FEBS Letters 468 (2000) 120–124 FEBS 23349

Different epitopes are required for gp130 activation by interleukin-6, oncostatin M and leukemia inhibitory factor

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Received 7 January 2000

Edited by Marco Baggiolini

Abstract Gp130 is the common signal transducing receptor subunit of interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor and cardiotrophin-1. IL-6 and IL-11 induce gp130 homodimerization whereas the others lead to the formation of heterodimers with LIFR or OSMR. Binding epitopes for IL-6 and IL-11 are located in the immunoglobulin-like domain and the cytokine binding module (CBM). Here we show that a gp130 mutant lacking domain 1, although unresponsive to IL-6 and IL-11, can still activate signal transducer and activator of transcription (STAT) transcription factors in response to LIF or OSM. Moreover, point mutations in the CBM of gp130 (F191E and V252D) that severely impair signal transduction in response to IL-6 and IL-11 differentially interfere with gp130 activation in response to LIF and OSM. Thus, epitopes involved in gp130 homodimerization are distinct from those leading to the formation of gp130/LIFR or gp130/OSMR heterodimers. These findings may serve as the base for rational design of gp130 antagonists that specifically interfere with bioactivity of distinct IL-6-type cytokines.

Key words: gp130; Cytokine; Receptor; Signal transduction; Ba/F3 cell

1. Introduction

Cytokines are highly potent mediators mainly involved in regulation of immune responses and hematopoiesis. Specificity of cytokine action is based on the molecular recognition of the ligand by its receptor. On the other hand, redundancy of cytokine action is caused by the fact that different cytokines utilize common receptor proteins to trigger the cytoplasmic signal transduction cascades. This redundancy is particularly pronounced in the family of interleukin (IL)-6-type cytokines that all use the transmembrane protein gp130 as signal transducing receptor component (for review, see [1]). IL-6 [2] and IL-11 [3], after binding to their specific α-receptors, induce gp130 homodimerization whereas leukemia inhibitory factor (LIF)[2], oncostatin M (OSM) [4], CNTF [5] and CT-1 [6] signal via a heterodimer of gp130 and a second signal transducing receptor protein, the LIFR. OSM can also signal via a gp130/OSMR heterodimer [7]. The α-receptors can be functionally replaced by the respective soluble receptors lacking the transmembrane and cytoplasmic parts. Activation of gp130 homodimers or gp130/LIFR as well as gp130/OSMR heterodimers results in the phosphorylation of transcription factors of the signal transducer and activator of transcription (STAT) family, mainly STAT3. Activated STAT3 dimers translocate into the nucleus and induce target gene expression [8].

Gp130, LIFR and OSMR belong to the family of hematopoietic cytokine receptors characterized by the presence of at least one cytokine binding module (CBM) (for review, see [9]). The extracellular part of gp130 has been predicted to consist of six individual domains: an Ig-like domain (D1), followed by a single CBM (D2 and D3) and three fibronectin type III-like domains (D4–D6, Fig. 1) [10]. The main epitopes involved in recognition of the gp130-homodimerizing cytokines have recently been defined. Besides critical residues in the CBM of gp130, the Ig-like domain is required for activation of gp130 in response to both IL-6 and IL-11 [11–13]. According to tertiary structure predictions, the ectodomain architecture of LIFR shows some similarities to the one of gp130 (Fig. 1). In this study using several gp130 mutants, we investigated requirements for gp130 activation by the cytokines LIF and OSM.

2. Materials and methods

2.1. Antibodies and expression vectors

The monoclonal antibodies B-P4 (gp130) and 10B2 (LIFR) were previously described [14,15]. Construction of gp130 wild-type and mutant expression vectors was described elsewhere [11,13]. The human full-length LIFR cDNA was cloned into pSBC-1 to yield the mammalian expression vector pSBC-LIFR as previously described [16].

