Differential Activation of Acute Phase Response Factor/STAT3 and STAT1 via the Cytoplasmic Domain of the Interleukin 6 Signal Transducer gp130

I. DEFINITION OF A NOVEL PHOSPHOTYROSINE MOTIF MEDIATING STAT1 ACTIVATION

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Interleukin-6 (IL-6) and γ-interferon (IFNγ) activate an overlapping set of genes via the Jak/STAT pathway. However, at least in human cells, a differential activation of STAT transcription factors was observed: IL-6 activates both acute phase response factor (APRF) and STAT3 and STAT1, whereas IFNγ leads only to STAT1 activation. All STATs cloned so far contain SH2 domains. Since all cytokine receptors using the Jak/STAT pathway were found to be tyrosine-phosphorylated after ligand binding, it has been proposed that specific phosphotyrosine modules within the cytoplasmic domain of the receptor chains recruit different STAT factors. We have analyzed by mutational studies and by phosphopeptide competition assays which of the tyrosine modules of the IL-6 signal transducer gp130 are capable of recruiting either APRF or STAT1. We found that two of the four tyrosine modules that are important for APRF activation also activate STAT1. For these modules, we propose the new consensus sequence YPX. We further present evidence that STAT1 is activated independently from APRF suggesting that gp130 contains multiple independent STAT binding sites. We compare the APRF and STAT1 activation motifs of gp130 with the STAT1 activation motif of the IFNγ receptor and demonstrate that the specificity of activation can be changed from APRF to STAT1 and vice versa by only two point mutations within a tyrosine module. These data strongly support the concept that the activation of a specific STAT is determined mainly by the phosphotyrosine module. The significance of these findings for other receptor systems is discussed.

Interleukin 6 (IL-6) is a multifunctional cytokine synthesized by many different cells after appropriate stimulation. It acts on a wide spectrum of target cells and exerts multiple functions during the immune response, hematopoiesis, neuronal differentiation, and the acute phase reaction (1-4).

IL-6 acts via a cell surface receptor complex composed of two subunits: an 80-kDa IL-6-binding protein (IL-6 receptor) and a 130-kDa signal transducing protein, gp130 (5-7). gp130 is also the signal transducing component of the receptors for leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, interleukin 11, and cardiotrophin 1 (8, 9). Binding of IL-6 to its receptor induces the homodimerization of two gp130 molecules, thereby transducing the signal into the cell (10). The 61-amino acid juxtamembrane region of the 277-amino acid cytoplasmic domain of gp130 was found to be sufficient for mediating a proliferative signal in transfected BAFB03 cells (11). This region contains two short segments referred to as "box 1" and "box 2" which are conserved between members of the hematopoietic cytokine receptor family. Three members of the Janus tyrosine kinase family, Jak1, Jak2, and Tyk2, are found to be constitutively associated with gp130 and are activated and autophosphorylated after IL-6 stimulation (12, 13). Recently, Tanner et al. (14) have shown that the conserved box 1 motif within gp130 is required for the association with Jak1 and Jak2. The Jak kinases are believed to mediate the tyrosine phosphorylation of gp130, thereby creating docking sites for SH2 domain containing signaling molecules (15). All STAT factors contain SH2 as well as SH3 domains (16). The SH2 domain has been shown to be involved in both activation and dimerization of STAT1 and STAT2 in response to interferons (17, 18). Two members of the STAT family of transcription factors, namely APRF (or STAT3) and STAT1, are rapidly phosphorylated on tyrosine in a number of cell types upon IL-6 stimulation, a process for which Jak kinases were known. However, the specific activating motifs remain to be identified.

