RESEARCH COMMUNICATION

Identification of a Leu-Ile internalization motif within the cytoplasmic domain of the leukaemia inhibitory factor receptor

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Leukaemia inhibitory factor (LIF) signals via a heterodimeric receptor complex comprised of the LIF receptor (LIFR) and the interleukin (IL)-6 signal transducer gp130. Upon binding to its cognate receptor LIF is internalized. In this study, we show that the LIFR is endocytosed independently of gp130. By using a heterochimaeric receptor system we identified a dileucine-based internalization motif within the cytoplasmic domain of the LIFR. Our findings suggest that a heterodimeric LIFR/gp130 complex and homodimeric gp130/gp130 complex are endocytosed via distinct internalization signals.

Keywords: dileucine, endocytosis, gp130, interleukin-6, LIF receptor.

INTRODUCTION

Leukaemia inhibitory factor (LIF) is an interleukin (IL)-6-type cytokine that elicits a pleiotropic spectrum of activities. It induces differentiation of mouse monocytic leukaemia M1 cells and suppresses the differentiation of embryonic stem cells [1,2]. Like IL-6, LIF stimulates acute phase protein synthesis in HepG2 cells. In addition, it induces a cholinergic phenotype in adrenergic neurons and enhances the survival and proliferation of embryonic sensory neurons and myoblasts [1,2]. LIF exerts its function via a heterodimeric transmembraneous receptor complex consisting of the LIF receptor (LIFR) and the IL-6 signal transducer gp130 [3]. Upon LIF binding, cytoplasmic tyrosine kinases of the Janus family (Jaks) are activated which phosphorylate the LIFR and gp130 leading to the recruitment of latent signal transducers and activators of transcription (STATs). STATs also become phosphorylated, dimerize and are translocated to the nucleus where they bind to enhancer elements within the regulatory regions of target genes [4].

After binding to its receptor, LIF is internalized and the LIFR is down-regulated [5]. Zhang et al. [6] have very recently demonstrated that the 88 C-terminal amino acids of the cytoplasmic domain of the LIFR are necessary for efficient internalization of LIF via the LIFR/gp130 complex. However, the nature of the internalization motif was not addressed in that study. For the IL-6 receptor, we have shown that its endocytosis and ligand-induced down-regulation is mediated by gp130 and depends on a dileucine internalization motif within the cytoplasmic domain of the signal transducer [7]. In addition, we demonstrated that activation of the Jak/STAT pathway is not necessary for internalization of gp130 to occur but that gp130 is endocytosed constitutively [8,9]. In order to identify the LIFR internalization motif we constructed chimaeric receptors consisting of the extracellular domains of the IL-5 receptor (IL-5R) α and β chains respectively, and the transmembrane and cytoplasmic parts of wild-type and mutant forms of the LIFR and gp130. This experimental approach allowed us to examine the internalization capabilities of the cytoplasmic tail of the LIFR and its mutants in COS-7 cells, independently of the endogenously expressed gp130. We also compared the internalization kinetics of wild-type human LIFR and gp130 in stably transfected Ba/F3 cells using flow cytometry.

MATERIAL AND METHODS

Materials

Restriction enzymes, T4-DNA ligase and Klenow enzyme were purchased from Boehringer Mannheim and New England Biolabs. Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany). Dulbecco's modified Eagle's medium (DMEM) was from GIBCO. Recombinant human IL-5 was prepared as described previously [10]. FITC-conjugated mouse IgG antibody was purchased from Dakopats. $Na^{125}I$ was purchased from ICN and ^{125}I-IL-5 was prepared using the Iodogen iodination agent (Pierce Chemicals) as described in [11].

Construction of expression vectors

Standard cloning procedures were performed as outlined by Sambrook et al. [12]. The construction of pSVL-based expression vectors (Pharmacia) coding for the receptor chimaeras IL-5Rα/gp130 and IL-5Rβ/gp130 has been described previously [13]. Starting from these constructs, the cDNA for the gp130

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; IL, interleukin; Jak, Janus kinase; LIF, leukaemia inhibitory factor; R, receptor; STAT, signal transducer and activator of transcription.

