al., [1993]) reporter plasmid together with either wild-type (wt) or Ser-727→Ala-727 mutant (m) of Stat3 expression constructs were treated with IFN-α for 6 hr; cell extracts prepared and luciferase assays carried out. Luciferase light units were plotted on the Y axis (open bars, untreated; closed bars, IFN-α-treated).

ulated gene factor 3 (ISGF-3) on the IFN-α response element can utilize Stat1β as well as Stat1α (Muller et al., 1993); Stat1β lacks the terminal 38 amino acids, including Ser-727, that are found in Stat1α. In addition, Stat2, which lacks the PMSP box (Figure 1), does not contain phosphoserine (Improta et al., 1994) (at least in U3A and 2fTGH cells) and appears to be the activating protein in the IFN-α-induced ISGF-3 complex; COOH-terminal truncation of Stat2, including loss of several acidic amino acids, eliminates IFN-α-induced transcription (S. A. Qureshi and J. E. D., unpublished data).

Serine phosphorylation has been reported to increase transcriptional activation by a number of different proteins (Hunter and Karin, 1992). For example, the cAMP response element–binding (CREB) proteins are phosphorylated on serine after induced increases in cAMP (Hoeffler et al., 1988; Gonzalez and Montminy, 1989; Sassone-Corsi, 1994). The catalytic subunit of protein kinase A is thought to be the responsible enzyme (Hoeffler et al., 1988) for phosphorylating CREB. The ternary complex factor and the serum response factor that bind the c-*fos* promoter are phosphorylated on serine in response to polypeptides owing to activated serine kinases, probably involving the p42/p44 MAP kinases (Treisman, 1994) or the pp90^{rsk} kinase (Rivera et al., 1993), respectively. Also, AP-1 is serine phosphorylated after phorbol ester treatment of cells (Karin and Smeal, 1992), possibly owing to a newly described kinase, Jnk (Hibi et al., 1993), which is related to the MAP kinases. In all these cases, the serine phosphorylation increases the effectiveness of the transcription factors already resident in the nucleus (Figure 7).

A possible mechanism by which serine phosphorylation generally increases transcription has recently been described. A very large (265 kDa) protein, CREB-binding protein (Chrivia et al., 1993), which may act as a coactivator, binds to phosphorylated CREB or AP-1 protein much better than it does to unphosphorylated proteins (Arias et al., 1994; Kwok et al., 1994). Thus, there may be a general requirement for many resident nuclear transcription factors to be phosphorylated on serine to be maximally active in the assembly of active transcription complexes.

We would draw a distinction (Figure 7) between transcriptional activation through serine phosphorylation of resident nuclear factors and those factors, like the STATs, that are only transiently activated and present briefly in the nucleus in response to polypeptide ligand activation (Shuai et al., 1992; Zhong et al., 1994b). Increased serine kinase activity triggered through Ras–MAP kinase activation, cAMP elevation, protein kinase C activation, or activation of serine kinase pathways from the cytokine receptors themselves might result in affecting any serine phosphate–sensitive transcriptional factor resident in the nucleus at the time of a wave of serine kinase activity and might affect ongoing transcription by changing the rate. However, specific induction of genes from a transcriptional ground state near zero might not be accomplished alone by serine phosphorylation, but might require specific receptor-kinase activation of proteins such as the STATs, which are first phosphorylated in the cytoplasm before dimerization and nuclear translocation. As we show, of course, further increase (or probably decrease, in some cases) in transcriptional activity could await serine phosphorylation of the STAT. The possibility of obligatory participation of a protein that does not depend primarily on a serine phosphorylation preserves the specificity inherent in the protein–protein interaction of the ligand–receptor complex, and some such mechanism would seem to be required if different polypeptide ligands do indeed trigger different patterns of gene activity from previously quiescent genes.

