Differential Activation of Acute Phase Response Factor/Stat3 and Stat1 via the Cytoplasmic Domain of the Interleukin 6 Signal Transducer gp130

II. Src HOMOLOGY SH2 DOMAINS DEFINE THE SPECIFICITY OF STAT FACTOR ACTIVATION*

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Distinct yet overlapping sets of STAT transcription factors are activated by different cytokines. One example is the differential activation of acute phase response factor (APRF, also called Stat3) and Stat1 by interleukin 6 and interferon-γ. Interleukin 6 activates both factors while, at least in human cells, interferon-γ recruits only Stat1. Stat1 activation by interferon-γ is mediated through a cytosolic tyrosine motif, Y440, of the interferon-γ receptor. In an accompanying paper (Gerhardt, C., Heesel, B., Sasse, J., Hemmann, U., Landgraf, C., Schneider-Mergener, J., Horn, F., Heinrich, P. C., and Graeve, L. (1996) J. Biol. Chem. 271, 12991-12998), we demonstrated that two tyrosine motifs within the cytoplasmic part of the interleukin 6 signal transducer gp130 specifically mediate APRF activation while two others can recruit both APRF and Stat1. By expressing a series of Stat1/APRF domain swap mutants in COS-7 cells, we now determined which domains of Stat1 and APRF are involved in the specific recognition of phosphorytrosine motifs. Our data demonstrate that the SH2 domain is the sole determinant of specific STAT factor recruitment. Furthermore, the SH2 domain of Stat1 is able to recognize two unrelated types of phosphorytrosine motifs, one represented by the interferon-γ receptor Y440DKPH peptide, and the other by two gp130 YPXQ motifs. By molecular modeling, we propose three-dimensional model structures of the Stat1 and APRF SH2 domains which allow us to explain the different binding preferences of these factors and to predict amino acids crucial for specific peptide recognition.

Most interleukins, colony-stimulating factors, and interferons bind to plasma membrane receptors which are members of the hematopoietic receptor superfamily (1). These cytokines regulate cellular functions and gene expression via various intracellular signaling cascades of which the so-called JAK-STAT1 pathway has recently attracted considerable attention (2). This pathway has first been established for interferon (IFN) signaling. The transcription factors Stat1α, Stat1β, and Stat2, formerly known as p91, p84, and p113 components, respectively, of the IFN-stimulated gene factor-3 complex were shown to be activated by tyrosine phosphorylation in response to IFN-α (3) and Stat1 also by IFN-γ (4, 5). Subsequent to their phosphorylation, STAT factors homo- or heterodimerize, translocate to the nucleus, and bind to regulatory DNA elements of target genes. STAT factors contain putative SH3 and SH2 domains in their carboxy-terminal parts as well as potential leucine zipper-like α-helical structures toward their amino termini (6). The SH2 domains seem to be involved in both the activation process and the dimerization of the STATs (7). A centrally located portion of Stat1 has recently been demonstrated to represent its DNA-binding domain (8). Tyrosine phosphorylation of STATs is most likely catalyzed by members of the JAK family of protein-tyrosine kinases (9). To date, four members of that family, Jak1, Jak2, Jak3, and Tyk2, have been cloned, of which Jak1 and Tyk2 have been shown to be essential for IFN-α signaling while Jak1 and Jak2 are required in the IFN-γ pathway (10-12).

We and others have previously established that the JAK-STAT pathway also plays an important role in the signal transduction of cytokines of the interleukin 6 (IL-6) family. IL-6, a pleiotropic cytokine involved in hematopoiesis, regulation of immune responses, and the acute phase reaction binds to a plasma membrane receptor complex that consists of an IL-6 receptor, gp80, and a signal transducing component, gp130 (13, 14). Binding of IL-6 to its receptor induces the dimerization of gp130 (15), activation of the gp130-associated protein-tyrosine kinases Jak1, Jak2, and Tyk2 (16, 17), and the phosphorylation of gp130 at tyrosine residues (18). We have previously shown that IL-6 triggers the rapid activation and tyrosine phosphorylation of a latent transcription factor, acute phase response factor (APRF), which is implicated in the induction of multiple acute phase protein and immediate-early genes (16, 19-22). APRF has been proven to be a member of the STAT family and therefore cytokines that activate APRF in the presence of IFN-γ might also activate Stat1 and Stat2, or vice versa, in the absence of IFN-γ. It is therefore possible that the SH2 domains of Stat1 and APRF are involved in the interaction of the STAT family members with the IL-6 receptor gp130.