2.2. Transfection and analysis of COS-7 cells

COS-7 cells were transiently transfected using the DEAE-dextran method as described. Cells were cultivated for 48 h prior to stimulation. Mock transfection was performed using 20 μg pSVL vector. All other cells were transfected with 10 μg pSBC-LIFR and 10 μg of the respective gp130 construct in pSVL. 48 h after transfection, COS-7 cells were analyzed for receptor expression by FACS as described [11,13]. For gp130 or LIFR expression, B-P4 and 10B2, respectively, were used as primary antibodies. *R*-phycoerythrin-conjugated goat anti-mouse IgG Fab-fragment was used as a secondary antibody.

2.3. Transfection and analysis of Ba/F3 cells

Ba/F3-gp130 cells and Ba/F3-LIFR cells were transfected by electroporation as described [11,16]. To obtain Ba/F3 cells expressing the LIFR together with gp130 or the gp130F191E point mutant, Ba/F3-LIFR cells were coelectroporated with 28 μg of the respective gp130

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Abbreviations: CBM, cytokine binding module; D, domain; LIF, leukemia inhibitory factor; OSM, oncostatin M; s, soluble; STAT, signal transducer and activator of transcription

PII: S0014-5793(00)01205-9

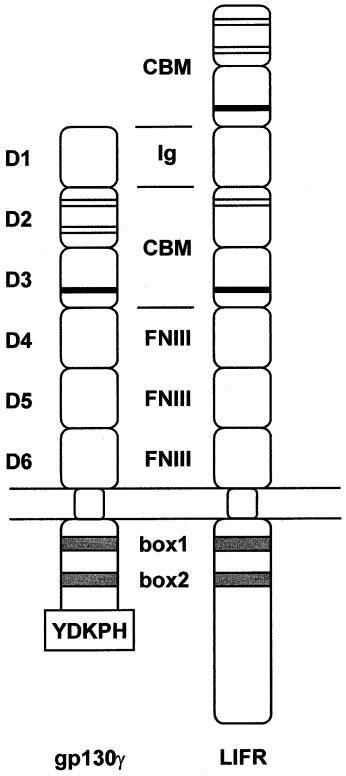

Fig. 1. Schematic representation of LIFR and gp130γ. The predicted structural organization of gp130 and LIFR is shown. Black lines indicate conserved cysteine residues, black bars the WSXWS motifs present in the CBMs. Further domains are assigned as Ig-like (Ig) or fibronectin type III-like (FNIII). Gp130 domains are numbered from domain 1 (D1) to domain 6 (D6). In the cytoplasmic part of gp130γ, the amino acid residues following the Janus kinase binding sites (box 1 and box 2, gray boxes) were replaced by the motif YDKPH of the interferon-γ receptor that strongly and specifically activates STAT1.
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expression vector and 2 μg of pSV2neo as described. Selected Ba/F3 clones were screened for the presence of membrane-bound gp130 and LIFR proteins by flow cytometry using the same antibodies as for analysis of COS-7 cells.

2.4. Electrophoretic mobility shift assay (EMSA)

48 h after transfection, COS-7 cells were stimulated for 15 min at 37°C with OSM (4 ng/ml) or LIF (20 ng/ml) or the combination of IL-6 (12.5 ng/ml) and sIL-6R (500 ng/ml) or left unstimulated. Stably transfected Ba/F3 cells were stimulated for 15 min at 37°C with OSM (50 ng/ml) or LIF (50 ng/ml) or the combination of IL-6 (25 ng/ml) and sIL-6R (1 μg/ml) or left unstimulated. Preparation of nuclear extracts and EMSAs were performed as described [17]. A double-stranded oligonucleotide derived from the c-fos promoter (m67SIE; 5′-GATCC GGGAG GGATT TACGG GGAAA TGCTG-3′) [18] was used as ^{32}P-labelled probe. Gels were analyzed using a PhosphorImager (Molecular Dynamics) as well as conventional autoradiography.

