Mapping of Stat3 serine phosphorylation to a single residue (727) and evidence that serine phosphorylation has no influence on DNA binding of Stat1 and Stat3

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ABSTRACT

During their polypeptide ligand-induced activation Stats (signal transducers and activators of transcription) 1 and 3 acquire, in addition to an obligatory tyrosine phosphorylation, phosphorylation on serine which boosts their transactivating potential [Wen, Z., Zhong, Z. and Darnell, J. E. Jr. (1995) Cell 82, 241–250]. By examining phosphopeptide maps of wild-type and mutant protein we show here that the Stat3 serine phosphorylation, like the Stat1 serine phosphorylation, occurs on a single residue, serine 727. Neither the DNA binding of Stat1 nor Stat3 is demonstrably affected by the presence or absence of the serine phosphorylation. Thus the earlier demonstration that transcription is enhanced by the presence of the serine 727 residue likely occurs after DNA binding. These findings do not agree with earlier claims of excess serine to tyrosine phosphorylation in activated Stats 1 and 3 or to claims of more stable DNA binding of serine phosphorylated Stat dimers

INTRODUCTION

Polypeptides when bound to their cognate cell surface receptors cause activation of latent cytoplasmic transcription factors termed STATs (signal transducers and activators of transcription) (1). The Stat molecule becomes bound by its -SH2 group to the internal domain of an activated tyrosine phosphorylated receptor kinase complex and itself becomes phosphorylated on tyrosine either by a Janus kinase associated with a receptor lacking intrinsic tyrosine kinase activity or by a receptor that has a tyrosine kinase domain. The tyrosine phosphorylation is obligatory for STAT dimerization, nuclear translocation and subsequent DNA binding. A second induced phosphorylation event on serine of a Stat molecule can also enhance the transcriptional stimulus when a STAT dimer acts in the nucleus (2). For example, during IFN-γ stimulation of fibroblast cells, Stat1α also becomes phosphorylated on a single residue, serine 727. If cells contain wild-type Stat1α there is a 4–5-fold greater transcriptional induction than if they contain a mutant protein, Stat1α (S727A). This mutant which has a single serine residue substituted by alanine exhibits no serine phosphorylation either constitutive or induced. The Ser727 of Stat1 has demonstrated in vivo function; neither the induction by interferon-γ (IFN-γ) of antiviral state or of growth arrest of cells occurs in cells with the S727A mutation (3,4). Aside from this direct demonstration of specific serine phosphorylation, other evidence of serine phosphorylation of Stats 3 and 5 has been presented (2,5–7). For example, ligand-induced tyrosine phosphorylated Stat3 exhibits a time dependent slower migration in electrophoresis consistent with the acquisition of phosphorylation on serine (5,6). In accord with a possible serine phosphorylation of Stat3, the slower electrophoretic migration is inhibited by H7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride], a serine/threonine kinase inhibitor. However, the site(s) of serine phosphorylation in Stat3 has not yet been directly demonstrated. In addition, conflicting evidence about the role of serine phosphorylation in Stat3 function has been described. In one report the protein was thought not to bind DNA in the absence of serine phosphate (5) whereas in other reports this was found not to be the case (2,6,8,9). In addition, it has also been claimed that increased serine phosphorylation of Stat1 occurs in monocytes in a molar excess compared with the single phosphotyrosine leading to greater affinity for Stat1 DNA binding sites, particularly for weaker binding sites (10). In this report we examine further the serine phosphorylation of Stat3 from epidermal growth factor (EGF)-stimulated cells and show that, like Stat1, mutation to alanine of a single serine residue (also residue 727 in Stat3) completely abolishes EGF-induced serine phosphorylation. However, on four different Stat3 DNA binding sites for which the activated protein has varying affinity, the binding of mutant and wild-type protein is indistinguishable. Finally, we re-examined the DNA binding of wild-type Stat1α and the Stat1αs (S727A) mutant both by gel shift competition analysis with strong and weak DNA binding sites and by varying the protein input in gel shift assays. We conclude that phosphorylation of serine 727 does not contribute to a changed DNA binding affinity for Stat1.

MATERIALS AND METHODS

Cell culture, antibodies and reagents

COS-1 cells, U3A-Stat1α and U3A-Stat1αs cells (2) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum (Hyclone Laboratories Incorporated).