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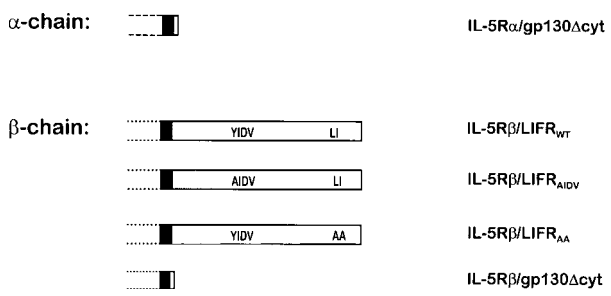

Figure 1 Schematic representation of IL-5R/gp130 and IL-5R/LIFR chimaeras

The chimaeras are composed of the extracellular part of the IL-5 receptor α- or β-chain respectively, and the transmembrane (filled box) and cytoplasmic parts (open box) of the signal transducer gp130Δcyt or LIFR. LI and AA represent the wild-type and the mutated dileucine motif (at position 210/211). YIDV and AIDV represent the wild-type or mutated tyrosine-motif (at position 115) of the LIFR respectively. Numbering starts at the first cytoplasmic amino acid (1) and ends at the C-terminus (238).

transmembrane and cytoplasmic part was exchanged with the corresponding sequences of the LIFR cDNA, that were generated by PCR using Vent DNA polymerase containing 3′–5′ proofreading exonuclease activity (New England Biolabs). The plasmid pDC302huLIF/R (kindly provided by Dr. I. M. Kerr, Imperial Cancer Research Fund, London, U.K.) served as a template. Oligonucleotides used for amplification were 5′-GGAATTCAGTATGTATGTGGTGACAA-3′ (sense) and 5′-CGGGATCCTGTTAATCGTTTGGTTTG-3′ (antisense). Underlined letters indicate restriction sites for *Eco*RI and *Bam*HI. PCR products were cut with the restriction enzymes *Eco*RI and *Bam*HI and ligated into the pSVL vector coding for the extracellular domains of the IL-5βR. The point mutants IL-5Rβ/LIFR$_{AIDV}$ and IL-5Rβ/LIFR$_{AA}$ were generated using mutagenic primers 5′-CAGGTTATTGCCATCGATGTTCA-GTCGATGTATCAGCC-3′ (sense) and 5′-CTGAACATCGA-TGGCAATAACCTGTGCAGTCCC-3′ (antisense) in the case of IL-5Rβ/LIFR$_{AIDV}$ and 5′-GCTCCATTAATAGTCGACAA-TTTGCGGCTCCTCCTAAAGAT-3′ (sense) and 5′-CAAAT-TGTCGGGAATTAATGGAGC-3′ (antisense) in the case of IL-5Rβ/LIFR$_{AA}$. The chimaeric IL-5Rβ/LIFR cDNA in the pSVL vector was used as a template. All constructs were verified by sequencing. All chimaeric constructs used are schematically depicted in Figure 1.

Cell culture and transfections

COS-7 cells (A.T.C.C. CRL 1651) were grown in DMEM supplemented with 10% (v/v) fetal-calf serum, streptomycin (100 mg/l) and penicillin (60 mg/l). Ba/F3 cells, a murine pre-B lymphocyte line, were cultured in DMEM containing 10% (v/v) fetal-calf serum and 5% (v/v) conditioned medium from X63Ag-653 BPV-mIL-3 myeloma cells as a source of IL-3 [14]. All cells were grown at 37 °C and 5% CO_2 in a water-saturated atmosphere.

Transient transfections of COS-7 cells were carried out using the Electro Square Porator T820 from BTX, San Diego, CA, U.S.A. For internalization studies, 2×10^6 COS-7 cells were co-transfected with 3 μg of pSVL vector containing the IL-5Rα/gp130 mutant cDNAs and with 27 μg of pSVL vector containing the IL-5Rβ/LIFR wild-type or mutant cDNAs or IL-5β/gp130Δcyt cDNA. For immunofluorescence studies, 2×10^6 COS-7 cells were each transfected with 30 μg of the respective cDNAs. All transfections were carried out using one pulse of 99 μs length and 1500 V. Cells were used for studies 2 or 3 days after transfection when reaching confluence.