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§The abbreviations used are: JAK, Janus kinase; STAT, signal transducer and activator of transcription; IL, interleukin; IFN, interferon; IFN-γ R, interferon-γ receptor α-chain; Epo, erythropoietin; EpoR, erythropoietin receptor; APRF, acute phase response factor; EMSA, electrophoretic mobility shift assay; SH2, Src homology domain 2; SH3, Src homology domain 3; SIE, sis-induced element.
is now also called Stat3 (23–25). Furthermore, we have demonstrated that, in addition to APRF (Stat3), Stat1 is also activated in response to IL-6 (16).

Further insight into the mechanisms of STAT activation originated from our observation that IL-6 induces the transient association of the STATs with the signal transducer gp130 (16). This finding suggested that the phosphorylation of STAT factors occurs at the cytokine receptor cytoplasmic parts. In fact, recent data indicate that the activation of Stat1 by IFN-γ relies on the interaction of its SH2 domain with a phosphotyrosine motif, Y440DKPH, of the IFN-γ receptor α chain (IFN-γR) (26, 27). Similarly, the activation of IL-4-STAT (Stat6) by IL-4 requires specific phosphotyrosine motifs in the IL-4 receptor α subunit (28). In the case of gp130, Stahl et al. (29) have reported that four tyrosine motifs conforming to the consensus sequence YXXQ are all able to independently mediate APRF (Stat3) activation. Recent studies from our laboratory have demonstrated that two of these YXXQ motifs (Y767RHQ and Y814FKQ) give rise to the specific activation of APRF (Stat3) while the other two (Y905LPQ and Y915MPQ) are capable of mediating the activation of both APRF (Stat3) and Stat1 (56). The sequences of the latter motifs, however, do not resemble the IFN-γ Y440DKPH motif. This raised the question of how Stat1 can be activated through two distinct types of phosphotyrosine motifs. Is the Stat1 SH2 domain able to recognize different motifs on its own or are additional domains of Stat1 involved?

By use of a series of Stat1/Stat3 chimeras we now demonstrate that the Stat1 SH2 domain is both required and sufficient to direct the activation through both the IFN-γR Y440DKPH motif and the gp130 YXQ motifs. A domain swap mutant in which the SH2 domain of APRF (Stat3) was replaced by the one of Stat1 exhibited the same activation specificity as wild-type Stat1. This finding demonstrates that solely the SH2 domain defines the interaction of STATs with gp130, and that the SH2 domain of Stat1 is able to interact with distinct types of phosphotyrosine motifs. Furthermore, we present three-dimensional model structures of the Stat1 and APRF (Stat3) SH2 domains which were built by using known SH2 domain structures as templates. Based on these models we are now able to explain the different specificities of the two STAT factors for receptor phosphotyrosine motifs. In addition, these models allow us to predict amino acids within the SH2 domains that are crucial for specific STAT factor/cytokine receptor interactions.

**EXPERIMENTAL PROCEDURES**

**Materials—** Enzymes were purchased from Boehringer Mannheim, DMEM and antibiotics from Life Technologies, Inc., and fetal calf serum from Seromed (Berlin, Germany). Oligonucleotides were synthesized by Eurogentec (Lüttich, Belgium), Biometra (Göttingen, Germany), and MWG-Biotech (Ebersberg, Germany). Radiochemicals were from Amer sham. Recombinant human erythropoietin (Epo) was a generous gift of Boehringer Mannheim. Antiserum to Stat1 and Stat3 have been described previously (24, 30). A cDNA coding for the murine Epo receptor (EpoR) was provided by H. Lodish (Cambridge, MA). M2 monoclonal antibodies to the FLAG epitope (sequence DYKDDDDK) were from Kodak/ICL.

Construction of Expression Vectors for Stat1, Stat3, and Chimeric STAT Proteins—Sall sites were introduced 3′ of the stop codons into murine APRF (Stat3) and human Stat1 cDNAs that were cloned into pBluescriptII (Stratagene) vectors as described previously (19) with the following modifications. Protein-DNA complexes were separated by electrophoresis on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25 TBE for 4 h. The gels were fixed for 30 min in 10% acetic acid, 10% methanol in water, dried, and autoradiographed, or analyzed using a PhosphorImager (Molecular Dynamics). For supershift analysis of STAT/DNA complexes nuclear extracts were incubated in the gel shift mixture with antisera at a final dilution of 1:200 at room temperature for 30 min. The m67SIE-oligonucleotide probe was then added and the EMSA was performed.

**Immunoblots—** Cells were lysed as described previously (16), and equal amounts of cellular protein were separated by SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to polyvinylidene difluoride membranes using a semi-dry electroblotting apparatus, and antigens detected by incubation with the appropriate primary and horseradish peroxidase-coupled secondary antibodies. The membranes were developed using an enhanced chemiluminescence kit (ECL, Amersham).

