Comparative study on the phosphotyrosine motifs of different cytokine receptors involved in STAT5 activation

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Abstract Several cytokines and growth factors activate transcription of their target genes via the JAK/STAT signalling pathway. It has been shown that the interaction between SH2 domains of STAT factors and receptor phosphotyrosine residues plays an essential role in the specific recruitment of STATs. For STAT5, however, the importance of receptor tyrosines is still controversial. Using a chimeric receptor system in COS-7 cells, we studied the activation of STAT5 through the interleukin-6 signal transducer gp130. In contrast to previous reports, we did not detect gp130-mediated STAT5 activation. However, STAT5 activation was achieved when tyrosine motifs of other cytokine receptors were fused to the membrane-proximal part of gp130. The comparison of the relative potency of different tyrosine motifs revealed that hydrophobic amino acids, preferentially leucine, in positions +1 and +3, and an aspartate residue in position −1 or −2 with respect to the tyrosine are likely to be required for efficient STAT5 recruitment. In summary, we show here for the first time that phosphotyrosine motifs can confer the ability to activate STAT5 to a heterologous receptor.

Key words: Cytokine receptor; STAT5; Tyrosine phosphorylation; Signal transduction; gp130

1. Introduction

A variety of cytokines, growth factors, and hormones bind to structurally related receptors of the hematopoietin receptor superfamily, the members of which are characterized by four conserved cysteine residues and a WSXWS motif in their extracellular domains [1]. Ligand binding to these receptors results in homo- or heterodimerization of the receptor molecules [2–4]. Subsequently cytoplasmic protein kinases which belong to the JAK family of tyrosine kinases become activated. JAK1, JAK2, JAK3, and TYK2 [5] are constitutively associated with the membrane-proximal portions of cytokine receptor cytoplasmic domains (box 1/2) [6,7]. After activation they phosphorylate the receptor molecules and a number of intracellular proteins, including the members of a family of transcription factors known as STATs (signal transducers and activators of transcription) [8]. All STAT factors contain a

SH2 domain which enables them to homo- or heterodimerize after tyrosine phosphorylation, with the SH2 domain of one STAT molecule binding to the phosphotyrosine residue of the other STAT [9]. STAT dimers then translocate to the nucleus where they bind to the promoter regions of their target genes [10–12]. Up to now, seven members of the STAT family of transcription factors have been cloned from mammalian cells, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. Furthermore, a STAT-like factor, d-STAT or Marelle, has recently been detected in insect cells [13].

Interestingly, several STAT factors can be activated by various cytokines which signal via distinct JAK kinases. For example, erythropoietin, prolactin, growth hormone, and IL2 all trigger the phosphorylation and activation of STAT5. The erythropoietin, growth hormone, and prolactin receptors are associated with JAK2 while the IL2 receptor is known to recruit JAK1 and JAK3 [14]. On the other hand, IL4 does not activate STAT5 although the IL4 receptor is associated with JAK1 and JAK3, too. In view of these data, the question arises of how specificity in signalling via the JAK-STAT pathway is achieved.

The experimental finding that the cytoplasmic parts of cytokine receptors not only contain a JAK binding domain, but also selectively bind different kinase substrates, provides a possible explanation of how specific STAT factors can be activated by specific cytokines. For the signal transducing subunit of the receptor complexes for IL6-type cytokines, gp130, it has been shown that the receptor phosphotyrosine motifs YXPQ are able to bind STAT1 and STAT3, whereas the motifs YXXQ recruit STAT3 only. In both cases binding of the STATs leads to their activation by the receptor-associated JAK kinases [15,16].