Ba/F3-gp130 cells were stably transfected by electroporation of 28 μg of pSVLgp130 and 2μg of pSV2neo into 3.5×10^6 cells in 0.8 ml of medium at 200 V and for 70 ms. LIFR cDNA was introduced using the same conditions with the bicistronic expression vector system pSBC in which the first cistron contained the LIFR cDNA and the second cistron contained the hygromycin resistance gene, hygromycin B phosphotransferase [15]. Selection with G418 (1 mg/ml) or hygromycin (0.5 μg/ml) in IL-3-conditioned medium was initiated 24 h after transfection. Selected Ba/F3 clones were screened for the presence of cell surface gp130 or LIFR by flow cytometry.

Flow cytometry

Cell surface expression of LIFR and gp130 and endocytosis of antibodies directed against gp130 and LIFR were quantified by flow cytometry as described previously [16]. Ba/F3 cells stably expressing human LIFR and gp130 were harvested, washed, and resuspended in cold binding medium (DMEM/0.2% BSA/20 mM Hepes, pH 7.0). Cells (1×10^6) in 200 μl were incubated for 60 min at 4 °C with 2 μg/ml of a LIFR-specific monoclonal antibody [17] or the gp130-specific monoclonal antibody B-R3 [18]. The cells were washed once at 4 °C, resuspended in prewarmed medium and incubated at 37 °C for the indicated times. The cells were then rapidly cooled and fixed in 2% (v/v) paraformaldehyde for 20 min on ice and washed twice in PBS. Subsequently, cells were resuspended in PBS supplemented with 5% (v/v) fetal-calf serum and 0.1% sodium azide; the receptor-bound antibodies were visualized using a 1:100 dilution of an R-phycoerythrin-conjugated anti-mouse IgG-F(ab′)$_2$ (Dianova) for 30 min on ice. Cells (2×10^4 cells/sample) were analysed using a FACScalibur (Becton Dickinson). Mean fluorescence intensity was recorded and used in the calculation of percentage antibody binding. Specific binding was calculated by subtracting the base line cell fluorescence intensity, which was determined with cells incubated solely with the secondary antibody, from the fluorescence intensity obtained with both primary and secondary antibodies. For each time point, t, the percentage of internalized antibody was calculated as follows: $100 \times (1 - \text{fluorescence intensity at time } t/\text{fluorescence intensity at time 0})$. All experiments were performed at least three times.

Immunofluorescence studies

Approximately 10^5 COS-7 cells grown on coverslips for 2 days were incubated for 2 h at 4 °C with a monoclonal antibody to the extracellular domain of the IL-5Rβ chain (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). After removing unbound antibody, cells were shifted to 37 °C for 30 min. Cells were washed twice with PBS, fixed with 2% (v/v) paraformaldehyde, permeabilized with 0.2% saponin and incubated with a 1:200 dilution of an FITC-conjugated mouse IgG antibody. Coverslips were mounted on slides with Mowiol™ 4-88 (Calbiochem Corp.) and analysed using an immunofluorescence microscope. A 100× oil lens was used to photograph the cells.

Internalization assay for IL-5R/LIFR and IL5R/gp130 chimaeras

Cells ($\sim 1 \times 10^5$ per well) transfected with the chimaeric constructs or with an empty control vector were incubated with 1 nM ^{125}I-IL-5 for 2 h at 4 °C. Internalization was initiated by rapidly warming the cells to 37 °C without removing unbound

ligand. After different times of incubation, cells were set on ice and washed three times with PBS containing 1 mM magnesium chloride and 0.1 mM calcium chloride. Surface-bound ^{125}I-IL-5 was removed by subjecting the cells to PBS/HCl, pH 3.0, 0.1 % BSA, for 2 min (acid wash) followed by an additional wash with PBS. The cells were further incubated with 1 μg/ml of proteinase K (Fluka) in PBS for 5 min to remove residual surface bound ^{125}I-IL-5. This treatment released more than 90 % of surface-bound ligand. Internalized ^{125}I-IL-5 was determined after lysis of the cells in 1 M NaOH. Specific binding and internalization was calculated by subtracting the values obtained with control vector transfected cells from the values obtained with cells expressing the chimaeric receptors.