In the IFNγ receptor α-chain a single tyrosine residue, Tyr440, was found to be functionally important for signal transduction and binding of STAT1 to the receptor (25, 26). Mutational studies demonstrated that the important sequence in the IFNγ receptor is YDXXH, with tyrosine, aspartic acid, and histidine all being crucial for stimulation of major histocompatibility class I expression and IRF-1 induction.
Although STAT1 activation is also triggered via gp130, there is no sequence within the cytoplasmic tail of gp130 resembling the YDKPH motif of the IFN-γ receptor. Therefore, the question arose, how STAT1 recruitment can be triggered by IL-6. Three alternative mechanisms can be envisioned: (i) STAT1 is recruited directly via specific phosphorysine modules of gp130, (ii) STAT1 associates with gp130 only indirectly via bound APRF, and (iii) STAT1 associates directly with an activated Jak kinase. In the present study, we therefore identified by DNA binding competition assays with synthetic phosphopeptides and by mutational analysis the specific tyrosine motifs required for APRF and STAT1 activation through gp130. We found that APRF can be activated independently via four tyrosine modules but that only two of these were able to mediate STAT1 activation. The specificity of the activation sequences can be changed by two point mutations in both directions. The identification of a novel phosphorysine motif within the cytoplasmic domain of gp130 important for STAT1 activation strongly suggests that STAT1 transiently associates with gp130 independent of APRF.

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim (Mannheim, Germany). Oligonucleotides were either synthesized with the Gene Assembler (Pharmacia, Freiburg, Germany) or purchased from MWGBiotech (Ebersberg, Germany) or Eurogentec (Liege, Belgium). Biotra (Gottingen, Germany) or MWG Biotech (Ebersberg, Germany) or purchased from Eurogentec (Liege, Belgium). Biomatra (Gottingen, Germany) or MWGBiotech (Ebersberg, Germany). Recombinant human IL-6 was prepared as described by Arcone et al.

Calf serum was from Seromed (Berlin, Germany). Recombinant human erythropoietin has been kindly provided by J. Burg and K. H. Sellinger (Boehringer Mannheim, Penzberg, Germany). Recombinant human erythropoietin has been kindly provided by J. Burg and K. H. Sellinger (Boehringer Mannheim, Penzberg, Germany). Recombinant human erythropoietin has been kindly provided by J. Burg and K. H. Sellinger (Boehringer Mannheim, Penzberg, Germany). Recombinant human erythropoietin has been kindly provided by J. Burg and K. H. Sellinger (Boehringer Mannheim, Penzberg, Germany).

**Cell Culture**

COS-7 cells (ATCC CRL 1651) were grown in Dulbecco's modified Eagle's medium and HepG2 cells in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, streptomycin (100 mg/liter), and penicillin (60 mg/liter).

**Expression Vectors**

The construction of the pSVL-EgAβ—A pBluescript-vector containing the cDNA for the Eg-chimera was digested with Bgl II and BamHI sites and the sequence encoding 7 amino acids around tyrosines 759 and 767 of gp130 and 440 of the IFNγ receptor were inserted into pBl30. These constructs were digested with Sphl and BamHI and ligated with the FLAG-oligonucleotide (see above). The constructs were subcloned into pSVL-Eg as described.

Construction of pSVL-EgY915stop—An oligonucleotide coding for the Y915 motif containing a NsiI and Sall site were introduced in the NsiI/SalI-digested plasmid. The mutants were subcloned into pSVL-Eg as described.

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Preparation of Nuclear Extracts from HepG2 and COS-7 Cells

Nuclear extracts from HepG2 cells were prepared as described previously (22). Nuclear extracts of COS-7 cells were prepared as described by Andrews and Faller (29). The amount of protein was measured with a Bio-Rad™ protein assay.

**Electrophoretic Mobility Shift Assay (EMSA)**

EMSA were performed as described previously (22) with the following modifications. For nuclear extracts from COS-7 cells a gel shift incubation buffer without KC1 was used. We used a double-stranded oligonucleotide mutated SIE-gp130 plasmid from the c-fos promoter (m67SIE: 5'-GAT CGG GGA GGG ATT TAC GGG GAA ATG CTG-3') (30). The protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE at 20 V/cm for 4 h. Gels were fixed in 10% methanol, 10% acetic acid, and 80% water for 1 h, dried, and autoradiographed.

For the supershift assay, nuclear extracts, gel shift mixture, and antisera (in a final dilution of 1:500) were incubated for 30 min at room temperature. Then the double-stranded 32P-labeled m67SIE-oligonucleotide was added and the EMSAs were performed.