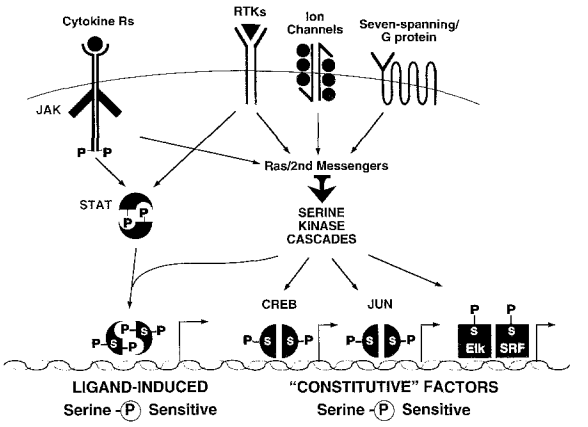

Figure 7. STAT-Directed Transcription: Necessity of Tyrosine Phosphorylation and Link to Serine Kinases

A schematic diagram of transcriptional activation by induced transcription factors contrasting those factors that are constitutively in the nucleus and are activated by serine phosphorylation with factors like the STATs, which require cytoplasmic activation as well as serine phosphorylation.

Experimental Procedures

Cell Culture and Antibodies

U3A cells, NIH 3T3, and COS cells were grown in DMEM supplemented with 10% calf serum (Hyclone Laboratories Incorporated). For serum starvation experiments, cells were deprived of serum (0%) for 14 hr. Stable cell lines were selected with G418 (300 μg/ml; GIBCO BRL) in DMEM supplemented with 10% calf serum. Individual clones were then selected and expanded in the presence of G418 (100 μg/ml). Anti-Stat1C and Stat3C sera were raised in rabbit as previously described (Schindler et al., 1992a, 1992b; Zhong et al., 1994a, 1994b). Anti-phosphotyrosine antibody PY20 was purchased from Transduction Laboratories. IFN-γ (7 ng/ml; a gift from Amgen) and PDGF (100 ng/ml; obtained from the National Institutes of Health) treatments of cells were for 20 min.

Plasmid Constructions

The STAT mammalian expression plasmids were made by inserting the coding region into the NotI and ApaLI (Stat1α, Stat1αs, wild-type, and Ser-727→Ala-727 mutant of Stat3) or NotI (Stat1β) sites of the expression vector RC/CMV (Invitrogen). The 3 × Ly6E GAS–LUC and 4 × IRF-1 GAS–LUC were made by subcloning three copies of Ly6E GAS (Khan et al., 1993) or four copies of IRF-1 GAS (Sims et al., 1993) oligonucleotides, respectively, into the BamHI site of TK–LUC (Shuai et al., 1993a, 1993b). pCMVβ, a β-galactosidase expression plasmid, was purchased from Invitrogen. Oligonucleotide-directed mutagenesis was performed according to a protocol described in Ausubel et al. (1994).

Phosphopeptide Mapping and Phosphoamino Acids Analyses

Cells were starved for 2 hr in phosphate-free DMEM (GIBCO, BRL)

with or without 10% calf serum dialyzed against a HEPES buffer (1.8 mM $CaCl_2$, 5 mM KCl, 0.8 mM $MgSO_4$, 110 mM NaCl, 44 mM $NaHCO_3$, and 25 mM HEPES [pH 7.3]). The labeling was carried out with 0.5 mCi/ml of [^{32}P]orthophosphate (DuPont) for 2.5 hr in medium otherwise lacking phosphate. Cell lysates were prepared and precleared with preimmune serum and protein A–agarose (Oncogene Science). The Stat1α proteins were immunoprecipitated with anti-Stat1C serum, subjected to 7.5% SDS–PAGE and autoradiography. The ^{32}P-labeled Stat1α proteins were recovered after SDS–PAGE, digested with trypsin (100 ng/ml; Worthington Biochemical Corporation) in 50 mM NH_4HCO_3. Phosphopeptides were separated by electrophoresis at pH 3.5, followed by chromatography on thin-layer cellulose plates (Kodak) and visualized by autoradiography (Walaas and Nairn, 1989). Individual phosphopeptides were eluted from TLC plates with pyridine and hydrolyzed in 6 M HCl at 110°C for 90 min. Phosphoamino acids were determined by electrophoresis at pH 1.9 and then at pH 3.5 on the TLC plate (Walaas and Nairn, 1989).

In Vitro Phosphorylation Assay

Synthetic peptide (5 μg) was phosphorylated by 5 μl of purified Xenopus MAP kinase (a gift from Dr. J. Maller, University of Colorado School of Medicine) in 27 mM Mg acetate, 20 mM HEPES (pH 7.4), 0.1 mM ATP, and 10 μCi of [γ-^{32}P]ATP (DuPont) at 30°C for 20 min and separated by the two-dimensional technique on a thin-layer cellulose plate. The ^{32}P-labeled peptide was then eluted from the TLC plate and subjected to phosphopeptide mapping analysis.