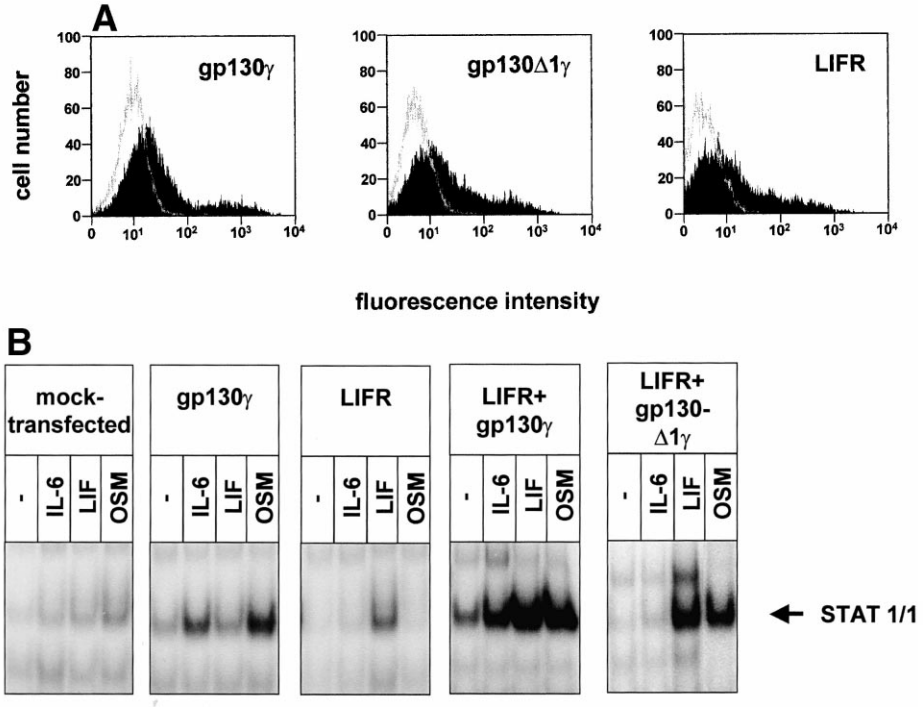

Fig. 2. STAT1 activation in COS-7 cells transiently transfected with LIFR and either gp130γ or gp130Δ1γ in response to various cytokines. (A) 48 h after transfection, cells were analyzed for receptor surface expression by FACS analysis. Cells were incubated with gp130 antibody B-P4 (left and central panel) or with LIFR antibody 10B2 (right panel) followed by PE-conjugated secondary antibody (black histograms). As a negative control, mock-transfected cells were treated in the same way (gray open histograms). Representative surface expressions of the receptors are shown. (B) 48 h after transfection, cells were stimulated for 15 min with IL-6 (12.5 ng/ml in the presence of 500 ng/ml sIL-6R), LIF (20 ng/ml) or OSM (4 ng/ml) or left unstimulated (−) as indicated. Nuclear extracts were prepared and activated STAT1 homodimers were detected by EMSA after binding to a labelled oligonucleotide probe (m67SIE). A representative of three independent experiments is shown.

3. Results and discussion

3.1. The Ig-like domain of gp130 is required for signal transduction in response to IL-6, but not in response to LIF or OSM

Using a gp130 with the STAT recruitment motifs replaced by the strongly and exclusively STAT1 activating YDKPH motif derived from the interferon-γ receptor (gp130γ, Fig. 1), we established a system that allows the study of gp130 mutants in COS-7 cells on the background of low levels of endogenous gp130 [11,13]. To assess the role of the Ig-like domain of gp130 for receptor activation in response to IL-6, LIF or OSM, COS-7 cells were transfected with LIFR in combination with gp130γ or a construct lacking D1 (gp130Δ1γ). Surface expression of receptors was analyzed by FACS. Both gp130 variants appear at the cell surface in comparable amounts (Fig. 2A, left and central panels). Similarly, surface expression of transfected LIFR was verified (Fig. 2A, right panel).