**Transfection**

Transfection of cells was carried out using the Gene Pulser™ from Bio-Rad Laboratories (Munich, Germany). 2 x 10^6 cells in 0.8 ml of DMEM were co-transfected with 10 µg of pSVL vector containing either the STAT1 or APRF cDNA plus 20 µg of pSVL vector containing EpoR/gp130 chimera cDNAs using a voltage of 230 V and a capacity of 960 µF. Three days after transfection, cells were used for additional studies.

**Synthesis of Nonphosphorylated and Tyrosine-phosphorylated Peptides**

All peptides were synthesized as amides on a multiple peptide synthesizer (Abimed AMS 422, Langenfeld, Germany) according to the standard Fmoc machine protocols using TentaGel S RAM resin (50 µm) (Rapp Polymere, Tuebingen, Germany) and PyBOP (benzotriazole-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) activation (Novabiochem, Bad Soden, Germany). Side chain protection groups were Tisyl (Asn, Gin, His, Cys), Pmc (Arg), tert-butyl (Ser, Thr, Tyr, Asp, Glu), and t-Boc (Lys, Trp). For the synthesis of the phosphorylated peptides, Fmoc-Tyr(PO3H2)-OH (Novabiochem, Bad Soden, Germany) was used. The peptides were cleaved from the resin and deprotected by treatment with 750 µg of phenol, 250 µl of ethanedithiol, 500 µl of thioanisole, 500 µl of water in 10 ml of trifluoroacetic acid for 4 h. After precipitating the peptides with cold tert-butyl methyl ether, the samples were washed six times with tert-butyl methyl ether, dissolved in 5% acetic acid, and freeze-dried. All products were purified to greater than 80% purity by preparative high performance liquid chromatography.

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RESULTS

In order to elucidate through which tyrosine module STAT1 is activated upon IL-6 stimulation, we first used a phosphopeptide competition assay. Such an approach has recently been used successfully in studying the activation of IL-4 STAT/STAT6 (31). Since the SH2 domain of STAT factor is believed to mediate both, binding to the receptor at phosphotyrosine sites and dimerization with a second phosphorylated STAT factor (18), incubation of a STAT dimer with a large molar excess of relevant phosphotyrosine peptides should lead to the dimer dissociation and thereby prevention of its binding to the specific DNA element.

We examined by electrophoretic mobility shift assays (EMSAs) the inhibitory effects of several synthetic peptides containing each one of the six tyrosine residues of the intracellular domain of gp130 in a nonphosphorylated and a phosphorylated form (Fig. 1A). In addition, the following peptides were analyzed: Yγ, which contains tyrosine 440 of the IFNγ receptor (32); YAPRF, which contains tyrosine 705 of APRF believed to play a critical role in dimerization (19); YRHE, which contains tyrosine 767 but has a point mutation from glutamine (Q) to glutamic acid (E) at position +3 and Yn, which contains the identical amino acids as peptide Y767 but in a random formation (Fig. 1A).

Nuclear extracts from HepG2 cells stimulated with IL-6 were analyzed using an oligonucleotide with high affinity to both APRF and STAT1 (19, 30). EMSAs of control extracts show three complexes (Fig. 1B, left lanes) which are (from top to bottom), APRF homodimers, APRF-STAT1 heterodimers, and STAT1 homodimers (19). Four of the six phosphopeptides from gp130 were able to specifically inhibit the formation of DNA-protein complexes: Y767, Y814, Y905, and Y915 (Fig. 1B), whereas Y683 and Y759 had no effect (data not shown). Peptides Y767 and Y905 were effective already at the lowest concentration used (30 μM), phosphopeptides Y814 and Y915 inhibited the DNA binding of STATs only when used at higher concentrations. The phosphopeptide YRHE, which has the same sequence as Y767 except for a point mutation Gln→Glu at position +3 showed almost no competition indicating that the glutamine in position +3 from tyrosine 767 is important for APRF activation. Peptide Yn, from the IFNγ receptor preferentially abolished the association with the DNA of STAT1 and only at high concentrations reduced that of APRF. Interestingly, the phosphopeptide from APRF (YAPRF) also competed STAT1 and APRF DNA binding. Random peptide Yn showed no competition, nor did the nonphosphorylated peptides (Fig. 1B, right lanes).