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Anti-Stat1C, Stat1M and Stat3C sera were raised in rabbit as previously described (11,12). Anti-phosphotyrosine antibody PY20 was purchased from Transduction Laboratories. Human IFN-γ and mouse EGF were gifts from Amgen and Dr Stanley Cohen (Vanderbilt University School of Medicine), respectively.

Expression plasmids and transfection

The Stat mammalian expression constructs were made by inserting the coding region of Stat3 into the expression vector RC/CMV (Invitrogen) (2). Transfection was done by calcium phosphate–DNA precipitates (13) with either wild-type or S727A mutant of RC/CMV-Stat3 (25 µg) on COS-1 cells in a 10 cm dish. The precipitates were left on the cells for 12 h, washed with phosphate buffered saline (PBS) and incubation continued for an additional 30 h in DMEM with 10% calf serum. Finally, cells were deprived of serum (0%) in DMEM for 15 h prior to EGF treatment and 32P-orthophosphate labeling.

Immunoprecipitation and Western blot

Cell extracts were prepared as previously described (2). For immunoprecipitation, whole cell lysates were incubated with indicated antibodies on ice for 1 h and rocked at 4°C with protein A–agarose beads (Oncogene Science) for an additional 2 h. The beads were then washed with ice-cold whole cell lysate buffer (14), NaCl/EDTA buffer [0.5 M NaCl, 10 mM KCl, 20 mM HEPES (pH 7.9), 1 mM EDTA and 0.5% NP-40] and PBS. For Western blot, proteins were separated on 7.5% SDS–PAGE gel and transferred to Nitrocellulose membrane (Scheicher and Schuell). The membrane was probed with either anti-phosphotyrosine antibody PY20 (1:2000), anti-Stat1M (1:2000) or anti-Stat3C (1:4000) serum according to standard methods (13). For reprobing experiments, membranes were stripped in 0.5 M NaCl/0.5 M CH3COOH solution at room temperature for 10 min, washed with TBST buffer [20 mM Tris (pH 7.6), 137 mM NaCl, 0.1% Tween 20] and stained with another indicated antibody. Immunoblots were developed with chemiluminescence reagent (ECL, Amersham).

Electrophoretic mobility shift assays (EMSA)

EMSA of nuclear extracts was carried out on 4%, 29:1 polyacrylamide:biacrylamide gel as described previously (15) with 32P-labeled m67 (double-stranded 5′-GTCGACATTCTTCCGGATCGTCGA-3′), interferon regulatory factor-1 (IRF-1) (double-stranded 5′-GATCGATTCTCCTGGGAATTCTC-3′), and Ly6E (double-stranded 5′-ATTCGATTCTCCTGGGAATTCTC-3′). An indicated excess amount of unlabeled Ly6E or guanylate-binding protein (GBP) (double-stranded 5′-ATTCGATTCTCCTGGGAATTCTC-3′) oligonucleotide was added in the indicated binding reaction for gel shift competition assay (Comp.). The dilution of nuclear extracts were done with nuclear extraction buffer (2).

RESULTS AND DISCUSSION

Since we do not have cells that lack Stat3 completely, as we had available for Stat1 (2,17,18), we examined the phosphorylation of Stat3 in COS-1 cells, which have a low amount of endogenous Stat3. We had shown earlier that overexpression of the desired protein could be achieved after transfection with a plasmid encoding either the wild-type Stat3 protein or the Stat3 S727A mutant and the overexpressed Stat3 could be activated by EGF (2,11). To study the induction of serine and tyrosine phosphorylation of Stat3, transfected COS-1 cells were labeled with 32PO4 for 2 h and treated or left untreated with EGF for 30 min. (A) Cells were lysed and protein extracts were incubated with anti-Stat3C serum, separated on SDS–PAGE, and the 32P-labeled Stat3 was visualized by autoradiography. Lane 1: Stat3W; lane 2: Stat3W + EGF; Lane 3: Stat3M; Lane 4: Stat3M + EGF. (B) The 32P-labeled Stat3 bands were excised from the gel and digested with endoproteinase ASP-N and trypsin. The digests were then subjected to two dimensional analysis on TLC plates. a: Stat3W; b: Stat3W + EGF; c: Stat3M; d: Stat3M + EGF. (C) Phosphopeptide 1, 2 and 3 were eluted from the TLC plates and applied for phosphoamino acid analysis. Lane 1: standard phosphoserine, phosphothreonine and phosphotyrosine; lane 2: phosphopeptide 1; lane 3: phosphopeptide 2; lane 4: phosphopeptide 3.