RESULTS

We have recently demonstrated that the IL-6 signal transducer gp130 is internalized constitutively and that ligand binding does not increase its endocytosis [9]. We first analysed whether the LIFR is internalized independent of its association with gp130. Ba/F3 cells which stably express either human gp130 (Ba/F3-gp130) or the human LIFR (Ba/F3-LIFR) were incubated with monoclonal antibodies to the respective receptor chain at 4 °C and after shifting to 37 °C internalization was monitored by flow cytometry (Figure 2). As expected, surface gp130 was internalized with a half-life of about 15 min. This is in agreement with previous findings [19]. Notably, the LIFR was internalized even more rapidly than gp130 (Figure 2). Since Ba/F3-LIFR cells lack gp130 this finding strongly suggests that the LIFR can be endocytosed independently of gp130. Thus, the cytoplasmic domain of the LIFR is expected to contain an internalization motif of its own.

Inspection of the amino acid sequence of the LIFR cytoplasmic tail revealed two likely candidates for an internalization motif, a putative tyrosine motif $\mathbf{Y}_{115}$IDV and a putative dileucine motif SRQF$\mathbf{L}_{210}\mathbf{I}_{211}$PP. In order to test whether any of these or both are essential for endocytosis of the LIFR we replaced tyrosine 115 or leucine 210/isoleucine 211 by alanine residues, each by site-directed mutagenesis (Figure 1).

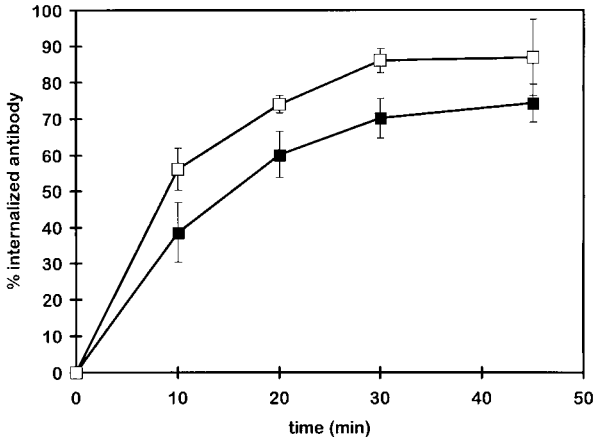

Figure 2 Endocytosis of wild-type gp130 and LIFR as measured by flow cytometry

Kinetics of internalization of LIFR- and gp130-specific monoclonal antibodies in Ba/F3 cells stably transfected with LIFR (□) or gp130 (■) respectively. The cells were incubated for 60 min at 4 °C with LIFR- or gp130-specific monoclonal antibodies. The cells were incubated at 37 °C for the indicated times, rapidly cooled and fixed in paraformaldehyde. The receptor-bound antibodies were detected using an R-phycoerythrin-conjugated anti-mouse IgG-F(ab′)$_2$. The cells were analysed using a FACScalibur. Mean fluorescence intensity was recorded and used in the calculation of percentage antibody binding. Specific binding was calculated as described in the Material and methods section. Data are the means ± S.D. of three independent experiments.