To more precisely identify the tyrosine residues of gp130 necessary for STAT1 recruitment, we constructed a series of chimeric receptors consisting of the extracellular domain of the erythropoietin receptor (EpoR) and transmembrane and cytoplasmic parts of gp130 (Fig. 2). These chimeric receptors allowed us to study the activation of the STAT factors independently of endogenous gp130 in transiently transfected COS-7 cells which do not express the EpoR. The expression of the different chimeric receptors as analyzed by Western blotting using an antibody to the FLAG epitope was found to be comparable (data not shown). Three days after transfection, cells were stimulated with Epo, nuclear extracts were prepared and analyzed by EMSAs.

To first investigate whether both STAT1 and APRF activa-
tion can be triggered through the EpoR/gp130 chimera (Eg), we compared the STAT factor activation by Epo in transfected COS-7 cells with the one stimulated by an IL-6-soluble IL-6R complex in untransfected cells. Stimulation via the endogenous gp130 in nontransfected COS-7 cells by soluble IL-6R and IL-6 resulted in the formation of three DNA-protein complexes, designated a, b, and c corresponding to APRF homodimers, APRF-STAT1 heterodimers, and STAT1 homodimers, respectively (Fig. 3) (19, 33). After transient transfection of COS-7 cells with cDNAs coding for the chimera containing the complete cytoplasmic domain of gp130 (Eg) and murine APRF, complex a and, to a minor extent, complex b were formed (Fig. 3). Both disappeared after preincubation with APRF antiserum, whereas treatment with STAT1 antibodies had no effect. Nuclear extracts from COS-7 cells expressing the Eg-chimera and STAT1 showed only complex c. Anti-APRF treatment had no effect, whereas incubation with anti-STAT1 led to a supershift. From these results we conclude that both APRF and STAT1 can be activated via the Eg-chimera. However, our observations indicated that the endogenous levels of the two STAT factors in COS-7 cells are quite different. Stimulation of endogenous gp130 preferentially activated STAT1 but only small amounts of APRF. This is in contrast to the typical IL-6 response in HepG2 and other cells (Fig. 1B). Similarly, in COS-7 cells that were transfected with the Eg-chimera alone, preferential STAT1 activation was observed. Also, Wen and colleagues (34) found only low endogenous amounts of APRF in COS-7 cells. Therefore, all subsequent studies were performed with either APRF or STAT1 coexpression.

To investigate which of the tyrosine residues within the cytoplasmic domain of gp130 are responsible for the activation of APRF and/or STAT1, we performed coexpressions of the different chimeras (Fig. 2) with either APRF or STAT1. In control cells which were transfected with the pSVL vector and either APRF- or STAT1-cDNAs, no activation of STAT factors was detected (Fig. 4). The full-length chimera (Eg) was able to activate both APRF and, to a minor extent, STAT1. In contrast, the deletion mutant EgΔ5, which lacks the two carboxyl-terminal tyrosine residues of gp130 resulted in the activation of exogenous APRF, whereas coexpressed STAT1 could not be recruited. Deletion mutant EgΔ18 which includes only the box 1 and 2 regions of gp130 and therefore lacks the five carboxyl-terminal tyrosine residues, activated neither APRF nor STAT1. These results indicate that the last two tyrosine residues are required for STAT1 recruitment.