In the case of STAT5, the discussion about whether its activation depends on receptor phosphotyrosine residues is still unresolved. STAT5 was originally discovered as mammary gland factor in mammary epithelial cells, where it is activated by prolactin [17]. Several other cytokines and growth factors also lead to the activation of STAT5, e.g. IL2, erythropoietin, GM-CSF, and growth hormone. For the erythropoietin and growth hormone receptors, and the IL2 receptor β-chain, it has been shown that specific tyrosine residues which become phosphorylated after ligand binding are necessary for the activation of STAT5 [18–23]. On the other hand, it has been described that STAT5 activation via gp130 occurs independently of any receptor phosphotyrosine residues [24]. Some investigators reported that in the case of growth hormone receptor phosphotyrosines do not play a role in STAT activation, either [25].

In order to analyze activation of STAT5 via gp130 and to

determine how STAT5 is recruited to cytokine receptors and whether receptor phosphotyrosine residues are necessary for its activation, we created a series of chimeric receptors based on a truncated receptor which is not able to activate STAT factors on its own and of tyrosine motifs taken from different cytokine receptors which are able to mediate STAT5 activation. In our experimental system the ability to activate STAT5 could be transferred to the truncated receptor construct by different tyrosine modules which showed some homology, indicating that receptor tyrosine motifs play an important role for the activation of STAT5. Activation of STAT5 through gp130, however, could not be observed.

2. Materials and methods

2.1. Reagents

Restriction enzymes and T4-DNA ligase were purchased from Boehringer Mannheim (Mannheim, Germany). Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany). The monoclonal antibody M2 against the FLAG epitope (N-DYKDDDDK-C) was from Kodak (New Haven, CT, USA). DMEM and DMEM/F12 mix were from Gibco (Eggenstein, Germany), fetal calf serum from Seromed (Berlin, Germany). Recombinant human IL6 was prepared as described by Arcone et al. [26]. Its specific activity was 1×10^6 B-cell stimulatory factor-2 units/mg protein. Prolactin was purchased from Sigma (Deisenhofen, Germany). Recombinant human erythropoietin was kindly provided by Drs. J. Burg and K.-H. Sellinger (Boehringer Mannheim, Penzberg, Germany). Erythropoietin receptor cDNA was a generous gift of Dr. H. Lodish (Boston, MA, USA).

2.2. Cell culture

COS-7 cells (ATCC CRL 1651) were grown in Dulbecco's modified Eagle's medium at 5% CO_2 in a water-saturated atmosphere. Cell culture media were supplemented with 10% fetal calf serum, streptomycin (100 mg/l) and penicillin (60 mg/l).

2.3. Expression vectors

Standard cloning procedures were performed as outlined by Sambrook et al. [27]. Construction of the expression vectors pSVL-Eg-Flag and pSVL-ΔB-Flag has been described previously [16]. For the construction of pSVL-Eg-YCTFP, -YLSLQ, -YSTVV, -YIDVQ, -YLVLD, -YVEIH, -YLSLP, and -YTSIH, oligonucleotides coding for the different tyrosine motifs and containing an NsiI and a SalI site were introduced in the NsiI and SalI digested pB130ΔS plasmid described previously [16]. The mutants were subcloned into pSVL-Eg as outlined by Gerhartz et al. [16].

2.4. Transfection

Cells were transfected by electroporation with the Gene Pulser from Bio Rad Laboratories (Munich, Germany). 2×10^6 cells in 0.8 ml DMEM were cotransfected with 20 μg of pECE vector containing STAT5A cDNA and 10 μg of pSVL vector containing erythropoietin receptor/gp130 chimera cDNAs or prolactin receptor cDNA, or 10 μg of pXM vector containing erythropoietin receptor cDNA using a voltage of 230 V and a capacity of 960 μF.

2.5. Preparation of nuclear extracts

Nuclear extracts of HepG2, H35 and COS-7 cells were prepared as described by Andrews and Faller [28]. Protein concentrations were measured by the Bio Rad protein assay.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described previously [16]. We used double-stranded ^{32}P-labeled probes: the bicore probe, which consists of two core elements of the α2-macroglobulin promoter in a 14 bp span (5'-AGC TGC AGT TCT GGG AAA GTC CTT AAT CCT TCT GGG AAT TCT-3'), and a mutated SIE oligonucleotide of the c-fos promoter (m67SIE: 5'-GAT CCG GGA GGG ATT TAC GGG GAA ATG CTG-3') [29]. 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.