Figure 1. Phosphopeptide map and phosphoamino acid analyses of Stat3. COS-1 cells were transiently transfected with expression vector constructs with either wild-type (Stat3W) or S727A mutant (Stat3M) of Stat3. Cells were labeled with 32PO4 for 2 h and treated or left untreated with EGF for 30 min. (A) Cells were lysed and protein extracts were incubated with anti-Stat3C serum, separated on SDS–PAGE, and the 32P-labeled Stat3 was visualized by autoradiography. Lane 1: Stat3W; lane 2: Stat3W + EGF; Lane 3: Stat3M; Lane 4: Stat3M + EGF. (B) The 32P-labeled Stat3 bands were excised from the gel and digested with endoproteinase ASP-N and trypsin. The digests were then subjected to two dimensional analysis on TLC plates. a: Stat3W; b: Stat3W + EGF; c: Stat3M; d: Stat3M + EGF. (C) Phosphopeptide 1, 2 and 3 were eluted from the TLC plates and applied for phosphoamino acid analysis. Lane 1: standard phosphoserine, phosphothreonine and phosphotyrosine; lane 2: phosphopeptide 1; lane 3: phosphopeptide 2; lane 4: phosphopeptide 3.
Figure 2. DNA binding and tyrosine phosphorylation of wild-type and S727A mutant of Stat3. (A) After EGF treatment, nuclear extracts were prepared from cells transfected with either wild-type (lanes 1–3, 7–9, 13–15, 19–20 and 23–25) or mutant (lanes 4–6, 10–12, 16–17, 21–22 and 26–28) Stat3 for gel shift with four different 32P-labeled DNA probes (M67: lanes 1–6 and 19–22; APRE: lanes 7–12; IRF-1: lanes 13–18; Ly6E: lanes 23–28). Preimmune (pi) (lanes 2, 5, 8, 11, 14, 17, 24 and 27) or anti-Stat3C (3C) (lanes 3, 6, 9, 12, 15, 18, 20, 22, 25 and 28) serum was added in the DNA binding reaction. (B) Whole cell lysates from EGF-treated cells expressing either wild-type (W: lane 1) or mutant (M: lane 2) of stat3 were precipitated with anti-Stat3C serum and probed with anti-phosphotyrosine antibody PY20.

Stat1 phosphopeptides (2), we did not obtain clear two-dimensional peptide maps, either because of too large a released peptide with consequent poor mobility in two-dimensional analysis or because charged residues near to the trypsin cleavage site inhibited peptide bond cleavage. In any event in those analyses the putative large phosphopeptide released from wild-type protein that remained on the origin was virtually eliminated from the S727A mutant protein (data not shown). To more clearly examine the 32P phosphopeptide of Stat3 we used endoproteinase Asp-N and trypsin for digestion which, from Stat3, should yield a serine phosphopeptide of 7 amino acids (DLPMSPR) and a tyrosine phosphopeptide of 10 amino acids (DPGSAAPYLK). A typical analysis of wild-type Stat3 and Stat3 S727A mutant phosphoprotein from EGF-treated or untreated cells is shown in Figure 1. In the first step (Fig. 1A) the precipitation and isolation of a specific 32P-labeled Stat3 band, a clear 3–5-fold increase in phosphorylation was evident in both wild-type and mutant proteins upon EGF treatment. In peptide digests of the 32P-labeled Stat3 band from cells transfected with wild-type Stat3 there were two clear spots from untreated cells (labeled 1 and 2, Fig. 1B, a) which upon secondary amino acid analysis proved to be phosphoserine (Fig. 1C), whereas in the digests from EGF-treated cells expressing wild-type Stat3 the same two spots (labeled 1 and 2) were significantly increased and accompanied by a third weaker spot (labeled 3, Fig. 1B, b) which proved to be phosphotyrosine (Fig. 1C). In digests of the S727A mutant protein there was no radioactivity recovered in the peptide maps from untreated cells. The digests from Stat3 of EGF-treated cells expressing S727A protein however contained the phosphotyrosine spot (Fig. 1B, d) but no serine phosphorylation.