**Molecular Modeling of SH2 Domains—** For manipulation and graphic representation of protein structures, the programs WHAT IF (34) and GRAMM (35) were used on an Indyg02 SGI computer. Energy minimizations were performed under vacuum conditions with the GROMOS program library (W.F. van Gunsteren, distributed by BIOMOS Molecular Software B.V., Laboratory of Physical Chemistry, University of Groningen). The following SH2 domain sequences and structures were used as templates: phospholipase C-γ1, Brookhaven data bank entry code 2pld (36); phosphatidylinositol 3-kinase p85α subunit, code 2pnb (37); Syp protein-tyrosine phosphatase, code 1ayd (38); c-abl protein-tyrosine kinase, code Tab2 (39); v-scr protein-tyrosine kinase, codes 1spr and 1shk (40). Initial amino acid sequence alignments of Stat1 and APRF (Stat3) with sequences of known structures was according to Shuai et al. (7) with minor changes due to structural requirements derived from the known SH2 structures. The sequential alignment of APRF (Stat3) codons 697–699 into GCG GAT CCA (silent mutation) and Stat1 codons 693–695 into CTG GAT CCC (thereby replacing glycine 695 by proline), respectively. The introduction of an XbaI site into p8-Stat3-Sal changed codons 716–717 to TCT AGA and replaced threonines 716 and 717 by serine and arginine, respectively. The corresponding position in the Stat1 cDNAs (codons 715–717) carried a K3E change.

By using the authentic SphI (codons 324–326 in Stat1 and 328–330 in Stat3), TaqI (Stat3 codons 569–570), and Msel (Stat1 codons 565–566) sites, and the newly introduced BamHI and XbaI sites, chimeric STAT cDNAs were constructed by exchanging the respective DNA fragments within the pBluescript vector context. The cDNAs were then subcloned into pSVL. All constructs were sequenced throughout chimeric and mutated regions.

The "R6020" point mutation in the Stat1 SH2 domain was generated by site-directed mutagenesis (U.S.E. kit, Pharmacia), changing Stat1 codon 602 from CGG (arginine) to CAG (glutamine). Further details on expression vector construction are available upon request.

**Construction of EpoR/gp130 Expression Vectors—** An expression vector coding for a chimeric receptor which consists of the murine Epo receptor (EpoR) extracellular part and the human gp130 transmembrane and cytoplasmic parts was constructed as follows. Murine EpoR cDNA was subcloned into pBluescript, and a unique EcoRI site was introduced into codons 246–247 by insertion of a linker oligonucleotide at the nearby NheI site. An XbaI-EcoRI fragment containing the portion of the gp130 extracellular part was then cloned into the XbaI and EcoRI sites of pSVL-gp130 (31) thereby fusing EpoR codon 245 in frame to gp130 codon 605. Construction of the expression vectors pSVL-EgY-FLAG, pSVL-EgY440, pSVL-EgY767, pSVL-EgY841, pSVL-EgY905, and pSVL-EgY195 is described elsewhere (56).

**Transformation of COS-7 Cells—** COS-7 cells (ATCC CRL 1651) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml). Cells were transfected by electroporation with a single pulse at 230 V and 96 μF using a Gene Pulser™(Bio-Rad). Approximately 2 × 10^6 cells were cotransfected in 0.8 ml of medium with 10 μg and 20 μg of expression vectors for receptor chimeras and STAT proteins, respectively. After electroporation, the cells were grown to confluency (48 to 72 h) and stimulated by adding human recombinant Epo (7 units/ml) to the medium for 15 min.

**Electrophoretic Mobility Shift Assay (EMSA)—** Nuclear proteins were extracted as described (32). DNA binding of STAT factors was analyzed by EMSA using a double-stranded ^32^P-labeled m67SIE-oligonucleotide derived from the sis-inducible element of the c-fos promoter region (33). EMSA was carried out as described previously (19) with the following modifications. Protein-DNA complexes were separated by electrophoresis on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25 TBE for 4 h. The gels were fixed for 30 min in 10% acetic acid, 10% methanol in water, dried, and autoradiographed, or analyzed using a PhosphorImager (Molecular Dynamics).
the known structures is based on the direct superposition of their backbone coordinates. The alignment implies a large insertion within the CD loops of both STAT SH2 domains (see Fig. 7) for which the template structures could not serve as a model. We therefore did not include this region in our models.

Using the structures mentioned above as templates, residues were exchanged according to the alignment. For amino acid insertions or deletions in loop regions, data base searches implemented in the used software were performed. After exchanging all residues using a data base search approach (34), graphical inspection showed an already good packing of side chains in the hydrophobic core of the domains which subsequently was optimized by side chain rotations and energy calculations.

Binding of IFN-γR and gp130 phosphotyrosine peptides to the STAT SH2 domains was modeled by using the coordinates of peptides py111P (py, phosphotyrosine) and pyVPML bound to the phospholipase C-γ1 and v-src kinase SH2 domains, respectively (36, 40).