2.7. Western blots

Aliquots of cell lysates were electrophoresed on a 7.5% SDS-polyacrylamide gel and the proteins were transferred to a polyvinylidene difluoride membrane (Qiagen, Hilden, Germany). The membranes were reacted with the M2 monoclonal anti-FLAG antibody and then processed for chemiluminescent reactions.

3. Results

3.1. Lack of STAT5 activation via gp130 in transfected COS-7 cells

In order to establish a system suitable for the examination of STAT5 activation, we transfected COS-7 cells with expression vectors for STAT5A and for the murine prolactin and erythropoietin receptors, respectively. Three days after transfection cells were stimulated with erythropoietin or prolactin for 15 min, then nuclear extracts were prepared and analyzed by EMSAs. In the band shift assays we employed a synthetic oligonucleotide as diagnostic substrate consisting of two 'core' STAT binding sites of the rat α2-macroglobulin promoter [11] in a 14 bp span (bicore probe). A similar probe has been shown to bind STAT5 with high affinity [24].

The prolactin and erythropoietin receptors were both able to mediate STAT5A activation after stimulation with the respective hormone. In nuclear extracts of control cells transfected with STAT5A cDNA only, no activated STAT5 was detectable after stimulation with erythropoietin or prolactin (data not shown).

To study STAT5 activation as mediated by gp130, we used chimeric receptors consisting of the extracellular domain of the erythropoietin receptor and the transmembrane and cytoplasmic regions of gp130 (Fig. 1). These chimeric constructs permitted us to analyze the activation of STAT factors independently of endogenous gp130 [16]. To prove that the erythropoietin receptor/gp130 chimera (Eg) is functionally active, we stimulated transfected COS-7 cells with erythropoietin and compared the activation of endogenous STATs with the pattern of untransfected COS-7 cells stimulated with IL6 and
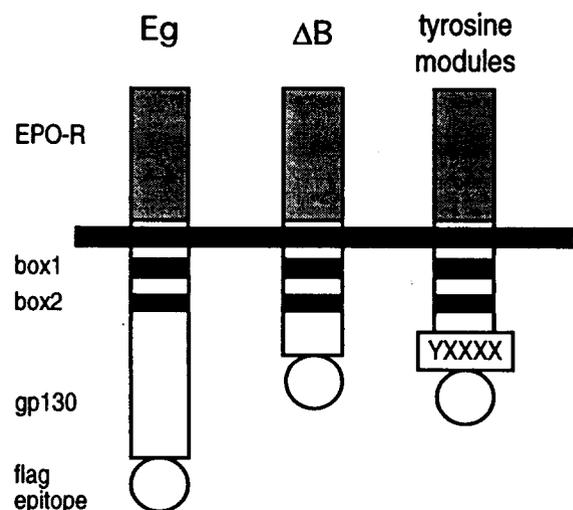

Fig. 1. Chimeric receptors. The chimeras consist of the extracellular domain of the erythropoietin receptor and the transmembrane and cytoplasmic parts of gp 130. They contain a FLAG epitope at their C-terminal ends. The 'tyrosine module' chimeras have been constructed with the aid of synthetic oligonucleotides. They have been sequenced throughout their synthetic regions.