From these results we conclude that induction of tyrosine phosphorylation proceeds normally in the S727A mutant of Stat3 and that there is significant EGF induction of serine phosphorylation of the wild-type version of this protein. Further, on a molar basis there is a great deal more serine phosphorylation than tyrosine phosphorylation in the total wild-type protein. Even though only a single time point was taken in these experiments, we know from examinations of Stat1 (Wen et al., unpublished) that tyrosine phosphorylation precedes serine phosphorylation and from earlier experiments with Stat3 (6) that the slower migrating Stat3 band in gel electrophoresis, previously taken as evidence for Stat3 serine phosphorylation, develops after tyrosine phosphorylation has already occurred. Thus we conclude that the two phosphorylation events are not coupled, that many more molecules get phosphorylated on serine than tyrosine and possibly that serine phosphorylation does not require recruitment to the membrane of the Stat protein as does tyrosine phosphorylation.

We believe it likely that a single serine, residue 727 of Stat3, is the major, probably the sole, target of serine phosphorylation in these cells because when this residue is changed to alanine there is no longer any discernible serine phosphorylation. Our explanation for the existence of two spots in the phosphopeptide analysis of Figure 1B is either that oxidation of methionine residue of this peptide (DLPMSPR) occurs in some peptides or perhaps, more likely, incomplete trypsin digestion. It has been reported that serine phosphorylation of peptides can inhibit proteolysis by trypsin (19). The protein was first digested by endoproteinase ASP-N which should result in release of peptide DLPMSPRTL followed by trypsin that would cleave after an arginine residue. The second step also might be inhibited because the phosphoserine
Figure 3. DNA binding and tyrosine phosphorylation of wild-type (Stat1α) and S727A mutant (Stat1αs) of Stat1. (A) After treatment of cells with (lanes 3–8 and 11–16) or without (lanes 1–2 and 9–10) IFN-γ for 25 min, nuclear extracts from U3A-Stat1α (lanes 1, 3, 5, 7, 9, 11, 13 and 15) or U3A-Stat1αs (lanes 2, 4, 6, 8, 10, 12, 14 and 16) cells were used for gel shift with either 32P-labeled IRF-1 (lanes 1–8) or Ly6E (lanes 9–16) probe. Preimmune (pi) (lanes 5–6 and 13–14) or anti-Stat1C (1C) (lanes 7–8 and 15–16) was included in the indicated DNA binding reaction. (B) Nuclear extracts from IFN-γ-treated (lanes 2–19) U3A–Stat1α (lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18) or U3A–Stat1αs (lanes 3, 5, 7, 9, 11, 13, 15, 17 and 19) cells were used for gel shift with 32P-labeled IRF-1 probe. Excess of unlabeled Ly6E (10 times: lanes 4–5, 50 times: lanes 6–7, 100 times: lanes 8–9 and 200 times: lanes 10–11) or GBP (50 times: lanes 12–13, 100 times: lanes 14–15, 200 times: lanes 16–17 and 500 times: lanes 18–19) oligonucleotide was added in the indicated DNA binding reaction. Lane 1 was a mixture of nuclear extracts from untreated U3A-Stat1α and U3A–Stat1αs cells. (C) Gel shift was carried out with either 32P-labeled M67 (lanes 1–10) or IRF-1 (lanes 11–20) probes using nuclear extracts from either U3A-Stat1α (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19) or U3A-Stat1αs (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20) cells. The nuclear extracts were diluted 1: 2 (lanes 5–6 and 15–16), 1:4 (lanes 7–8 and 17–18), or 1:8 (lanes 9–10 and 19–20) with nuclear extraction buffer or left undiluted (lanes 3–4) prior to the DNA binding reaction. Lanes 3–10 and 13–20 were nuclear extracts from IFN-γ-treated cells whereas lanes 1–2 and 11–12 were untreated samples. The Stat1–DNA complexes were indicated as GAF. (D) U3A-Stat1α (lanes 1–2) and U3A-Stat1αs (lanes 3–4) cells were treated with IFN-γ (lanes 2 and 4) or left untreated (lanes 1 and 3). Protein extracts were precipitated with anti-Stat1C serum and stained with anti-phosphotyrosine antibody PY20 (upper panel). The blot was then stripped of the PY20 antibody and probed with anti-Stat1M serum (lower panel).

is only two residues from the cleavage site. Thus the phosphopeptide was probably incompletely digested by trypsin thus leading to two phosphopeptide spots (putatively DLPMSPR and DLPMSPRTL) rather than one in the phosphopeptide analysis map. That mutation of a single serine removes all phosphoserine from the protein strongly supports these arguments.