**RESULTS**

Differential Activation of STAT Factors by Chimeric Receptors in COS-7 Cells—In many cell types, IL-6 gives rise to the activation of both APRF(Stat3) and Stat1 while, at least in human cells, IFN-γ activates Stat1 only. To determine which domains of Stat3 and Stat1 define this differential pattern of activation, we constructed expression vectors coding for a series of Stat3/Stat1 chimeric molecules as schematically shown in Fig. 1A. Chimera Stat3/1 combined the amino-terminal half of Stat3 with the carboxyl-terminal half of Stat1. In the other chimeras, internal parts of Stat3 were replaced by the respective Stat1 sequences. In Stat3/1(D, SH3 + 2, Y) and Stat3/1(D, SH3 + 2), the DNA-binding, SH3, and SH2 domains in combination with and without the Stat1 tyrosine phosphorylation site, respectively, were from Stat1 while in Stat3/1(SH2) only the Stat3 SH2 domain was replaced by the one of Stat1 (Fig. 1A).

COS-7 cells were used to overexpress wild-type and chimeric STAT proteins by transient transfection. Due to the presence of the IL-6 gp130 signal transducer in these cells, endogenous STAT factors can be activated by the addition of IL-6 plus soluble IL-6 receptor to the medium (56). To selectively target the transfected cells, an expression vector was cotransfected with and without the Stat1 tyrosine phosphorylation site, respectively, were from Stat1 while in Stat3/1(SH2) only the Stat3 SH2 domain was replaced by the one of Stat1 (Fig. 1A).

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To determine whether the chimeric receptor Eg has the potential of activating both STAT factors in COS-7 cells, we next cotransfected Stat1α or Stat3 expression vectors. Coexpression of Stat1α yielded only slightly enhanced activation of the factor (Fig. 2). Overexpression of Stat3, however, which gave rise to strongly enhanced Stat3 levels as detected by immunoblotting, resulted in the appearance of three DNA-protein complexes upon Eg treatment (Fig. 2). Supershift analysis demonstrated that the predominant, slowest migrating band was only recognized by Stat1 antisera and hence are formed by Stat3 homodimers whereas the middle and high-mobility bands represent complexes containing Stat3-Stat1 heterodimers, and Stat1α homodimers, respectively (Fig. 3). We conclude from these experiments that the chimeric receptor Eg can give rise to the activation of both Stat1α and Stat3 in COS-7 cells.

We next asked whether usage of hybrid receptors allows us to reproduce the differential STAT activation in response to IL-6 and IFN-γ. Activation of Stat1 by IFN-γ has been shown to rely on phosphorylation of tyrosine residue 440 of the IFN-γR (26, 27). We therefore constructed an expression vector that...
expressed Eeg, whereas in Eeg only the differential responsiveness of Stat1 and Stat3 to IL-6 and IFNγ is reproduced by the action of the hybrid receptors Eeg and Eegγ in COS-7 cells.

Activation of Chimeric STAT Proteins—Next, the chimeric STAT proteins described above were coexpressed with Eeg. We had observed previously that chimeric Stat3/Stat1 molecules comprising the amino-terminal half of Stat1 comigrate with Stat1 while chimeras containing the Stat3 amino terminus comigrate with Stat3 in EMSA experiments. The lack of endogenous Stat3 activation in response to Eeg therefore allowed us to determine whether chimeras of the latter type were activated or not. Coexpression with Eeg of all hybrid STAT proteins shown resulted in the appearance of additional EMSA bands with mobilities different from that of homodimeric Stat1 (Fig. 3). None of the factors was activated without prior addition of Epo to the medium (data not shown). This finding indicated that all these STAT chimeras could be activated through Eeg.

To further corroborate that conclusion, supershift analysis using specific Stat1 and Stat3 antisera were performed. Expression of chimera Stat3/1 yielded three EMSA bands exhibiting mobilities identical with the ones observed upon wild-type Stat3 expression (Fig. 3). Preincubation with an antiserum to Stat3 did not affect any of these bands demonstrating that none was due to endogenous Stat3. However, an antiserum to the Stat1 carboxyl terminus shifted all three bands. This is in accordance with the presence of the epitope recognized by that antibody in the Stat3/1 chimera.

Expression and activation of chimera Stat3/D, SH3 + 2, Y produced a similar pattern of three bands (Fig. 3). This chimera contains part of the epitope recognized by the Stat3 antiserum but not the one recognized by Stat1 antiserum. Accordingly, the two bands with lower mobility were abolished by preincubation with Stat3 antiserum while the Stat1 antibody shifted the two bands of higher mobility. We conclude that, like Stat3, the chimeras Stat3/1 and Stat3/D, SH3 + 2, Y can form both homodimers and heterodimers with Stat1 upon activation.