In order to further investigate which tyrosine residue directs the activation of STAT1, chimeric molecules containing box 1 and box 2 plus one of the five distal tyrosine modules of gp130 were tested (Fig. 2). For comparison, a construct containing box 1 and box 2 of gp130 and the tyrosine module of the IFN-γ receptor was used (EgYγ440). Stimulation of chimeric receptor Egγ759 did not result in the activation of any of the two STAT factors (Fig. 4). Recently, it was shown that this tyrosine residue binds the phosphotyrosine phosphatase PTP1D/syp (24). In contrast, the chimeras containing tyrosine modules Y767 and
Y814 predominantly activated APRF. However, a weak activation of endogenous STAT1 also must have occurred since in both cases small amounts of the APRF/STAT1 heterodimer were formed. The chimeric receptors containing Y905 and Y915, however, were capable of stimulating both STAT proteins; nevertheless, they were still more active toward APRF (Fig. 4, lower panel). The last amino acid in gp130 is the glutamine in position +3 from tyrosine 915. To exclude that the amino acid residues of the FLAG epitope that is fused to the Y915 module in our construct artificially create an activation motif for STATs, we also constructed a chimeric receptor lacking this epitope. This protein showed the same activation pattern as EgY915 with the FLAG epitope (data not shown). Thus, our data show that the strong residues 767, 814, 905, and 915 recruit APRF, whereas tyrosines 905 and 915 can also recruit STAT1.

A mutant receptor EgY440 containing the tyrosine module of the IFNγ receptor crucial for the activation of STAT1 by IFNγ (25) stimulated DNA binding mainly of STAT1 (Fig. 4). Only in cells overexpressing APRF, also low amounts of APRF and APRF/STAT1 heterodimer were activated. This result demonstrates that the specificity of STAT activation is determined neither by the box 1 and 2 region nor by the kinases involved (Jak1 and Jak2 in both cases) but rather by the tyrosine motifs within the cytoplasmic domain of the signal transducer.

Stahl et al. (24) have recently suggested that the consensus sequence for APRF activation is YXXQ. For the STAT1 recruitment by the IFNγ receptor, the sequence YXXQ has been proposed (25). Both gp130 tyrosine modules, namely module Y905 and Y915, that are capable of activating STAT1 comprise the sequence YXQ. To further define the amino acid requirements of these different types of motifs, we introduced various point mutations. The tyrosine module Y814 was found to have the highest selectivity of activation toward APRF (Fig. 4). Since its sequence YFKQN varies by 3 amino acids from the sequence of the IFNγ module YDKPH which shows the best selectivity toward STAT1, we constructed chimeras containing point mutations within the Y814 module of gp130 or the Y440 module of the IFNγR (Fig. 5). Each module contained a centrally located tyrosine flanked on both sides by 5 amino acids of the authentic sequence of the gp130 or IFNγR, respectively. The motif Y814 (YFKQN) was mutated to YDKQN, YDKPN, and YDKQH. Likewise, we changed the IFNγR sequence (YD-KPH) into YFKPH, YDKQH, and YFKQH which corresponds to the 4-amino-acid motif of Y814 in gp130. When nuclear extracts from COS-7 cells expressing these chimeras were tested in EMSAs, we found that changing the Y814 motif (YFKQN) into YDKQN resulted in a major loss of activated APRF and a complete disappearance of the small amount of activated STAT1 (Fig. 6A). The mutation of YFKQN to YDKPN led to a chimeric receptor which caused reduced APRF but an enhanced STAT1 activation. However, full activation of STAT1 was obtained by the motif containing the double mutant YFKQN → YD-KQN. Therefore, by exchanging 2 amino acids downstream of the tyrosine at position +1 and +4 we were able to switch the selectivity completely from APRF to STAT1.

When the reverse exchanges were analyzed, it was found that mutation of Y440DKPH into Y440FKPH resulted in the loss of STAT1 activation, whereas the change of Y440DKPH into Y440DKQH had no effect (Fig. 6B). However, mutation of Y440DKPH into Y440FKQH which creates the 4-amino-acid sequence of Y814, resulted in a total change of the activation selectivity. The STAT1 homodimer could not be activated any longer, whereas the APRF homodimer was formed to the same extent as it was via authentic Y814.

