The Amino Acid Residues Immediately Carboxyl-terminal to the Tyrosine Phosphorylation Site Contribute to Interleukin 6-specific Activation of Signal Transducer and Activator of Transcription 3*

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Signal transducers and activators of transcription (Stat) proteins play an important role in signaling through a variety of cytokine and growth factor receptors. Each of the Stat proteins is activated in a ligand-specific manner. Only the Src homology 2 (SH2) domains of Stat1 and Stat2 are critical for the ligand-specific activation of interferon signaling. In this study we determined the domains in Stat3 protein that contribute to interleukin 6 (IL-6)-specific phosphorylation. Based on evidence that Stat3, but not Stat1, is activated in the presence of low levels of IL-6 and soluble IL-6 receptor, we constructed various swap mutants between Stat3 and Stat1 and examined their response to IL-6 after their transient expression into COS7 cells. The region upstream of the SH2 domain was exchangeable between Stat1 and Stat3, whereas the region carboxyl-terminal to the SH2 domain of Stat3 was critical to phosphorylation by IL-6. However, unlike Stat1 and Stat2 in interferon signaling, the swap mutant in which 5 amino acid residues just carboxyl-terminal to the tyrosine phosphorylation site (Tyr709) in Stat3 was replaced by the corresponding region derived from Stat1 was not phosphorylated in response to IL-6. Substituting 1 amino acid (Lys709) at position +4 relative to Tyr709 abolished the tyrosine phosphorylation of Stat3 in response to IL-6. Co-immunoprecipitation experiments demonstrated that these mutants were associated with gp130 at an extent similar to wild-type Stat3. Taken together, these results show that the amino acid residues immediately carboxyl-terminal to the tyrosine phosphorylation site are involved in IL-6-specific activation of Stat3.

Cytokines and growth factors mediate their biological effects through interaction with their receptors. Investigations into the transcriptional response to interferons have identified the Janus kinases (Jak)-signal transducers and activators of transcription (Stat) signaling pathway, which is in fact used by a variety of cytokines, including IL-2, IL-3, IL-5, granulocyte-macrophage colony-stimulating factor, prolactin, and thrombopoietin (16–21). Although Stat1 is activated by many cytokines and growth factors, it appears specific for IFN signaling in vitro. Stat1 and Stat2 are phosphorylated in response to IFN-α, whereas Stat1, but not Stat2, is phosphorylated in response to IFN-γ. Besides the IFN signaling systems, Stat1 is activated by signaling through various cytokine receptors (3–6), growth factor receptors (6–8), and the G protein-coupled receptor for angiotensin II (9, 10). Stat3 is phosphorylated by stimulation with cytokines using gp130 and gp130-related receptors such as IL-6, leukemia inhibitory factor (LIF), oncostatin M, ciliary neurotrophic factor, and granulocyte colony-stimulating factor, as well as EGF (11–13). Stat4 and Stat6 are phosphorylated in response to IL-12 and IL-4, respectively (14, 15). Stat5 is phosphorylated by a variety of cytokines, including IL-2, IL-3, IL-5, granulocyte-macrophage colony-stimulating factor, prolactin, and thrombopoietin (16–21).

The targeted disruption of the Stat genes in mice has revealed the involvement of each Stat protein in the cytokine signal pathways in vivo (22–27). Although Stat1 is activated by many cytokines and growth factors, it appears specific for IFN but retain responsiveness to LIF and remain LIF-dependent for undifferentiated growth (22), indicating that Stat1 does not play a distinctive role in LIF signaling. Stat6 plays an essential and specific role in IL-4 signaling (26, 27).