Expression and activation of chimeras Stat3/D, SH3 + 2 and Stat3/D, SH2 produced a different pattern of EMSA bands, with the predominant band comigrating with Stat3-Stat1 heterodimeric complexes (Fig. 3). This band was reduced by preincubation with antisera to Stat1 or Stat3 in both cases. Since the chimeras do not contain the epitope recognized by the Stat1 antibody, this finding demonstrates that the bands represent heterodimeric complexes of the chimeras with Stat1. Therefore, heterodimerization of these chimeras with Stat1 appears to play a role in their activation.

This view was further supported by the observation that complexes comigrating with Stat3 homodimers appeared when Stat1 levels were depleted by prior incubation with Stat1 antiserum (Fig. 3). In summary, we conclude from the above experiments that all chimeric STAT proteins constructed are functionally expressed in COS-7 cells, can be activated via the cytoplasmic part of gp130, and are able to bind DNA.

The SH2 Domain of Stat1 Solely Determines Activation through the IFNγ Y440DKPH Motif—Activation of all tested chimeric STAT proteins through the hybrid receptor Eeg reflects the ability of the gp130 cytoplasmic domain to recruit both Stat1 and APRF (Stat3). In contrast, Eegγ was expected to trigger the activation of only those STAT chimeras that contain the domain(s) of Stat1 involved in the interaction with the IFNγ phosphotyrosine motif.

We therefore next compared the activation of wild-type and

coded for a chimeric receptor, Eegγ440 (Fig. 1B) that also combined the EpoR extracellular part with transmembrane and membrane-proximal intracellular parts of gp130. However, in Eegγ440, the distal part of gp130 containing all tyrosine modules required for Stat1 activation (56) was replaced by the IFNγY440DKPH motif. Epo-stimulation of COS-7 cells expressing Eegγ440 resulted in a strong activation of endogenous Stat1α (Fig. 2). Coexpression of Stat1α or Stat3 did not significantly change the response to stimulation through Eegγ440 (Fig. 2, right panel). Therefore, even in the presence of overexpressed Stat3, as demonstrated by immunoblot analysis, Eegγ440 solely activated Stat1α. We conclude that the differential responsiveness of Stat1 and Stat3 to IL-6 and IFNγ is reproduced by the action of the hybrid receptors Eeg and Eegγ440 in COS-7 cells.
chimeric STAT proteins through Eg and EgYγ440 in COS-7 cells (Fig. 4). As already shown above, Stat1α was activated upon Epo stimulation of either receptor hybrid, both with and without overexpression of Stat1α protein. In contrast, only Eg supported efficient activation of Stat3. However, when the carboxyl-terminal part of Stat3 was replaced by the one of Stat1 (chimera Stat3/1) both receptor types were able to activate that protein. Similarly, chimeras Stat3/1[D, SH3 + 2, Y] and Stat3/1[D, SH3 + 2] were found to be activated through both Eg and EgYγ440 (Fig. 4). We conclude that neither the tyrosine phosphorylation site nor the adjacent carboxyl-terminal part of Stat1 participates in the specific recognition of phosphotyrosine motifs. Finally, chimera Stat3/1[SH2] was also found to be recruited by the IFN-γ R Y440DKPH motif (Fig. 5). This pattern was identical with the one obtained with Stat1. Therefore, the chimera Stat3/1[SH2] was activated via all motifs capable of Stat1 binding but not those which specifically mediate Stat3 activation. We conclude that the SH2 domain of Stat1 is the sole determinant of association with the gp130 YXPO motifs.

To prove that the putative SH2 domain of Stat1 acts as a bona fide SH2 domain when binding to gp130, we introduced point mutations replacing arginine 602 by glutamine in the chimeras Stat3/1 and Stat3/1[D, SH3 + 2, Y]. Stat1 arginine 602 corresponds to a conserved arginine that is located in the bona fide SH2 domain when binding to gp130, we introduced point mutations replacing arginine 602 by glutamine in the chimeras Stat3/1 and Stat3/1[D, SH3 + 2, Y]. Stat1 arginine 602 corresponds to a conserved arginine that is located in the SH2 domain, exhibits the same relative responsiveness to different receptor phosphotyrosine motifs as Stat1. Furthermore, these data prove that the Stat1 SH2 domain is able to recognize two entirely different phosphotyrosine motifs. We therefore asked whether it may be possible to build, based on the published coordinates of other SH2 domains, model Stat SH2 domains—Three-dimensional models of the Stat1 and APRF(Stat3) SH2 domains—Three-dimensional structures of SH2 domains of various proteins have been solved by NMR spectroscopy or x-ray. To date, however, no data on STAT SH2 domain structures are available. The phosphotyrosine motifs binding either the Stat1 or Stat3 SH2 domains (or both) are now well-defined by the data presented here and the accompanying paper (56). We therefore asked whether it may be possible to build, based on the published coordinates of other SH2 domains, model Stat SH2 structures which allow us to explain their distinct binding specificities and to predict residues crucial for ligand recognition and binding.