Tyrosine module Y905 (YLQ) is one of the two motifs which was able to recruit both STAT factors. It differs from Y814 (YFKQ) by 2 amino acids, a leucine at +1 and a proline at +2. To study the importance of these two residues, we constructed chimeric receptors with the mutations YLQ → YFQ and YLQ → YLKQ (Fig. 5). The mutant EgY905FQ activated APRF in a manner comparable to the authentic module; the STAT1 homodimer, however, was found to be reduced (Fig. 7). The mutant EgY905LKQ showed reduced APRF and an even more diminished STAT1 activation. This indicates that the hydrophobic leucine at position +1 and more importantly the

| Chimera | Y767 | Y814 | Y905 | Y915 | Y440 |
|---------|------|------|------|------|------|
| APRF    | +    | +    | +    | +    | +    |
| STAT1   | -    | +    | +    | +    | +    |

| Mutated tyrosine modules (Y814) |
|-------------------------------|
| Y814  | L P R Q Q |
| Y814DKQ | Y D K Q N |
| Y814DKP | Y D K P N |
| Y814DKQH | Y D K Q H |

| Mutated tyrosine modules (Y440) |
|-------------------------------|
| Y440FQ | P T S F G |
| Y440FKP | Y F K P H |
| Y440FKQ | Y F K Q H |

| Mutated tyrosine modules (Y905) |
|-------------------------------|
| Y905F | G M P K S |
| Y905FPQ | Y F P Q T |
| Y905KQ | Y L K Q T |

FIG. 4. Activation of APRF and STAT1 by Epo via the chimeric receptors. COS-7 cells were transiently transfected by electroporation with expression vectors for chimeric receptors and STATs as indicated. After 3 days, cells were stimulated with 7 units of Epo/ml, and nuclear extracts were prepared. EMSAs were performed with the labeled m67SIE probe.

FIG. 5. Mutated tyrosine modules of Y814, Yγ440, and Y905. The chimeric receptors were constructed as described under "Experimental Procedures." The tyrosine modules are located distal to the box 1 and 2 region.
Activation of APRF and STAT1 via Signal Transducer gp130

In this study we have identified the tyrosine-containing motifs in the cytoplasmic domain of the IL-6 signal transducer gp130 which are responsible for the differential activation of the transcription factors APRF and STAT1. It is currently believed that upon binding of IL-6 to its receptor a homodimerization of the signal transducer gp130 is induced whereby associated kinases of the Jak family become activated. These kinases then phosphorylate and activate each other which in turn leads to the tyrosine phosphorylation of the signal transducer. The phosphotyrosine residues within gp130 are thought to create docking sites for signaling molecules containing SH2 domains, such as the STAT factors. To date, two of the seven modules, namely Y767, Y814, Y905, and Y915, are able to recruit APRF. In COS-7 cells, this observation was made only after coexpression of APRF; endogenous APRF could only weakly be activated by a complex of IL-6 and the soluble IL-6 receptor (Fig. 3) or by the Eg-chimera (data not shown). This result is most likely due to the fact that COS-7 cells express only small amounts of APRF, in contrast to STAT1, which is activated even without cotransfection (Fig. 3).

We have also demonstrated that two of the four tyrosine modules, namely Y905 and Y915, are capable of prominently activating STAT1 (Fig. 4). In contrast, Stahl et al. (24) did not observe tyrosine phosphorylation of this transcription factor. Since tyrosine phosphorylation of STATs was shown to be a prerequisite for nuclear transport and binding to DNA (36), our finding is not in accordance with the one of Stahl et al. (24). One possibility to explain these differences might be that the assay used in our studies (EMSA) more sensitively measures active STAT1 compared to the detection of phosphotyrosines in a Western blot. Alternatively, different expression levels of endogenous STAT1 in COS-7 cells could be responsible for this discrepancy.

The ability of modules Y905 and Y915 to recruit STAT1 in the absence of activated APRF strongly suggests that STAT1 via its SH2 domain binds directly to the phosphorylated gp130 dimer and not indirectly via APRF. Our results also rule out the possibility that STAT1 recruitment occurs directly via an active Jak kinase since the mutant EgJ3 which still contains the kinase binding region showed no activation. Thus, we propose that the activation of APRF and STAT1 via the cytoplasmic domain of gp130 is mediated by multiple independent docking sites. It could be hypothesized that for the formation of heterodimers either both factors bind to the same signal transducer chain at different sites or different factors bind to corresponding sites within the dimerized gp130. Future studies have to address these questions.