To understand how the specificity of the signal is achieved after the interaction of a ligand with the corresponding receptor and subsequent activation of Jak kinases and Stat proteins, the molecular mechanism of their interaction has been investigated. In response to IFN-γ, Stat1 is activated by Jak1 and Jak2. A functionally critical membrane-distal tyrosine residue (Tyr100) in the IFN-γ receptor is a target of activated Jak kinases, and the sequence YpDKPH, containing the phosphotyrosine of the IFN-γ receptor, provides the specific association site for Stat1 (28). In IFN-α signaling, Leung et al. showed that Stat1 and Stat2 proteins might be sequentially phosphorylated in response to IFN-α, and that phosphorylated Stat2 might be required, so that unphosphorylated Stat1 can bind to the activated IFN-α receptor (29). For IL-6 signaling, gp130 becomes homodimerized when IL-6 binds the IL-6 receptor (30, 31).
Dimerization induces intermolecular phosphorylation and activation of the associated Jaks (Jak1, Jak2, and Tyk2), which then phosphorylate tyrosines on gp130 (32). The box3 region of gp130 contains XXXQ, motifs and it acts as a docking site that selectively binds Stat3, which is in turn phosphorylated by activated Jaks (33). Using chimeric constructs between Stat1 and Stat2, Heim et al. (34) have shown that the SH2 domain in Stat proteins plays a critical role in their ligand-specific phosphorylation of Stat proteins (34).

We questioned whether the same is true of other members of the Stat family. Here we performed structure-function analyses of Stat3 by generating a series of swap mutants between Stat3 and Stat1 and identified the region that contributes to the specificity of IL-6-dependent Stat3 tyrosine phosphorylation. We confirmed the importance of the SH2 domain in Stat3 and showed that, besides the SH2 domain, the specific activation of Stat3 by IL-6 is determined by the amino acid sequences present just carboxyl-terminal to the tyrosine phosphorylation site.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant human IL-6 and soluble IL-6 receptor were purchased from Genzyme (Cambridge, MA). The antibodies to Flag epitope and to phosphotyrosine were purchased from IBI (New Haven, CT) and Upstate Biotechnology (Lake Placid, NY), respectively.

Cells and Cell Culture—COS cells were grown on plastic culture dishes in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Myeloid leukemia M1 cells were cultured in Eagle's minimal essential medium supplemented with twice the normal concentrations of amino acids and vitamins and 10% fetal calf serum.

Plasmid Construction—Some constructs were generated by introducing new restriction enzyme sites, which did not alter the amino acid residue, by the polymerase chain reaction or by site-directed mutagenesis (Transformer site-directed mutagenesis kit, Clontech). Other constructs were directly produced by site-directed mutagenesis. The subcloned polymerase chain reaction products were completely sequenced. Some constructs were generated by introducing new restriction enzyme sites, which did not alter the amino acid residue, by the polymerase chain reaction or by site-directed mutagenesis (Transformer site-directed mutagenesis kit, Clontech). Other constructs were directly produced by site-directed mutagenesis. The subcloned polymerase chain reaction products were completely sequenced.

The constructs were tagged with the Flag epitope (Eastman Kodak Co.) at their NH2 termini and cloned into the mammalian expression vector pEF-BOS (35). The amino acid (aa) boundaries of the chimeric proteins were as follows: 3/1A has aa 1–235 of Stat1 and 240–771 of Stat3; 3/1B has aa 1–325 of Stat1 and 330–771 of Stat3; 3/1C has aa 1–443 of Stat1 and 450–771 of Stat3; 3/1D has aa 1–596 of Stat1 and 604–771 of Stat3; 3/1E has aa 597–751 of Stat1 and 1–704 of Stat3; 3/1F has aa 449–751 of Stat1 and 1–751 of Stat3; 3/1G has aa 694–751 of Stat1 and 1–698 of Stat3; 3/1H has aa 699–751 of Stat1 and 1–698 of Stat3; 3/1I has aa 705–715 of Stat3 substituted for 701–711 of Stat1; 3/1K has aa 705–710 of Stat3 substituted for 701–706 of Stat1; and 3/1L has aa 711–715 of Stat3 substituted for 701–711 of Stat1. Y705F was generated by site-directed mutagenesis converting the tyrosine 705 of Stat3 to phenylalanine, and K709E was generated converting lysine 709 of Stat3 to glutamate.