Fig. 7 shows the alignment of Stat1 and Stat3 sequences with template SH2 domains as used for molecular modeling.
All residues known to be involved in binding the phosphorylated tyrosine (43) are conserved in the STAT SH2 domains. Using this alignment, we were able to build models of the SH2 domains of the two STAT factors. For a 25-aa acid insertion in the CD loops, however, none of the known structures could serve as a template. Since the CD loops of other SH2 domains do not contribute to the specific recognition of phosphorysine motifs (43), it seemed rectified to omit this region from our models. Fig. 8 shows electrostatic potential maps of the Stat1 and Stat3 SH2 models. The positively charged (blue) binding pockets for phosphotyrosine are almost identical in both SH2 domains. It is evident from Fig. 8, however, that residues adjacent to the peptide binding groove differ significantly.

We next evaluated whether the model structures allow explanation of the different binding specificities of Stat1 and Stat3. First, binding of a peptide pYDKPH (where pY represents phosphotyrosine) which corresponds to the IFN-

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 motif specifically recruiting Stat1 was modeled (Fig. 8A). As one would expect for a peptide binding with high affinity to this domain (27), the sequence was easily fitted into the modeled Stat1 SH2 domain. Aspartate Y-1 is located in the vicinity of arginine 649 which is part of the βD strand. Lysine Y+2 is exposed to the solvent without making contacts with the SH2 domain while proline Y+3 inserts into a rather small hydrophobic pocket lined by tyrosines 651, 666, and 681, and proline 688. Furthermore, the model implies that a two-aa acid insertion in the BG loop of the Stat1 SH2 domain (compared to the Stat3 sequence) positions glutamate 686 in close proximity to histidine Y+4. These peptide-SH2 domain interactions are schematically illustrated in Fig. 9A. Taken together, our model predicts that major contributions to specific binding of this peptide originate from electrostatic interactions of amino acids Y+1 and Y+4 with residues of the Stat1 SH2 domain. This interpretation is in accordance with these experimental limitations by species being crucial for Stat1 activation through the IFN-γR, as has been proven experimentally by Greenlund et al. (26).

In the next step we similarly analyzed the interaction of the gp130 YXXQ motifs with Stat3. Peptide pYFKQN corresponding to Y814 of gp130 was fitted into the Stat3 SH2 model structure (Fig. 8B). Contacts predicted from this model are schematically shown in Fig. 8B. Interestingly, arginine 649 of Stat1 is replaced by methionine 655 in Stat3. The side chain of this methionine is located in the vicinity of phenylalanine Y+1. Again, lysine Y+2 is not involved in SH2 binding. However, glutamine Y+3 fits into a large pocket between the BG loop, αB helix, and the central β sheets. Within this pocket, the glutamine side chain is predicted to form hydrogen bonds with tyrosine 657, cysteine 687, serine 691, and glutamine 692. No obvious contact sites are proposed for asparagine Y+4.

The experimental data in this study defined a second motif, YXXQ, capable of binding the Stat1 SH2 domain. Attempts to fit peptide pYLPQT (Y905 of gp130) into the Stat1 SH2 model in a configuration analogous to binding of the above peptides failed entirely. The smaller binding pocket of the Stat1 SH2 model cannot accommodate the large glutamine side chain nor does it contain residues capable of forming hydrophobic bonds with that amino acid. We therefore searched for peptides with similar sequences bound by other SH2 domains. In fact, the v-src SH2 domain was previously shown to bind a peptide pYVPML of the platelet-derived growth factor receptor in a rather unconventional manner (40). The coordinates of that structure could successfully be applied to the interaction of peptide pYLPQT with the Stat1 SH2 model. A striking feature of the resulting configuration is that proline Y+2 although not itself being involved in contacting the SH2 domain is important for positioning glutamine Y+3 outside the actual peptide binding groove (Fig. 8C). As a result, the glutamine side chain does not enter the hydrophobic pocket but can still form hydrogen bonds with tyrosine 651 and glutamate 686 (Fig. 9C). The requirement for proline Y+2 to achieve that configuration is in

![Fig. 6. Mutation of arginine 602 within the Stat1 SH2 domain abolishes activation through gp130.](http://www.jbc.org/)