All modules of gp130 that were able to activate APRF contain the 4-amino acid sequence YXXQ (Ref. 24 and this study), the two modules which also efficiently activate STAT1 the sequence YQPQ. For STAT1 activation through the IFN-γR, the consensus sequence YDXXH was proposed (25). To demonstrate that these 4- or 5-amino acid sequences are not only necessary but also sufficient for determining the specificity toward APRF and STAT1, we introduced a number of point mutations into tyrosine modules Y814 and Y440 in order to change their activation characteristics. In both cases, the exchange of only 2 amino acids resulted in a complete reversal of specificity.

Introduction of a negative charge by aspartate at position +1 of Y814 in all cases strongly impaired APRF activation (Fig. 6A). Aspartate is found at this position in the IFN-γR, the consensus sequence YDXXH was proposed (25). To demonstrate that these 4- or 5-amino acid sequences are not only necessary but also sufficient for determining the specificity toward APRF and STAT1, we introduced a number of point mutations into tyrosine modules Y814 and Y440 in order to change their activation characteristics. In both cases, the exchange of only 2 amino acids resulted in a complete reversal of specificity.

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In accordance with the results of Stahl and co-workers (24), we found in this study that four of the six tyrosine modules of gp130, namely Y767, Y814, Y905, and Y915, are able to recruit APRF. In COS-7 cells, this observation was made only after coexpression of APRF; endogenous APRF could only weakly be activated by a complex of IL-6 and the soluble IL-6 receptor (Fig. 3) or by the Eg-chimera (data not shown). This result is most likely due to the fact that COS-7 cells express only small amounts of APRF, in contrast to STAT1 which is activated even without cotransfection (Fig. 3).

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In accordance with the results of Stahl and co-workers (24), we found in this study that four of the six tyrosine modules of gp130, namely Y767, Y814, Y905, and Y915, are able to recruit APRF. In COS-7 cells, this observation was made only after coexpression of APRF; endogenous APRF could only weakly be activated by a complex of IL-6 and the soluble IL-6 receptor (Fig. 3) or by the Eg-chimera (data not shown). This result is most likely due to the fact that COS-7 cells express only small amounts of APRF, in contrast to STAT1 which is activated even without cotransfection (Fig. 3).

We have also demonstrated that two of the four tyrosine modules, namely Y905 and Y915, are capable of prominently activating STAT1 (Fig. 4). In contrast, Stahl et al. (24) did not observe tyrosine phosphorylation of this transcription factor. Since tyrosine phosphorylation of STATs was shown to be a prerequisite for nuclear transport and binding to DNA (36), our finding is not in accordance with the one of Stahl et al. (24). One possibility to explain these differences might be that the assay used in our studies (EMSA) more sensitively measures active STAT1 compared to the detection of phosphotyrosines in a Western blot. Alternatively, different expression levels of endogenous STAT1 in COS-7 cells could be responsible for this discrepancy.

The ability of modules Y905 and Y915 to recruit STAT1 in the absence of activated APRF strongly suggests that STAT1 via its SH2 domain binds directly to the phosphorylated gp130 dimer and not indirectly via APRF. Our results also rule out the possibility that STAT1 recruitment occurs directly via an active Jak kinase since the mutant EgJ3 which still contains the kinase binding region showed no activation. Thus, we propose that the activation of APRF and STAT1 via the cytoplasmic domain of gp130 is mediated by multiple independent docking sites. It could be hypothesized that for the formation of heterodimers either both factors bind to the same signal transducer chain at different sites or different factors bind to corresponding sites within the dimerized gp130. Future studies have to address these questions.

All modules of gp130 that were able to activate APRF contain the 4-amino acid sequence YXXQ (Ref. 24 and this study), the two modules which also efficiently activate STAT1 the sequence YQPQ. For STAT1 activation through the IFN-γR, the consensus sequence YDXXH was proposed (25). To demonstrate that these 4- or 5-amino acid sequences are not only necessary but also sufficient for determining the specificity toward APRF and STAT1, we introduced a number of point mutations into tyrosine modules Y814 and Y440 in order to change their activation characteristics. In both cases, the exchange of only 2 amino acids resulted in a complete reversal of specificity.