Mock transfected cells did not respond to stimulation with the combination of IL-6 and sIL-6R or LIF or OSM (Fig. 2B, first panel). Cells transfected with gp130γ alone showed activation of STAT1 homodimers after stimulation with IL-6 and OSM, but not with LIF (Fig. 2B, second panel). COS-7 cells express on their cell surface low levels of endogenous OSMR, while no endogenous LIFR could be detected by FACS analysis (data not shown). This leads to the conclusion that in the case of OSM stimulation, STAT activation observed in cells transfected with gp130γ constructs is due to heterodimers of gp130 with OSMR.

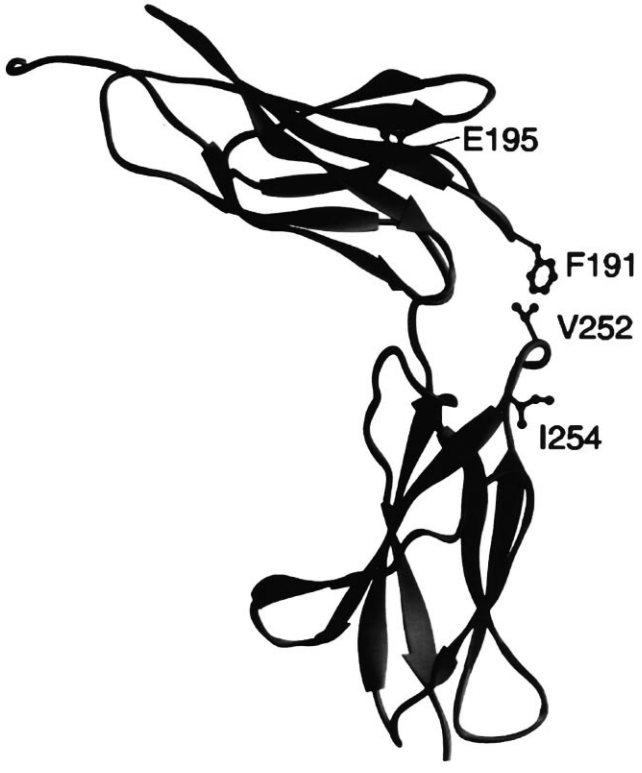

Fig. 3. Location of the point mutations in the CBM of gp130. Amino acid side-chains targeted by site-directed mutagenesis are shown in a ribbon presentation of the gp130 CBM based on the X-ray structure by Bravo et al. [20]. F191 is located in the E–F-loop of D2, E195 is located in strand F of D2 and V252 as well as I254 are located in the B–C-loop of D3.