The alignment used for molecular modeling of STAT SH2 domains. Stat1 and Stat3 sequences were aligned with SH2 domains of the following proteins: p1C, phospholipase C-γ1 (36); p85, phosphatidylinositol 3-kinase p85α subunit (37); syp, protein-tyrosine phosphatase Syp (38); abl, c-abl protein-tyrosine kinase (39); src, v-src protein-tyrosine kinase (40, 41). Secondary structure characteristics are given in top following the common nomenclature (37, 39, 40, 43). The brackets indicate the part of CD loop not included in our models. Asterisks represent the residues involved in phosphorytyrosyl binding (43). The open arrowhead indicates the amino acid in the βD sheet contacting residue Y+1. Residues interacting with amino acids Y+3 and Y+4 in our models are shown by closed circles.
accordance with our results obtained using point mutations of that phosphotyrosine motif (56). In conclusion, we propose from our models that the different binding specificities of the two STAT SH2 domains are due to only few deviations of their primary structures, i.e. amino acid exchanges in the βD sheet (Stat1 arginine 649 → Stat3 methionine 655), the carboxyl-terminal border of the αB helix (Stat1 tyrosine 681 → Stat3 cysteine 687), and the BG loop (Stat1 alanine 687 and proline 688 → Stat3 serine 691 and glutamine 692, respectively), and an 2-amino acid insertion within the Stat1 BG loop (arginine 683 and proline 684).

DISCUSSION

One mechanism by which STAT factors can be activated involves their SH2 domain-mediated binding to phosphorylated tyrosine residues within distal parts of cytokine receptors. This pathway has now been demonstrated to be responsible for the activation of Stat1 by IFNγ and Stat6 by IL-4 and is likely to be involved in Stat5 activation by IL-2 (27, 28, 45). SH2 domains were also shown to participate in the activation of Stat1 and Stat2 by IFNα (46). However, it is not yet known which parts of the IFNα receptor are involved. Interestingly, activation of Stat1 by IFNα seems to depend on the presence of Stat2 (47) indicating the existence of an additional, rather indirect, mechanism of Stat1 recruitment. Furthermore, for Stat5 activation by EpR and growth hormone, distal receptor parts may not be required (48, 49). How STAT factors can be activated in these cases is unknown although one might speculate that STATs associate with phosphotyrosine residues in either Jak tyrosine kinases themselves or in adaptor proteins.

As we have shown previously, IL-6-type cytokines induce the transient association of STATs with the IL-6 signal transducer gp130 (16). We have now dissected the mechanism of Stat1 and APRF(Stat3) activation through gp130 in more detail. In an accompanying paper (56), it was demonstrated that among the four gp130 tyrosine motifs recently shown as being involved in APRF(Stat3) activation (29), two motifs specifically recruit APRF(Stat3) whereas the other two can accommodate both factors. The latter motifs contain a proline in position Y+2, therefore conforming to the consensus YXPQ.

Here we have shown by expressing Stat1/Stat3 chimeras in COS-7 cells that solely the SH2 domains of Stat1 and APRF(Stat3) define the specificity of their interaction with different receptor motifs. Other domains of the STATs do not influence this selective activation. The pivotal role of the SH2 domains was most convincingly demonstrated by a APRF(Stat3) protein which instead of its own SH2 domain contained the one of Stat1 (chimera Stat3/1[SH2]). This protein showed two intriguing characteristics: firstly, it was activated through the IFN-γR Y440DKPH motif which otherwise specifically mediates Stat1 activation. Therefore, the Stat1 SH2 domain can direct another STAT factor to being activated via this receptor motif. An analogous result was recently generated using Stat1/Stat2 chimeras (46). Secondly, chimera Stat3/1[SH2] showed the same pattern of activation through gp130 phosphotyrosine motifs as APRF(Stat3) through the respective motifs which suggests that the role of Stat1 SH2 domain is not to direct Stat3 activation via APRF(Stat3) motifs but rather to alter the selectivity of Stat1 activation toward APRF(Stat3) motifs.
wild-type Stat1, i.e. it was activated via YXPO motifs but not motifs lacking the proline in position Y+2. Thus, an exchange of solely the SH2 domain converted Stat3 from a factor that is able to bind all gp130 YXXQ motifs into one which selectively associated only with YXPO motifs. In conclusion, these findings demonstrate that both Stat1 and APRF(Stat3) interact by their SH2 domains with the distal region of gp130. Alternate mechanisms for Stat1 activation, i.e. an indirect activation requiring APRF(Stat3) or a mechanism not involving distal receptor parts, are ruled out by our data.