| cytokine	IL6/sgp80		EPO		EPO		EPO		EPO		EPO	
stimulation	+	-	+	-	+	-	+	-	+	-	+	-
receptor	-		Eg		Eg		ΔB		ΔB		Eg-YLVLD	
STAT5	-		-		+		-		+		+	
probe	SIE		SIE		bicore		SIE		bicore		bicore	
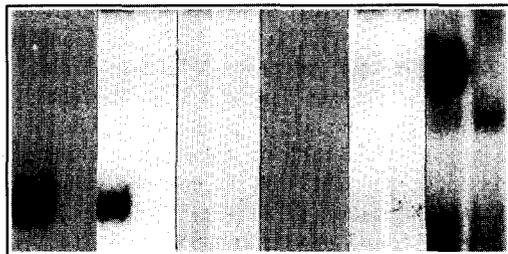
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 2. Activation of STAT factors via gp 130 and the Eg chimera. Untransfected COS-7 cells were stimulated with IL6 (100 B-cell stimulatory factor-2 units/ml) and soluble IL6 receptor (1 μg/ml). Nuclear extracts were prepared. The pattern of STAT activation was analyzed by EMSA and compared to that obtained via the Eg chimera in transfected cells (lanes 1–4). In addition ΔB, a truncated mutant of Eg, was examined with regard to its ability to activate STAT factors (lanes 7–10). As a positive control for STAT5 activation another chimeric receptor consisting of the truncated Eg chimera and the YLVLD tyrosine motif (Y343 of the erythropoietin receptor) was created. Y343 is known to be crucial for STAT5 activation via the erythropoietin receptor. COS-7 cells were transfected with Eg-YLVLD. Nuclear extracts were prepared after stimulation with erythropoietin (7 U/ml) and analyzed by EMSA (lanes 11,12).

soluble IL6 receptor. As shown by EMSAs using the high affinity SIE probe, in both cases comparable STAT activation could be observed (Fig. 2, lanes 1,3). The single band appearing after stimulation has previously been demonstrated to be STAT1 [16,30].

We then cotransfected COS-7 cells with expression vectors for STAT5 and the Eg chimera and stimulated with erythropoietin. Neither STAT5A (Fig. 2) nor STAT5B (data not shown) was detectably activated as analyzed by EMSAs with the bicore probe. This observation is in contrast to the findings of other investigators who could demonstrate STAT5 activation through gp130 [24]. These authors also described that the gp130-mediated activation of STAT5 did not depend on the tyrosine residues of the receptor. Therefore, we ana-

lyzed a carboxy-terminally truncated form of the Eg chimera which retained box 1/2 of gp130 but was devoid of the five carboxy-terminal tyrosine residues (see Fig. 1, ΔB). After stimulation with erythropoietin no activated STAT factors could be detected in EMSAs with the SIE and bicore probes (Fig. 2, lanes 7–10).

To demonstrate that the chimeric receptor is able to mediate STAT5 activation in principle, we added the tyrosine motif YLVLD to the carboxyterminally truncated form of the chimera. The motif YLVLD corresponds to Y343 of the erythropoietin receptor and was shown to be crucial for STAT5 activation by several authors [18–20]. Stimulation with erythropoietin led to a strong activation of STAT5 via this receptor mutant (Fig. 2, lanes 11,12). STAT5B was also activated although to a lesser extent (data not shown).

3.2. Tyrosine motifs mediate STAT5 activation

To examine the mode of STAT5 activation by other receptors, we constructed further chimeras (Fig. 1). Each of them consists of the extracellular domain of the erythropoietin receptor, the transmembrane and cytoplasmic regions of gp130, the latter of which was truncated after box 1/2, a synthetic oligonucleotide representing a tyrosine motif taken from a STAT5 activating receptor and a FLAG epitope. In many cases positions +1 and +3 within phosphotyrosine motifs have been found to determine the specificity of SH2 domain/phosphotyrosine interactions. Therefore, we searched for tyrosine motifs which bear some similarity with the YLVLD motif at positions +1 and +3 in a variety of cytokine receptors. COS-7 cells were cotransfected with expression vectors for STAT5A and for the different constructs. The expression of the different chimeric receptors as analyzed by Western blotting using an antibody to the FLAG epitope was found to be comparable (Fig. 3B). After stimulation of the transfected cells with erythropoietin for 15 min nuclear extracts were analyzed by EMSAs using the bicore probe.