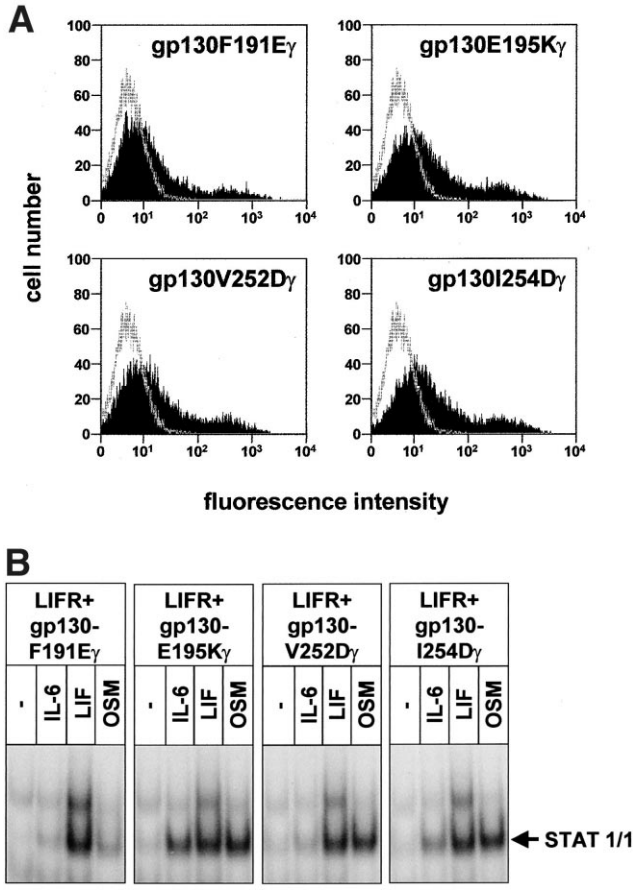

Fig. 4. STAT1 activation in COS-7 cells transiently transfected with LIFR and gp130γ point mutants in response to various cytokines. (A) 48 h after transfection, cells were analyzed for receptor surface expression by FACS analysis. Cells were incubated with gp130 antibody B-P4 followed by PE-conjugated secondary antibody (black histograms). As a negative control, mock-transfected cells were treated in the same way (gray open histograms). Analysis for LIFR surface expression was performed as described in Fig. 2A and gave comparable results as shown there. (B) 48 h after transfection, cells were stimulated for 15 min with IL-6 (12.5 ng/ml in the presence of 500 ng/ml sIL-6R), LIF (20 ng/ml) or OSM (4 ng/ml) or left unstimulated (−) as indicated. Nuclear extracts were prepared and activated STAT1 homodimers were detected by EMSA after binding to a labelled oligonucleotide probe (m67SIE). A representative of three independent experiments is shown.

To analyze the effect of the mutants in response to LIF, LIFR was cotransfected with the gp130γ constructs. Stimulation of LIFR and gp130γ-transfected cells with IL-6, OSM or LIF leads to a robust activation of STAT1 (Fig. 2B, fourth panel). The STAT1 activation in response to LIF or OSM is not due to overexpression of LIFR in these cells, as cells transfected with LIFR alone showed only a very weak response after LIF stimulation and no response after stimulation with OSM (Fig. 2B, third panel). When COS-7 cells were transfected with LIFR and gp130Δ1γ, the response to IL-6 was reduced to background levels whereas the LIF signal remained largely unaffected (Fig. 2B, fifth panel). The response to OSM in these cells was slightly reduced compared to cells stimulated with LIF. In cells transfected with