Introduction of a negative charge by aspartate at position +1 of Y814 in all cases strongly impaired APRF activation (Fig. 6A). Aspartate is found at this position in the IFN-γR, the consensus sequence YDXXH was proposed (25). To demonstrate that these 4- or 5-amino acid sequences are not only necessary but also sufficient for determining the specificity toward APRF and STAT1, we introduced a number of point mutations into tyrosine modules Y814 and Y440 in order to change their activation characteristics. In both cases, the exchange of only 2 amino acids resulted in a complete reversal of specificity.
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specificity from STAT1 to APRF by introducing a phenylalanine at position +1 and a glutamine at position +3. As mentioned above, position +1 does not tolerate a negatively charged residue. Within the four tyrosine motifs from gp130 and the two STAT factor motifs responsible for homo- or heterodimerization (APRF, Y705LKTK; STAT1, Y701IKTE) at this position either a hydrophobic amino acid (Phe, Leu, Met, Ile) or a positively charged arginine was found (Fig. 28). At position +2, a positive charge (histidine or lysine) or a proline was observed. Thus, for APRF activation, we propose the consensus sequence YX₉₋₁₀Xₙ₋₃PQ (h = hydrophobic, p = positive charge).

The most obvious difference between the two phosphotyrosine modules of gp130 that were capable of recruiting STAT1 and module Y₄₄₀ is the lack of charged amino acids in the gp130 modules. The mutations of residues at position +1 and +2 in Y₉₀₅ demonstrated that both contribute to the specificity for STAT1 and that the introduction of a positive charge (lysine) at position +2 destroys the affinity for STAT1. In the module Y₄₄₀, a lysine at this position is tolerated; however, only in combination with a negative charge at position +1. Thus for STAT1 activation via a module that also activates APRF, the consensus sequence YX₉₋₁₀Xₙ₋₃PQ is proposed. These interpretations are consistent with the models for the SH2 domains of APRF and STAT1 that are proposed. In conclusion, our results are consistent with the models for the SH2 domains of APRF and STAT1 and that the introduction of a positively charged residue at position +2 destroys the affinity for STAT1. In the module Y₄₄₀, a lysine at this position is tolerated; however, only in combination with a negative charge at position +1. Thus for STAT1 activation via a module that also activates APRF, the consensus sequence YX₉₋₁₀Xₙ₋₃PQ is proposed. These interpretations are consistent with the models for the SH2 domains of APRF and STAT1 that are proposed.

The studies with the synthetic phosphopeptides only partially confirmed the data found by transfection of the chimeric receptors. The gp130 phosphopeptides Y₇₋₇₆ and Y₉₋₉₀₅ most efficiently inhibited the DNA binding of both STAT factors. A differential activation of STAT1 by module Y₉₀₅ was as found by the studies of chimeras could not have been predicted from this result. Likewise, phosphopeptide Y₉₋₇₆ inactivated both STAT1 and APRF dimers, whereas the corresponding chimera activated only STAT1. Probably, these in vitro competition assays can measure only large affinity differences whereas already subtle differences may be important for the in vivo choice of STATs. Thus, studies of this kind have to be interpreted with care and should be confirmed by independent methods. Interestingly, the phosphopeptide containing Y₇₋₇₆ of APRF also is capable of dissociating the STAT dimers, suggesting that this phosphotyrosine residue is actually responsible for the dimerization.

In addition to IL-6, a number of other cytokines were recently described to activate APRF. A search in a protein database library for sequences that agree with the consensus sequences found for the activation of either APRF or APRF and STAT1 revealed appropriate motifs in the cytoplasmic domains of receptors for leukemia inhibitory factor, granulocyte colony-stimulating factor, interleukin 10 and 12, interferon-α, and thrombopoietin (Fig. 8). Interestingly, such a motif was also found in the β-chain of the mouse, but not the human IFNγR. This might explain why some authors (using murine cells) have reported that APRF is activated upon IFNγ stimulation, whereas in human cells this was not the case (23, 37).

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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: I. DEFINITION OF A NOVEL PHOSPHOTYROSINE MOTIF MEDIATING STAT1 ACTIVATION
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