As shown in Fig. 3A several tyrosine motifs were able to recruit STAT5A to the receptor and mediate its activation: the motifs YLVLD (erythropoietin receptor), YLSLP (GM-CSF receptor) and YLSLQ (IL2 receptor, β-chain) proved to be very efficient. A high level of STAT5A activation was also obtained with YVEIH (prolactin receptor) and YCTFP (IL2 receptor, β-chain). YIDVQ (LIF receptor) was found to be only weakly active, and no STAT5A activation could be de-

A

IL2-R β-chain YCTFP		IL2-R β-chain YLSLQ		LIF-R YIDVQ		GH-R YTSIH		GM-CSF-R β-chain YLSLP		PRL-R YVEIH		EPO-R YLVLD		gp130 YSTVV	
+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-

B

Eg   ΔB   ·   YSTVV   YLSLQ   YCTFP   YLSLP   YVEIH   YLVLD   YTSIH   YIDVQ

66kD —

46kD —
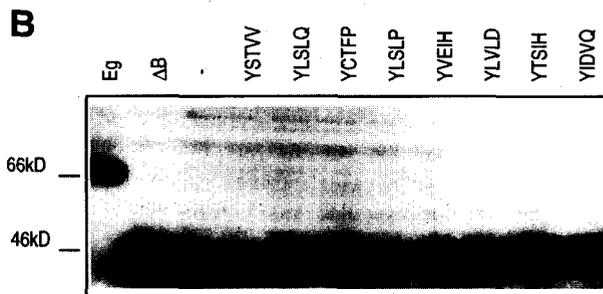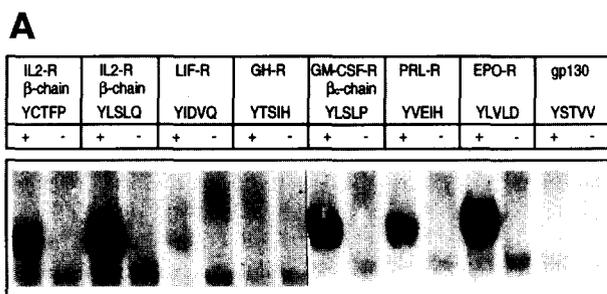
Fig. 3. A: STAT5 activation by chimeric receptors. COS-7 cells were transfected with expression vectors for STAT5A and different chimeric receptors. After 3 days the transfected cells were stimulated with erythropoietin (7 U/ml) for 15 min. Nuclear extracts were prepared and analyzed by EMSAs using the bicore probe. B: Expression of chimeric receptors in COS-7 cells. COS-7 cells were transfected with the cDNA for the respective chimeric receptors. After 3 days cell lysates were prepared and the expression of the constructs was checked by Western blotting using a FLAG antibody. The double bands seen in this figure correspond to differentially glycosylated forms of the chimeric receptors. Yoshimura et al. [34] showed for the erythropoietin receptor, whose extracellular part the chimeras share, that Golgi-processed erythropoietin receptor molecules differ from their precursors in molecular weight and that both forms are found in erythropoietin receptor expressing cells.

Table 1

Tyrosine module	Receptor	Activation of STAT5
DTYLVLD	EPO-R (Y343)	+++
QDYLSLP	GM-CSF-R, β_c-chain (Y882)	++(+)
DAYLSLQ	IL2-R, β-chain (Y510)	++(+)
DAYCTFP	IL2-R, β-chain (Y392)	++
LDYVEIH	PRL-R (Y496)	++
VIYIDVQ	LIF-R (Y974)	(+)
PDYTSIH	GH-R (Y595)	−
VQYSTVV	gp130 (Y759)	−

tected using the YTSIH (GH receptor) and YSTVV (gp130) constructs.

The identity of activated STAT5 was confirmed by immunoprecipitation with the phosphotyrosine antibody PY20, subsequent Western blotting and detection by a STAT5 antibody (Transduction Laboratories) (data not shown).