With the likelihood of a single phosphoserine residue in both Stats 1 and 3, we returned to the question of DNA binding by Stat
mutant proteins lacking serine 727. Figure 2A shows the induced DNA binding activity of nuclear extracts from EGF-treated COS-1 cells transfected with plasmids encoding either wild-type Stat3 (W in Fig. 2) or the Stat3 S727A mutant protein (M in Fig. 2). The prominent band is labeled SIS-inducible factor-A (SIF-A) because this DNA protein complex was originally identified as a SIS-induced factor and is known from other work to be a Stat3 homodimer (11,20,21). Antiserum tests show the complex to be Stat3: there is no reaction with pre-immune serum (pi) whereas the Stat3 carboxyl terminal antisera (3C) removed the SIF-A band. The m67 probe used in Figure 2 was first determined by Wagner et al. (22) to be the strongest binding version of various sequence arrangements from the c-fos promoter. Figure 2A shows that it binds wild-type and mutant protein equally well. Three other sequences are compared, APRE (23,24), IRF-1 (25), and Ly6E (26), that differ in binding affinity for Stat3 more than 10-fold. In every case the binding of wild-type and mutant are similar. Thus the two proteins bind to both high affinity and low affinity sites in an equivalent manner.

The inset on the right of Figure 2B shows the electrophoretic migration of tyrosine phosphorylated Stat3 protein in the extracts used to compare the DNA binding of the proteins. All of the tyrosine-phosphorylated, wild-type protein migrates more slowly than the mutant protein, indicating that the tyrosine phosphorylated molecules are totally serine phosphorylated in the examination in Figure 2A, strong evidence therefore that DNA binding is unaffected by serine 727 phosphorylation.

Finally, a similar but somewhat more extensive quantitative analysis of DNA binding by Stat1α and Stat1γ (S727A) was carried out. IFN-γ was used to induce active Stat1 proteins in U3A cells that were permanently complemented with either wild-type Stat1α or S727A mutant, Stat1γ (2). DNA binding in nuclear extracts was carried out with four different DNA probes for which Stat1 has a varying affinity. The induced band [labeled IRF-1 probe competed with two unlabeled weaker bands (Fig. 3 C, lanes 3 and 4). Competition experiments were then performed in parallel for wild-type and Stat1γ proteins bound to IRF-1 probe and competed with different amounts of an unlabeled probe (Fig. 3B). Figure 3B shows labeled IRF-1 probe competed with two unlabeled weaker binding sites, Ly6E (lanes 4 to 11) and GBP (lanes 12 to 19) (27). Again the wild-type and Stat1γ proteins bound indistinguishably. Finally, we tested the effect of varying the protein concentration of wild-type and Stat1γ mutant protein on DNA binding. As shown in Figure 3C, both wild-type and mutant Stat1 bound equally to m67 (lanes 1–10) and IRF-1 (lanes 11–20) probes at several different protein levels.

The results presented here argue strongly that serine 727, near the -COOH terminus and in the transactivation domain of Stats 1 and 3, is the principal if not the only site of IFN-γ and EGF-induced serine phosphorylation in Stats 1 and 3. In addition, the phosphorylation on serine 727 apparently has no effect on the DNA binding affinity of either Stat1 or 3. The C-terminus is required for transcriptional activation from Stats 1, 3, 4, 5 and 6 (2,18,28–31) and the addition of a phosphorylation to a serine in this region can be viewed as enhancing the normal function of the transcriptional activation domain. The protein(s) capable of recognizing this domain both with and without its serine phosphate are important targets for future research. While it may be true that in other cell types or in other Stats there are other serine residues that can be phosphorylated, we would urge caution in making that conclusion based on any results other than direct observation of phosphopeptides and by mutagenesis to prove the potential importance of other residues.

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