It is noteworthy that in contrast to human cells, IFNγ does activate both Stat1 and APRF(Stat3) in murine cells (50). The Y440KPH motif of the murine IFNγR, however, is identical with that of the human receptor. Furthermore, species-specific differences of the APRF(Stat3) protein cannot explain this discrepancy since we used the murine APRF(Stat3) cDNA in our studies. The phenomenon, however, may rely on a difference between the human and murine IFNγ receptor β chains. Neither the IFNγ receptor α chains nor the human IFNγ receptor β chain, accessory factor-1 (51), contain YXXQ motifs. In contrast, the cytosolic part of the murine IFNγ receptor β chain (52) contains a motif Y274WFQT which may give rise to APRF(Stat3) activation.

It is an interesting outcome of our studies that the Stat1 SH2 domain must be able to recognize at least two distinct phosphotyrosine motifs. The Y440KPH of the IFNγR requires the aspartate and histidine residues in positions Y+1 and Y+4, respectively, for efficient Stat1 recruitment (27). In contrast, in the YXPO motifs of gp130 the proline and glutamine residues are of importance (56). Therefore, these two types of motifs not only exhibit different sequences but their binding of Stat1 displays completely distinct sequence requirements.

It recently has been demonstrated that molecular modeling of SH2 domains can give valuable insights into the mode of specific SH2 domain-peptide binding and allows us to predict residues involved in the interactions (53). In order to understand the different preferences of Stat1 versus APRF(Stat3) on a more molecular level, we therefore built such models based on the coordinates of known SH2 structures. Although the overall structures of the Stat1 and APRF(Stat3) SH2 models turned out to be very similar, major changes appeared in the BG loop which participates in specific ligand recognition of other SH2 domains (43). Based on these models, we predict that the different binding specificities of the two STAT SH2 domains are due to only four amino acid exchanges, located in the βD sheet, the αB helix, and the BG loop, and a two-amino acid insertion in the BG loop of Stat1. One consequence of these differences is that aspartate Y+1 and histidine Y+4 of the IFNγR Y440 motif come into close contact to positively (arginine 649) and negatively charged (glutamate 686) residues, respectively, in the model structure proposed for the Stat1 SH2 domain but not for APRF(Stat3). Our models therefore allow us to explain why APRF(Stat3) is not activated through the IFNγR Y440 motif. Another consequence of the structural differences described above is the appearance of a small and rather hydrophobic binding pocket for the Y+3 residue in the Stat1 SH2 model. This pocket smoothly accommodates proline Y+3 of the IFNγR phosphotyrosine motif. In contrast, a larger binding pocket is predicted for the APRF(Stat3) SH2 domain. Several amino acids lining this pocket can form hydrogen bonds explaining the preference of this factor to bind motifs with a glutamine in position Y+3. Therefore, binding to the specific target motifs could be explained on the basis of our models.

The most challenging question, however, was how to explain the fact that the two gp130 YXPO motifs are able to independently recruit both factors in spite of the proposed structural differences of their SH2 domains as discussed above. In fact, our models completely rule out the possibility that these motifs are bound in a similar configuration by both SH2 domains. The small binding pocket of the Stat1 SH2 model would not allow insertion of a glutamine side chain. The requirement of proline Y+2 for binding of Stat1 (56) led us to propose that the configuration of YXPO binding to Stat1 rather resembles binding of the PDGF receptor peptide YVPL to the v-src SH2 domain (40) which shares the proline Y+2 residue with the YXPO motifs. Here, the kink introduced by proline Y+2 into the peptide backbone allows the Y+3 residue to be bound outside the actual binding pocket. This model convincingly explains the requirement of proline Y+2 for Stat1 binding. Other configurations may potentially fulfill the same criterion. However, a clear prediction of our model structures is that YDKPH and YXPO peptides must be differently configured upon binding.
to the Stat1 SH2 domain. This is supported by the experimental data which demonstrated different requirements for the binding to these two types of motifs, as discussed above.

Specificity of the JAK-STAT pathway is controlled at other levels as well. An important contribution of different JAK kinases was suggested by recent studies on human fibrosarcoma cells deficient in Jak1 (54). In such cells, IL-6 could no longer activate Stat1 while some (although reduced) APRF(Stat3) activation still occurred. Furthermore, STATs were shown to exhibit distinct preferences to bind various target DNA elements (55). Dimerization of STAT factors is thought to be defined by the mutual interaction of the SH2 domain of one factor with the phosphotyrosine of the other (7). Experiments presented here suggest that dimerization is also under a subtle structural control. Some of the chimeric STAT proteins showed a pronounced preference for heterodimerization with Stat1 over homodimerization. In fact, more recent data from our laboratory indicate that additional parts of the STAT proteins contribute to this process.

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Differential Activation of Acute Phase Response Factor/Stat3 and Stat1 via the Cytoplasmic Domain of the Interleukin 6 Signal Transducer gp130: II. Src Homology SH2 Domains Define the Specificity of STAT Factor Activation

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