DNA Transfection—COS cells were transfected using the DEAE-dextran method and cultured for 48 h in complete media before being deprived of serum for 5 h. The cells were then incubated for 15 min with cytokines or growth factors, and then the cells were lysed for immunoprecipitation. M1 cells were transfected via electroporation with the expression vector and pSV2neo at a 20:1 ratio, and neomycin-resistant clones were selected in growth medium containing 750 mg/ml of G418 (Life Technologies, Inc.).

Immunoprecipitation and Immunoblotting—Cells were solubilized with Nonidet P-40 lysis buffer (1.0% Nonidet P-40 and 10 mM Tris HCl, pH 7.6, containing 150 mM NaCl, 5 mM EDTA, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 5 mg/ml aprotonin, leupeptin, and pepstatin) and then centrifuged. The clarified lysates were incubated with 10 μg of M2 anti-Flag monoclonal antibody and protein A-Sepharose (Pharmacia Biotech Inc.), and then immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with M2 or anti-phosphotyrosine monoclonal antibody. Blots were visualized using the ECL detection system (Amersham Corp.) according to the manufacturer’s procedures.

RESULTS

We assessed the dose of IL-6 required to phosphorylate Stat1 and Stat3. To discriminate exogenous and endogenous Stats, we constructed expression vectors for Stat1 and Stat3, both of which were Flag epitope-tagged on their amino termini. These vectors were transiently transfected into COS cells and stimulated with increasing concentrations of IL-6 together with soluble IL-6 receptor. The cells were then lysed, immunoprecipitated with anti-Flag antibody, and immunoblotted with anti-phosphotyrosine antibody. Fig. 1A shows that at an IL-6 concentration of 25 ng/ml, Stat3 was already fully phosphorylated, whereas Stat1 was not at all or only weakly phosphorylated. As the concentration of IL-6 increased, Stat1 phosphorylation was augmented in a dose-dependent manner, although the amount of phosphorylation at 300 ng/ml IL-6 was still significantly lower than that of Stat3 at 25 ng/ml. In contrast, Stat3 was maximally phosphorylated at 25 ng/ml IL-6. These results demonstrate that only Stat3 is activated at a low concentration of IL-6, although Stat1 is activated at a very high concentration. Therefore, we used 25 ng/ml IL-6 to stimulate COS cells in the following experiments. We also examined the tyrosine phosphorylation of wild-type Stat3 and Stat1 in response to EGF. Both Stat3 and Stat1 were phosphorylated in COS cells exposed to EGF (Fig. 1B).

To determine which domains contribute to the IL-6-specific activation of Stat3 protein, we constructed several swap mutants at conserved amino acids between Stat3 and Stat1, termed 3/1A–3/1D (Fig. 2). In contrast to 3/1A, the amino-terminal region of Stat3 was substituted by the comparable region of Stat1. The constructs 3/1B and 3/1C contained Stat1 residues in place of Stat3 aa 1–329 and 1–449, respectively. In the
To examine whether only the SH2 domain is sufficient for IL-6-specific phosphorylation of Stat3 and Stat1, we constructed swap mutants in which the carboxyl-terminal regions of Stat3 were replaced by the comparable regions of Stat1 (Fig. 4). In the construct 3/1E, the carboxyl-terminal region containing the DNA binding domain (aa 330–771) of Stat3 was substituted by that of Stat1. In the construct 3/1F, the region containing the SH3 and downstream domains (aa 454–771) was replaced by that of Stat1. Fig. 5 shows that IL-6 did not activate the 3/1E and 3/1F mutants. In contrast, EGF phosphorylated both of them. Together with the results shown in Fig. 3, these data indicated that the carboxyl-terminal region containing the SH2 and its downstream domains provide the specificity for Stat3 phosphorylation mediated by IL-6 but not by EGF.