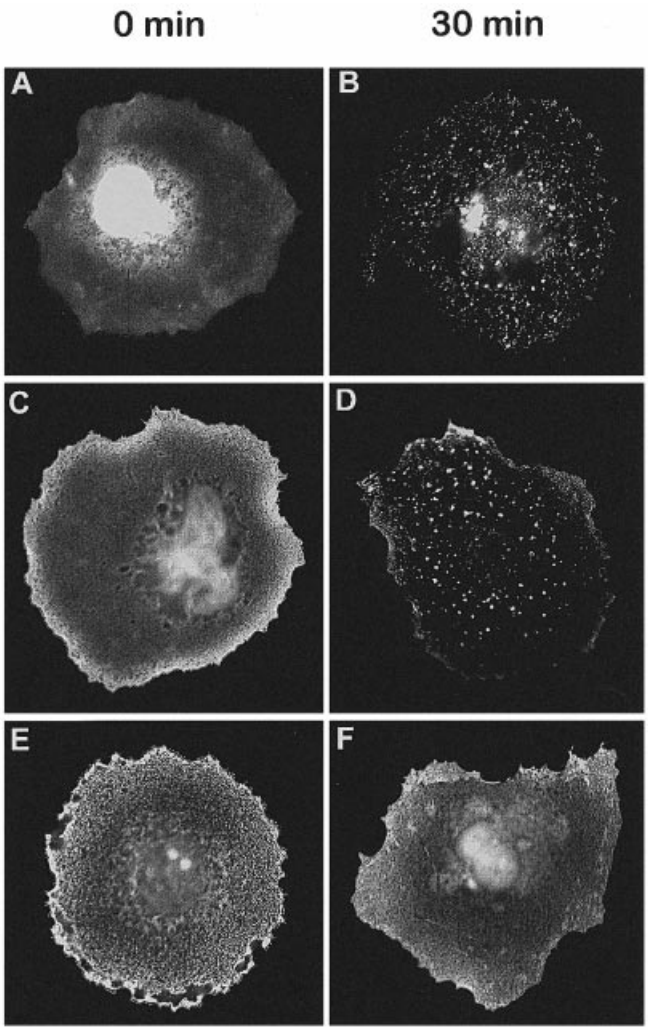

Figure 3 Internalization of monoclonal antibodies by COS-7 cells expressing IL-5Rβ/LIFR chimaeras

(**A** and **B**) IL-5Rβ/LIFR$_{WT}$, (**C** and **D**) IL-5Rβ/LIFR$_{AIDV}$, and (**E** and **F**) IL-5Rβ/LIFR$_{AA}$. Three days after transfection, COS-7 cells were incubated with an IL-5Rβ chain-specific monoclonal antibody for 2 h at 4 °C. Prebound antibodies were internalized for 30 min at 37 °C (**B**, **D** and **F**). Cells were fixed, permeabilized, and stained with an FITC-conjugated mouse IgG antibody. Representative optical cuts are shown.

To examine these mutated cytoplasmic tails we took advantage of a chimaeric receptor system recently established in our laboratory [13]. This system is comprised of the extracellular domains of the IL-5Rα and the IL-5Rβ-chains to which the transmembrane and cytoplasmic parts of gp130 and the LIFR were fused (Figure 1), and allows us to study independent mutations in one or both cytoplasmic chains of the heterodimeric gp130/LIFR complex, in cells that endogenously express gp130 [8]. We first analysed surface expression of the chimaeric receptor chains in single transfected COS-7 cells using a monoclonal antibody directed against the IL-5Rβ chain. The IL-5Rβ/LIFR$_{WT}$ chimaera was clearly detected at the cell surface (Figure