4. Discussion

Several cytokines are capable of inducing STAT5 phosphorylation via kinases of the JAK family, which are constitutively associated with the cytoplasmic tail of cytokine receptors and become activated after ligand-induced dimerization of the receptor molecules. Depending on the cytokine receptor with which a specific JAK kinase is associated, tyrosine phosphorylation of different STAT factors by this kinase can occur. It is currently believed that the specificity of STAT activation is determined by receptor tyrosine residues which are also phosphorylated by JAK kinases. The receptor phosphotyrosines represent docking sites for the SH2 domains of the respective STAT factors.

For STAT1 and STAT3 it has already been described that distinct receptor tyrosine motifs mediate differential activation of these two transcription factors [15,16]. Here we have shown that the ability to selectively activate STAT5 can be transferred by the addition of tyrosine motifs derived from STAT5-activating receptors to a chimeric receptor construct (Fig. 1, ΔB) which cannot recruit STAT factors on its own. Substantial sequence similarity exists among the different tyrosine modules that conferred the ability to activate STAT5. In the tyrosine motifs which mediate STAT5 activation most efficiently, YLVLD (Y343 of the erythropoietin receptor), YLSLQ (Y510 of the IL2 receptor β-chain), and YLSLP (Y882 of the GM-CSF receptor β-chain), leucine is found at positions +1 and +3 with respect to the tyrosine residue. Other amino acids with aliphatic side chains are part of the motifs YVEIH (Y509 of the prolactin receptor) and YIDVQ (Y974 of the LIF receptor), which also recruit STAT5 with YVEIH being more effective than YIDVQ. Surprisingly, the motif YCTFP (Y392 of the IL2 receptor β-chain) is also able to activate STAT5 in spite of a hydrophilic cysteine at position +1 (Table 1). It is to be noted that all five motifs mediating efficient STAT5 activation contain an acid residue (aspartate) in position −1 or −2 while the motif YIDVQ (Y974 of the LIF receptor), which is least effective, does not (see Table 1). Therefore, this residue may also contribute to STAT5 recruitment to the receptor.

Y343 of the erythropoietin receptor has been described to

be crucial for STAT5 activation via erythropoietin by different authors [18–20]. Using truncated erythropoietin receptor constructs and receptor molecules in which tyrosine residues had been point-mutated, it was shown that Y343 is sufficient for STAT5 phosphorylation, but controversy exists with respect to the question whether this tyrosine is also necessary for the recruitment of STAT5. Gobert et al. [20] found that Y401 of the erythropoietin receptor (YTILD) is able to activate STAT5, too, and can replace Y343. Interestingly, this motif contains a glutamic acid in position −1. On the other hand, Quelle et al. [19], using phosphopeptide competition assays, could not demonstrate that the phosphorylated YTILD-motif impairs dimerization of STAT5. They also showed that the modules pYLVVS (Y431) and pYVACS (Y479) are efficient competitors. In contrast to these observations Gobert et al. [20] found that Y431 and Y479 are not able to activate STAT5. Damen et al. [18], for their part, describe Y343 as necessary for the recruitment of STAT5 at low erythropoietin concentrations, but state that high concentrations of the growth factor lead to receptor tyrosine independent activation of STAT5, suggesting a loss of specificity under these conditions. Thus, the importance of Y343 of the erythropoietin receptor for STAT5 phosphorylation is widely accepted. However, the contribution of other tyrosine residues, of which Y431 (YLVVS) resembles our modules more closely than Y401 (YTILD) or Y479 (YVACS), is evaluated controversially by authors using either phosphopeptide competition assays or band shift assays as method of investigation.

In accordance with our results Gaffen et al. [31] and Friedmann et al. [22] showed by analysis of receptor mutants and phosphopeptide competition assays that Y392 (YCTFP) and Y510 (YLSLQ) are crucial for the activation of STAT5 by the IL2 receptor β-chain. It is remarkable that the two motifs do not show obvious sequence homology, but it should be noted that in both cases the two amino acids on the N-terminal side of the tyrosine residue are identical: DAYLSLQ and DAYCTFP. This may be a further indication that amino acids N-terminal of the tyrosine may play a role in STAT5 activation. Alternatively, STAT5 may be activated by different motifs, as has been shown for STAT1 which is recruited by a motif YXPQ in gp130 and the LIF receptor, whereas YDKPH represents the respective motif in the IFNγ receptor [15,16,32].