Cell Extracts and Immunoblot

Cells were lysed in hypotonic buffer (20 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM Na_3VO_4, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 3 mg/ml aprotinin, 1 mg/ml Pepstain, 20 mM NaF, and 1 mM DTT) with 0.2% NP-40 on ice for 10 min. After centrifugation at 4°C (13,000 rpm in microfuge) for 1 min, 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 with rocking for 30 min, and the supernatants were collected after centrifugation at 4°C (13,000 rpm) for 10 min. Whole-cell lysates were prepared as described previously (Shuai et al., 1992). Immunoprecipitation and Western blots were carried out by standard methods (Ausubel et al., 1994). Anti-Stat1C and anti-Stat3C sera were diluted 1:1000 for Western blots. For anti-phosphotyrosine blots, the precipitates were resolved on 7.5% SDS–polyacrylamide gel and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were then probed with anti-phosphotyrosine antibody PY20 (1:1000).

EMSA

Nuclear extracts were used for EMSA, which was carried out on 4% 29:1 polyacrylamide:bisacrylamide gels as described previously (Fried and Crothers, 1981) with ^{32}P-labeled IRF-1 GAS (Sims et al., 1993) or M67 SIE (Wagner et al., 1990) oligonucleotides as probes.

Luciferase Assay

Transfection was done by calcium-phosphate method (Ausubel et al., 1994) with individual RC/CMV–Stat1 or RC/CMV–Stat3 constructs, mixed with 3 × Ly6E GAS–LUC (9 μg), a luciferase reporter construct under the control of three GAS sites from the promoter of the Ly6E gene, or 4 × IRF-1 GAS–LUC (9 μg), a luciferase reporter construct with four GAS sites from the promoter of the IRF-1 gene, respectively, and a pCMVβ, a β-galactosidase expression plasmid (2 μg; Invitrogen). Precipitates were left on the cells (10 cm dish) for 8 hr. Afterward, cells were washed with PBS and grown in DMEM supplemented with 10% calf serum. After 12–16 hr, cells were left untreated or treated with IFN-γ (7 ng/ml) for 6 hr, harvested, and assayed for luciferase activity according to the Promega protocol. Luciferase light units of extracts from untransfected cells were used as basal level, and each transfection was normalized to concomitant β-galactosidase expression from a control-transfected vector (Ausubel et al., 1994). Each sample was performed in triplicate in a single experiment and repeated in four different experiments with similar results.

Northern Blot Analysis

U3A Stat1α or U3A Stat1αs was grown in DMEM supplemented with 10% calf serum in the presence of G418 (100 μg/ml) and treated with IFN-γ for 3 hr or left untreated. Poly(A)$^+$ RNA was selected by the Micro-FastTract Kit (Invitrogen), separated on 1% formaldehyde–agarose gel, and bound to ζ-probe membrane (Bio-Rad). Full-length cDNA of human IRF-1 or GAPDH was labeled with [γ-^{32}P]ATP using random hexamers (Sambrook et al., 1989) as probes. Hybridization was performed at 65°C in 0.25 M Na_2HPO_4, 7% SDS solution for 16 hr. The membrane was then washed at room temperature with 2 × SSC for 10 min and at 65°C with 2 × SSC, 0.1% SDS for 20 min, 1 × SSC, 0.1% SDS for 20 min, 0.5 × SSC, 0.1% SDS for 20 min, and 0.1 × SSC, 0.1% SDS for 40 min.

COS Cell Transfection Experiments

COS-1 cells were grown as a monolayer in DMEM with 10% calf serum. Transfection was done by calcium phosphate–DNA precipitates (Ausubel et al., 1994), with RC/CMV construct expressing either wild-type Stat3 or the Ser-727→Ala-727 mutant of Stat3 (20 μg). Precipitates were left on the cells (10 cm dish) for 8–10 hr, washed with PBS, and grown in DMEM supplemented with 10% calf serum. After 30 hr of transfection, the cells were deprived of serum for 14 hr and treated with mouse EGF (100 ng/ml) for 15 min. Extracts were prepared for gel mobility shift assay and immunoblot analysis. In this case, the nuclear extracts were used for EMSA and the immunoprecipitations were done with the mixture of cytoplasmic and nuclear extracts from the same samples.

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