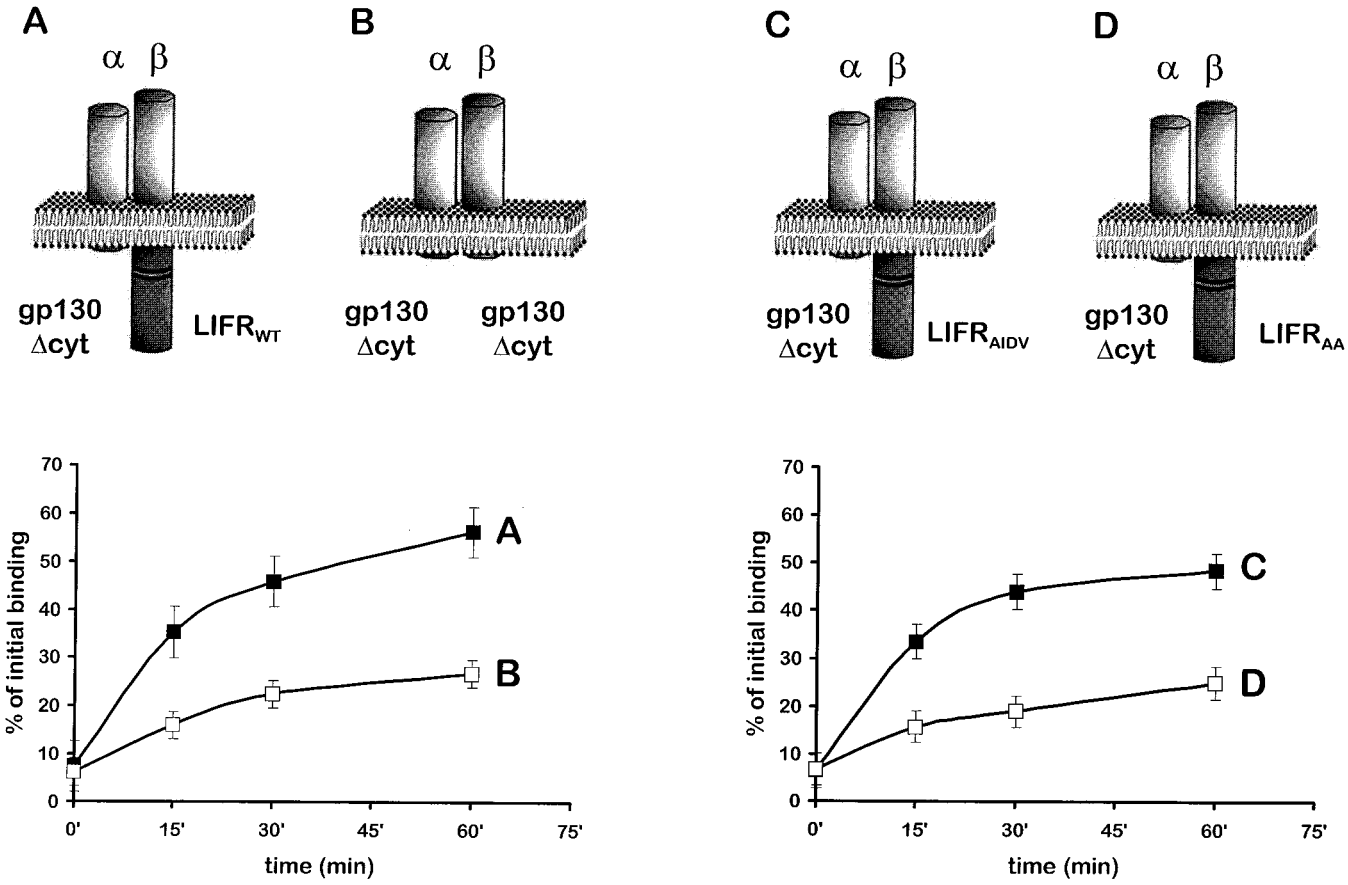

Figure 4 Internalization of surface-bound ^{125}I-IL-5 via chimaeric receptor complexes

(Top) Schematic diagrams of the chimaeric receptor complexes. (Bottom) Internalization of IL-5 via chimaeric receptors. Two or three days after transfection, cells were washed twice with ice-cold PBS. After incubation with 1 nM ^{125}I-IL-5 for 2 h at 4 °C, cells were warmed to 37 °C for the indicated times without removing unbound ligand. The cells were then set on ice and washed three times with PBS containing 1 mM magnesium chloride and 0.1 mM calcium chloride. Surface-bound and internalized ^{125}I-IL-5 were determined as described in the Material and methods section. Binding to mock-transfected cells was less than 7.5% of binding to chimaeric IL-5 receptor expressing cells; non-specific internalization was less than 15% of internalization via IL-5 receptor chimaeras. The values obtained for binding and internalization by mock-transfected cells were subtracted. Data are expressed as a percentage of the amount of IL-5 bound at time point 0. Data are the means ± S.D. for four independent experiments. Details of (**A**), (**B**), (**C**), and (**D**) are given in the text.

3A). The same was found for the chimaeras with either the tyrosine motif (IL-5Rβ/LIFR$_{AIDV}$) or the dileucine motif (IL-5Rβ/LIFR$_{AA}$) replaced by alanine (Figures 3C and 3E). When the antibodies were allowed to be internalized for 30 min at 37 °C, indirect immunofluorescence revealed a prominent punctate intracellular staining pattern (Figures 3B and 3D) in IL-5Rβ/LIFR$_{WT}$ and IL-5Rβ/LIFR$_{AIDV}$ transfected cells. In sharp contrast, in IL-5Rβ/LIFR$_{AA}$ transfected cells after 30 min the antibody was still mainly located at the cell surface (Figure 3F). These findings point to the dileucine sequence being the internalization motif. As expected, the IL-5Rα/gp130Δcyt and the IL-5Rβ/gp130Δcyt chimaera were also located at the cell surface and were not internalized (results not shown).