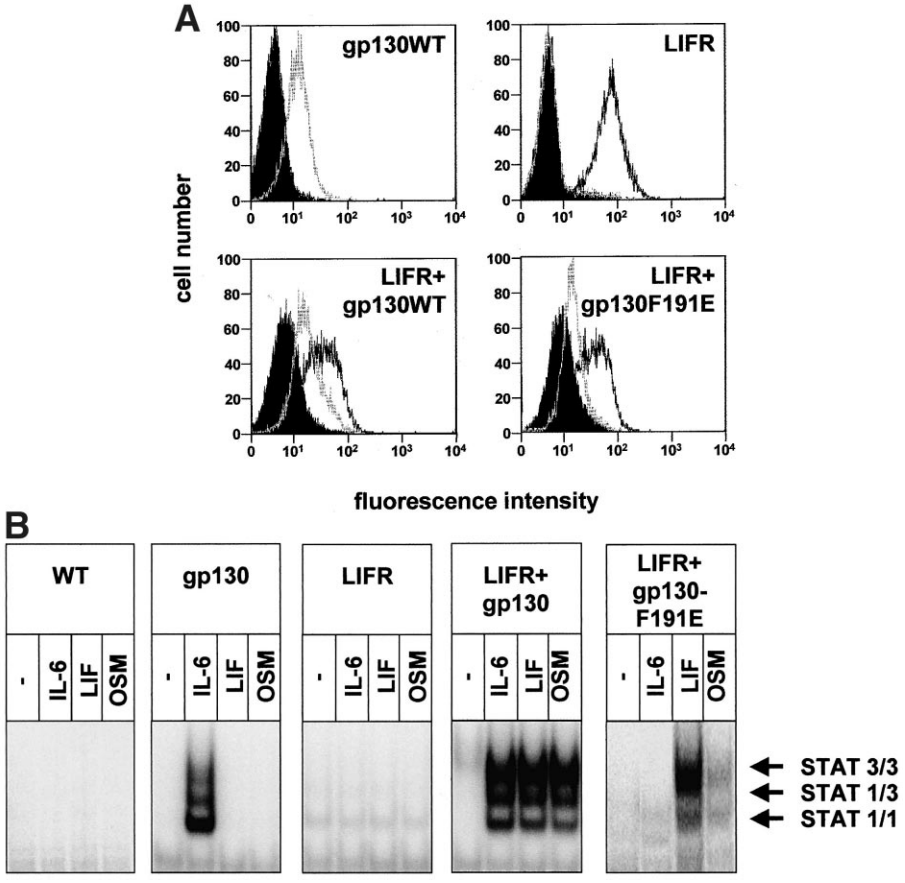

Fig. 5. STAT1 and STAT3 activation in Ba/F3 cells stably transfected with LIFR in combination with gp130 or the mutant gp130F191E in response to various cytokines. (A) Ba/F3 cells stably transfected with gp130, LIFR, or the combination of either LIFR and gp130 or LIFR and gp130F191E were analyzed for receptor surface expression by FACS analysis. Cells were incubated with gp130 antibody B-P4 or with LIFR antibody 10B2 followed by PE-conjugated secondary antibody (light and dark gray open histograms, respectively). As a negative control, cells were incubated with PE-conjugated secondary antibody alone (black histograms). (B) Stably transfected Ba/F3 cells were stimulated for 15 min with IL-6 (25 ng/ml in the presence of 1 μg/ml sIL-6R), LIF (50 ng/ml) or OSM (50 ng/ml) or left unstimulated (−) as indicated. Nuclear extracts were prepared and activated STAT dimers were detected by EMSA after binding to a labelled oligonucleotide probe (m67SIE). A representative of three independent experiments is shown.

gp130Δ1γ alone, the response to OSM was comparable to that of cells transfected with gp130Δ1γ and LIFR (data not shown).

The Ig-like domain of gp130 plays a crucial role in the formation of the signal transducing complex in response to IL-6 as well as IL-11 [12,13]. The model of the hexameric IL-6 receptor complex assigns as binding partners for the three binding sites on IL-6 the CBM of IL-6R for site I, the CBM of gp130 for site II and the Ig-like domain of a second gp130 molecule for site III, with the Ig-like domains functioning as bridges between the two IL-6/IL-6R/gp130 trimers in this complex [19]. Signal transduction in response to LIF is independent of the Ig-like domain of gp130. Site II of LIF is postulated to interact with the gp130 CBM whereas site III contacts the LIFR Ig-like domain. A similar trimeric complex has been suggested for the signal transducing complex of gp130 and OSMR [12], as OSMR also contains an Ig-like domain located N-terminal of the CBM. Therefore, the Ig-like domain of gp130 is not required for receptor complex formation in response to both LIF and OSM.

3.2. *Residues in the gp130 CBM critical for IL-6 responsiveness differentially contribute to activation by LIF or OSM*

When the importance of amino acids F191 and V252 in the gp130 CBM for receptor activation in response to IL-6 and IL-11 was established [11,13], no structural data on the gp130 ectodomain were available. In the mean time, the crystal structure of the gp130 CBM [20] as well as the solution structure of D3 have been solved [21]. Fig. 3 shows the positions of the amino acids selected for mutagenesis in the X-ray structure of gp130 confirming their location predicted from our molecular model [22].

COS-7 cells were transfected with the gp130γ point mutants gp130F191Eγ, gp130E195Kγ, gp130V252Dγ or gp130I254Dγ in combination with LIFR. Transfected cells were analyzed by FACS, indicating that the introduced point mutations do not interfere with cell surface expression of receptor mutants (Fig. 4A). As the antibody B-P4 used for detection of gp130 mutants recognizes an epitope within the membrane-proximal part (D4–D6), recognition of gp130 by this antibody is not altered due to introduction of the point mutations.