For the prolactin and GM-CSF receptors no receptor tyrosine motifs crucial for STAT5 activation have been described so far. We have shown in this paper that Y509 of the prolactin receptor and Y882 of the GM-CSF receptor β-chain are sufficient to mediate the activation of STAT5. Further studies with receptor mutants need to be done in order to find out whether the mentioned motifs are also necessary for the recruitment of STAT5 by the respective receptor.

Interestingly, the YIDVQ motif of the LIF receptor mediates weak STAT5 activation, suggesting that STAT5 might play a role in LIF signal transduction. Further studies are needed to elucidate this possibility.

The two tyrosine motifs YTSIH (Y595 of the growth hormone receptor) and YSTVV (Y759 of gp130), which are not able to mediate STAT5 activation, differ from most other modules in the hydrophilic amino acid at position +1 (threonine or serine, respectively). This finding suggests that the amino acid at position +1 is of special importance within the motif recognized by the SH2 domain of STAT5.

For the growth hormone receptor, Hansen et al. [21] showed that Y534 (YFCEA), Y566(YITTE), and Y627 (YVSTD) are able to mediate STAT5 activation independently of each other. In accordance with our results the amino acid at position +1 is hydrophobic in all three motifs, but hydrophilic residues are found at position +3. These findings also provide evidence for a hydrophobic residue at position +1 being more important than one at position +3. Smit et al. [33] reported that Y333 (YKPDF) and Y338 (YNDDS) of the murine growth hormone receptor may also be able to mediate STAT5 activation. Although these motifs are totally unrelated to those described herein, they share the aspartate in position −2. These authors also note that this residue might be important and speculate that non-SH2-type interactions might be involved.

The failure of YSTVV (Y759 of gp130) to activate STAT5 is not surprising since no STAT activation could be achieved with the receptor construct containing the complete cytoplasmic domain of gp130 in COS-7 cells. These findings, however, contrast with the results of Lai et al. [24], who could demonstrate STAT5 activation by a receptor construct consisting of the extracellular domain of the G-CSF receptor instead of the erythropoietin receptor used in our experiments and the intracellular part of gp130 in COS-1 cells. In addition, these authors found STAT5 activation by gp130 to be independent of receptor tyrosine residues by using a truncated mutant of their receptor construct. This contradiction to our data could be explained by a possible influence of the extracellular domain of a chimeric receptor on intracellular signalling. Alternatively, the assay system of Lai et al. [24] may measure STAT5 activation with greater sensitivity. Thus, the definite role of STAT5 in IL6 signal transduction needs to be clarified by further experiments. The inability of the cytoplasmic part of gp130 to recruit STAT5 in our experimental system allowed us to analyze phosphotyrosine motifs from other cytokine receptors with the aid of mutants of the Eg chimera.

In summary, we conclude from our experimental findings that the specificity of STAT5 activation in the JAK/STAT signalling pathway is mediated by receptor phosphotyrosine motifs. These motifs have a modular character, as the ability to activate STAT5 can be transferred to receptor constructs not capable of recruiting STAT factors by addition of a tyrosine motif of another receptor which activates STAT5. In the motifs sufficient to mediate STAT5 activation, leucine is repeatedly found at positions +1 and +3 relative to the tyrosine residue, but also other hydrophobic amino acids, especially those with aliphatic side chains, seem to be acceptable. From these data we derive the consensus sequence YZXZ for STAT5 activation with Z standing for a hydrophobic amino acid, preferably an aliphatic one, with the addition that acid residues in position −1 or −2 may also be important. The chimeric receptor system described here is likely to prove highly valuable in determining the exact requirements of STAT5 activation.

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