We and other groups have demonstrated that the SH2 domain is critical for Stat3 phosphorylation because a single amino acid substitution of arginine to glutamine in the SH2 domain completely abolished Stat3 phosphorylation. We examined whether only the SH2 domain is sufficient for IL-6-specific phosphorylation. We constructed the swap mutant, 3/1G, in which the region carboxyl-terminal to the SH2 domain (aa 699–771) was replaced by that of Stat1. These results showed that the amino-terminal region upstream of the SH2 domain is exchangeable between Stat1 and Stat3.

We found that the region upstream of the SH2 domain is exchangeable between Stat1 and Stat3. COS-7 cells were transfected with wild-type Stat3, Stat1, and the chimeric Stat3/1 proteins described in Fig. 2. Serum-deprived cells were incubated for 15 min at 37 °C with or without 25 ng/ml recombinant human IL-6 and 25 ng/ml soluble recombinant human IL-6 receptor. Cells were lysed and immunoprecipitated with the anti-Flag antibody M2. Immunoprecipitates were Western blotted using the monoclonal anti-phosphotyrosine antibody 4G10 (top panel) or the anti-Flag antibody M2 (bottom panel).

To exclude the possibility that these results were specific to COS cells, we generated permanent M1 clones that harbor wild-type Stat3 and several swap mutant expression vectors. M1 is a mouse myeloid leukemia cell line that differentiates into macrophages in response to IL-6. M1 cells did not respond to EGF, but wild-type Stat3 and 3/1L were phosphorylated by stimulation with IL-6 and soluble IL-6 receptor whereas, 3/1K was not (Fig. 7). As in COS cells, the 5 amino acid residues carboxyl-terminal to Tyr705 were critical for the IL-6-specific phosphorylation of Stat3 in M1 cells.

To examine the reason why IL-6 did not tyrosine phosphorylate the carboxy-terminal swap mutants in response to IL-6, we studied their association with gp130. COS cells were cotransfected with the expression vectors for wild-type Stat3 or its mutants and a gp130 expression vector. After stimulation by the corresponding region of Stat1 in 3/1K. Five downstream amino acids (aa 711–715) adjacent to the critical 5 amino acids were replaced by the corresponding region of Stat1 in 3/1L. Fig. 6B shows that EGF and IL-6 phosphorylated 3/1L, whereas only EGF phosphorylated 3/1J and 3/1K. The amino acid differences between the 5 amino acids critical for IL-6-specific phosphorylation of Stat3 and the corresponding 5 amino acids of Stat1 consists of 3 residues: +1 (Leu → Ile), +4 (Lys → Glu), and +5 (Phe → Leu). Since the substitution at +4 seemed to be the biggest difference, we changed +4 lysine to glutamate in Stat3 by site-directed mutagenesis (mutant K709E). EGF, but not IL-6, phosphorylated this mutant. Therefore, the +4 lysine is necessary for the IL-6-specific activation of Stat3.
Amino Acid Residues in IL-6-specific Activation of Stat3