Cells transfected with gp130F191Eγ and gp130V252Dγ showed no STAT activation upon stimulation with IL-6/IL-6R complexes. In gp130I254Dγ-transfected cells, the response is reduced while gp130F195Kγ-transfected cells served as a positive control (Fig. 4B). These findings are in line with the previously defined role of these amino acids in receptor activation by IL-6 or IL-11 [13]. None of the introduced point mutants severely affected signal intensity upon LIF stimulation. Upon OSM stimulation, gp130F195Kγ, gp130V252Dγ and gp130I254Dγ mutants show STAT activation comparable to that after LIF stimulation. The gp130F191Eγ mutant however does not respond to OSM. In cells transfected with the gp130γ mutants alone, the OSM response was comparable to that of cells cotransfected with gp130γ mutants and LIFR (data not shown). Thus, residues in the CBM required for gp130 activation in response to IL-6 and IL-11 are not necessarily crucial for gp130 activation in response to LIF or OSM.

To analyze whether the observed response of gp130 point mutants after stimulation with LIF or OSM depends on the cellular environment, Ba/F3 cells were stably transfected with LIFR, gp130 or a combination of either LIFR and gp130 or LIFR and the gp130F191E point mutant, respectively. Ba/F3 cells express gp130 or LIFR only after transfection of the respective cDNAs (Fig. 5A). Untransfected cells or cells transfected with LIFR alone do not show activation of STAT factors in response to any of the analyzed cytokines (Fig. 5B, first and third panel). Cells transfected with gp130 alone only respond to IL-6/sIL-6R but not to LIF or OSM (Fig. 5B, second panel). Cells expressing the LIFR together with gp130 show a prominent STAT activation after stimulation with all three cytokines analyzed (Fig. 5B, fourth panel). In contrast, cells expressing LIFR in combination with the gp130F191E mutant show a considerable STAT activation only after stimulation with LIF but no response to stimulation with IL-6/sIL-6R or OSM (Fig. 5B, fifth panel). These findings are in accordance with the observations made with transiently transfected COS-7 cells.

According to our model, site II of IL-6 interacts with the CBM of one gp130 in the signal transducing complex [22]. LIF and OSM are as well postulated to bind to the gp130 CBM. Thus, different requirements exist for binding of the analyzed cytokines to the gp130 CBM. In a previous study, monoclonal antibodies against the gp130 ectodomain that selectively interfere with the activation of gp130 by IL-6-type cytokines have been characterized [14]. The selectivity of some of these antagonistic antibodies that maps to the gp130 CBM (Müller-Newen, unpublished observation) can be explained in the light of our present findings.

The CBM is the structural hallmark of hematopoietic cytokine receptors [9]. These receptors are either monospecific (e.g. EpoR, GHR, G-CSFR) or recognize several ligands (gp130; the common β-chain of the receptors for GM-CSF, IL-3 and IL-5; the common γ-chain of the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15). In the case of the common β-chain, it has also been found that several mutations in the CBM differentially interfere with ligand recognition [23]. Thus, although the role of the CBMs in ligand binding is evolutionary conserved, the molecular details of ligand recognition diverge. These findings may serve as a base for the development of antagonists that specifically block the activity of a distinct cytokine that signals via one of the promiscuous cytokine receptors.

Recently, it has been suggested that gp130 may exist in preformed homo- or heterodimers (with LIFR or OSMR) on the cell surface which are then activated by the ligand [24]. In this model, such dimers would be a mandatory requisite for the induction of signal transduction. According to this scenario, the mutations of gp130 presented here would not only influence ligand binding but also prevent the preformation of homo- but not of heterodimers. Further studies are needed to give a more detailed insight into the activation mechanism of these receptors.

Acknowledgements: This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB542), and the Fonds der Chemischen Industrie (Frankfurt a. M).

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