In order to obtain more quantitative data, internalization studies using ^{125}I-IL-5 were performed. In these studies, COS-7 cells were transfected with an IL-5Rα/gp130Δcyt chimaera and one of the above mentioned IL-5Rβ/LIFR constructs or, as a control, an IL-5Rβ/gp130Δcyt chimaera. In a recent study we have demonstrated that IL-5R/gp130 chimaeras with and without a cytoplasmic tail bind IL-5 with similar affinity to the wild-type IL-5R complex [13]. A heterodimeric complex consisting of the IL-5Rα/gp130Δcyt and the IL-5Rβ/gp130Δcyt chimaeras internalized IL-5 inefficiently, since both cytoplasmic chains were lacking (Figure 4B). When the IL-5Rβ/gp130Δcyt was replaced by the IL-5Rβ/LIFR$_{WT}$, a significant increase in IL-5 endocytosis was observed (Figure 4A). A comparable effect was observed when the IL-5Rβ/gp130$_{WT}$ was co-expressed with the IL-5Rα/gp130Δcyt [8]. When the two mutated LIFR chimaeras were tested in this system, the IL-5Rβ/LIFR$_{AIDV}$ mutant internalized IL-5 as efficiently as the LIFR$_{WT}$ chimaera (compare Figures 4A and 4C). In contrast, the IL-5Rβ/LIFR$_{AA}$ behaved like the gp130Δcyt chimaera, indicating that this LIFR mutant has lost its internalization capability (Figure 4D).

DISCUSSION

Two new findings are presented in this study. First, the LIFR is internalized independent of its ligand, and therefore also independent of an association with the IL-6 signal transducer gp130. Secondly, the LIFR cytoplasmic domain contains a di-leucine type internalization motif at position 210/211. A di-leucine based motif has recently also been characterized in the cytoplasmic tail of gp130 [7], the common signal transducing component of all IL-6-type cytokines, i.e. of LIF, oncostatin M,

IL-11, ciliary neurotrophic factor and cardiotrophin-1. Two kinds of signalling complexes have been described so far, firstly, gp130 homodimers form upon binding of IL-6 or IL-11 to their respective receptor α-chains, secondly, gp130 heterodimers with either the LIFR or the oncostatin M receptor form after stimulation with LIF, oncostatin M, ciliary neurotrophic factor and cardiotrophin-1 [4]. Homodimers of the LIFR or the oncostatin-M receptor have not been demonstrated so far. Therefore, theoretically, all possible homodimeric or heterodimeric receptor complexes formed upon IL-6-type cytokine binding could be internalized via gp130. Such a concept was supported by our finding that a single dileucine motif within a dimeric gp130 is sufficient to mediate its endocytosis [8]. However, the data reported here and in a recent study by Zhang et al. [6] clearly demonstrate that the LIFR is internalized independent of gp130. Zhang et al. studied the internalization of LIF via wild-type gp130 and various LIFR mutants containing truncations of its cytoplasmic part. When expressed alone the LIFR was already capable of internalizing LIF [6], which like our data (Figures 2 and 3) supports the notion that endocytosis of LIFR is not dependent on signalling. An LIFR/gp130 complex displayed an increased internalization rate which was dependent on the 88 C-terminal amino acids of the cytoplasmic domain of the LIFR [6]. The Leu-Ile motif (Figure 4) identified in the present study is located within this region.

What is the biological relevance of this redundancy of signals for endocytosis within IL-6-type cytokine receptors? One possibility is that there exist receptor complexes containing the LIFR that do not contain gp130. Such complexes have not been described so far but cannot be excluded at the moment. A second possibility is that endocytosis via two independent internalization motifs is more efficient. In the case of gp130 homodimers this is unlikely, since we recently demonstrated that the internalization rates of ligated (dimeric) and non-ligated (monomeric) gp130 are the same [9]. In contrast, for the LIFR/gp130 heterodimer it was found that its internalization rate is increased compared to the rate for the LIFR alone [6]. A third possibility is that the surface expression of the LIFR has to be regulated independently from that of gp130 and therefore needs its own trafficking signals. A fourth possibility is that the identified internalization motif has additional functions not addressed in this study. For a number of receptors it has been observed that internalization motifs overlap with sequences determining basolateral localization in polarized cells [20]. The LIFR is expressed in a number of polarized cells such as hepatocytes and epithelial cells of the endometrium [21], and it is possible that leucine 210 and isoleucine 211 of the cytoplasmic part of the LIFR are also part of a basolateral targeting sequence. Future studies will address this question.

The LIFR internalization motif contains an isoleucine in the second position. However, such a deviation from the canonical dileucine motif has also been found in other internalization signals, e.g. in that of the major histocompatibility complex class II invariant chain [22] and the lysosomal integral membrane protein, LIMP-II [23]. Also, mutagenesis of the gp130 dileucine motif revealed that an isoleucine at either position is still functional [7].

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