In HepG2 cells at a low IL-6 concentration, SIF-A (a Stat3 homodimer) was the predominant DNA binding complex induced, whereas at higher IL-6 concentrations, SIF-B (a Stat3 and Stat1 heterodimer) and SIF-C (a Stat1 homodimer) were also formed. Although Stat1 is activated by IL-6, Stat3 plays a critical role in the biological effects exerted by IL-6. In myeloid leukemia M1 cells that undergo terminal differentiation and growth arrest in response to IL-6, overexpression of the dominant negative forms of Stat3 prevented both growth arrest and terminal differentiation (38, 39). On the contrary, despite its activation by factors other than IFNs, Stat1 is essential for IFN signaling, because Stat1-deficient mice display a complete lack of responsiveness to either IFN-α or IFN-γ but respond normally to other cytokines that activate Stat1 in vitro (22, 23). To date, how Stat1 is activated at high concentrations of IL-6 and what the function of Stat1 is in IL-6 signaling remain to be clarified. We and other investigators showed that gp130 is immunoprecipitated by activated Stat3 but not by Stat1, even when phosphorylated at high concentrations of IL-6 (data not shown). Stat1 may be activated by forming a heterodimer with Stat3 through docking to Stat3 phosphotyrosine, as demonstrated by the activation of Stat1 in IFN-α signaling (29). Alternatively, Stat1 may associate with gp130 in such a degree that recruitment cannot be detectable by immunoprecipitation assay (40) or may directly associate with activated Jak kinases and become phosphorylated. In fact, Gupta et al. (41) have reported that the Stat1 SH2 domain mediates an obligate interaction with the Jak kinases, indicating that overstimulation causes the direct binding of Stat1 with Jak protein kinases and subsequent phosphorylation by Jak5. Furthermore, very high erythropoietin levels activate Stat5 even when an erythropoietin receptor mutant lacks all intracellular tyrosines (42).

The amino-terminal region is conserved in Stat proteins and has multiple functions. The amino terminus of Stat1 is important in modulating its phosphorylation, possibly by interacting with a phosphatase (43). The domain also contributes to the co-operative DNA binding and enables the Stat proteins to recognize variations of the consensus site (44). Deletion mutants of Stat1 lacking the amino-terminal conserved region were not phosphorylated by IFN-α or IFN-γ, and the helical repeat within their amino-terminal regions is necessary for Stat1 phosphorylation (45, 46). Our findings that these regions are exchangeable between Stat3 and Stat1 suggested that the amino-terminal region does not function as a specificity determinant in their phosphorylation.

In this study, we showed that the carboxyl-terminal region, including the SH2 domain, determines the specificity between Stat3 and Stat1. Heim et al. (34) have also demonstrated that the SH2 domain plays a critical role in the selective activation

**DISCUSSION**

There is some discrepancy among studies of Stat protein activation by IL-6 treatment. Some have shown that only Stat3 is activated (11, 13), whereas others have found that both Stat3 and Stat1 are activated (36, 37). In addition to IFNs, Stat1 is tyrosine-phosphorylated by many factors, such as EGF, platelet-derived growth factor, LIF, IL-6, IL-10, colony-stimulating factor 1, and angiopoietin-1. However, the doses of these factors were not considered in these studies. In this study, Stat transient expression in COS cells and immunoblotting showed that only Stat3 is phosphorylated at low concentrations of IL-6, whereas high concentrations activated both Stat3 and Stat1. The dose-dependent selectivity of Stat activation has been shown by means of an electrophoretic mobility shift assay (6).

**FIG. 4.** Schematic representation of chimeric Stat proteins in which the carboxyl-terminal regions of Stat3 were substituted by those of Stat1. Open bars, parts originated from Stat3; shaded bars, Stat1. The abbreviations and numbers are the same as those described in the legend to Fig. 2.

**FIG. 5.** The region carboxyl-terminal to the SH2 domain of Stat3 is important for phosphorylation by IL-6 but not by EGF.

COS-7 cells were transfected with the chimeric Stat3/1 proteins described in Fig. 4. Cells were stimulated without or with recombinant human IL-6 and soluble IL-6 receptor (6) or recombinant human EGF (E) as described in the legend to Fig. 3. Cells were lysed and immunoprecipitated with the anti-Flag antibody M2. Immunoprecipitates were Western blotted using the monoclonal anti-phosphotyrosine antibody 4G10 (top panel) or the anti-Flag antibody M2 (bottom panel).

with IL-6 and soluble IL-6 receptor, the lysates were immunoprecipitated with anti-Flag antibody and then blotted with anti-phosphotyrosine and anti-Flag antibodies, respectively (Fig. 8). Anti-Flag antibody co-precipitated gp130 in COS cells transfected with the vector expressing wild-type Stat3, indicating that wild-type Stat3 associates with gp130. In COS cells transfected with the vector expressing wild-type Stat1 and stimulated with IL-6 and soluble IL-6 receptor, Stat1 was neither phosphorylated nor associated with gp130.

In contrast, all the swap mutants, 3/1J, 3/1K, 3/1L, and K709E, co-immunoprecipitated gp130, although only 3/1L was phosphorylated, as shown in Fig. 6B (Fig. 8). These results indicate that the 5 amino acid residues carboxyl-terminal to Tyr705 were critical for the IL-6-specific phosphorylation.
of Stat1 and Stat2 in response to IFNs. Changing the Stat1 SH2 domain to that of Stat2 prevented IFN-\(\gamma\) activation, whereas changing the SH2 domain of Stat2 to that of Stat1 allowed phosphorylation by IFN-\(\gamma\). It is most probable that the exchange of the SH2 domains altered the specific association of Stat proteins with the receptor complexes. In fact, the SH2 domain of each Stat protein interacts with a specific amino acid sequence containing phosphotyrosines in the receptors. The consensus sequence YXXQ in the cytoplasmic domain of gp130 is the critical determinant by which Stat3 is activated (33). The consensus sequence (Y440DKP) of the IFN-\(\gamma\)-chain may provide a binding site for Stat1 (28). Stat6 binds two tyrosine phosphopeptides (GYPKAFS and GYPKPFQ) derived from the intracellular domain of the IL-4 receptor \(\alpha\)-chain.

Heim et al. (34) demonstrated in the same study that the Stat1 chimeric constructs harboring the Stat2 sequence sur-
rounding the tyrosine phosphorylation site can be normally activated in response to IFN-α or IFN-γ. Therefore, they concluded that swapping the tyrosine phosphorylation site and its surrounding amino acids between Stat1 and Stat2 did not change the specificity of activation by IFN-α and IFN-γ. However, this study showed that the amino acid sequence carboxyl-terminal to Tyr705 in Stat3 contributes to IL-6-specific Stat3 activation. The chimeric protein in which the amino acid sequence immediately carboxyl-terminal to Tyr705 in Stat3 was replaced by the corresponding sequence derived from Stat1 was associated with gp130 but not activated, indicating that the SH2 domain is sufficient for Stat3 to bind gp130 but some additional factors are required for the phosphorylation of Stat3. When overexpressed, Jak5 equally activates all Stat proteins without ligand stimulation, so the Jak kinase has been regarded as having no specificity for substrate between Stat families (47, 48). We also confirm that our recombinant Stat3/1 proteins were all activated by co-expressed Jak1 (data not shown). In the case of receptor-mediated phosphorylation, some topological constraints in the receptor-Jak-Stat complex may hinder the access of Stat3/1 to the catalytic domain of Jak kinase. Kirshnan et al. (49) also showed that there might be some favorable geometry of the receptor-Jak-Stat complex to allow Jaks to phosphorylate Stat2 in IFN-α signaling.

All swap mutants between Stat1 and Stat3 were equally associated with the EGF receptor and phosphorylated Stat1 and Stat3 are both activated through the EGF receptor (4, 6, 8, 50). Studies using the EGF receptor deletion mutants showed that two intracellular tyrosine residues (Tyr1066 and Tyr1068) are necessary for Stat activation (51) and may provide binding sites for Stats. Although EGF stimulates Jak phosphorylation on tyrosine residues (52), whether the receptor activates Stats directly or through Jaks is in dispute. However, a study using Jak1-defective cells has revealed that the kinase activity of the receptor but not Jak1 is critical for Stat activation (53). Thus in EGF both the mechanism of recruitment and phosphorylation might be different from gp130. It remains to be established whether the amino acid sequence surrounding the tyrosine phosphorylation site is required for the activation of other Stats in other cytokine and growth factor signaling.

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