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# The cytoplasmic domain of the interleukin-6 receptor gp80 mediates its basolateral sorting in polarized Madin-Darby canine kidney cells

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## SUMMARY

**The IL-6 receptor complex is expressed in different polarized epithelial cells such as liver hepatocytes and intestinal cells. It consists of two subunits: gp80, which binds the ligand, and gp130, which is responsible for signal transduction. In stably transfected Madin-Darby canine kidney (MDCK) cells we have studied the localization of the human IL-6 receptor subunits and found that gp80 and gp130 are predominantly expressed at the basolateral membrane. Analysis of MDCK cells expressing truncated forms of gp80 or gp130 showed that loss of the cytoplasmic domains results in apical delivery. Expression of deletion mutants of gp80 in MDCK cells led to the identification of two discontinous motifs responsible for basolateral sorting: a membrane-proximal tyrosine-based motif (YSLG) and a more membrane-distal dileucine-type motif (LI). Activation of signal transducer and activator of transcription-3 (STAT-3) only occurred via basolaterally located gp80, suggesting that endogenous gp130 is also constrained to the basolateral plasma membrane. Our identification of a basolateral sorting signal within the cytoplasmic region of gp80 for the first time attributes a function to this domain.**

Key words: Interleukin-6, Basolateral sorting, Epithelium, Polarity, Jak/STAT, MDCK cell

## INTRODUCTION

Interleukin-6 (IL-6) is a multifunctional cytokine that acts on a wide spectrum of different target cells and exerts multiple functions during the immune response, hematopoiesis, neural differentiation and the acute phase reaction (for a recent review, see Heinrich et al., 1998). IL-6 acts via a receptor complex consisting of an 80 kDa binding protein (gp80, IL-6R) and a 130 kDa signal transducer, gp130 (Hibi et al., 1990; Taga et al., 1989; Yamasaki et al., 1988). Ligand-induced dimerization of gp130 results in the activation of associated Janus kinases (JAK) that phosphorylate the signal transducer, thereby creating docking sites for signal transducers and activators of transcription (STAT)-3 and STAT-1. STATs become tyrosine-phosphorylated, dimerize and translocate to the nucleus, where they bind to enhancer elements within the promoter region of IL-6-responsive genes (Heinrich et al., 1998).

After binding of IL-6 to gp80, the ligand/receptor complex is rapidly internalized and degraded intracellularly, resulting in the loss of IL-6 binding sites at the plasma membrane (Zohlnhöfer et al., 1992). Internalization of IL-6/gp80 complexes was found to be dependent on a dileucine-type internalization motif within the cytoplasmic tail of the signal transducer gp130 (Dittrich et al., 1994, 1996). Whereas the cytoplasmic part of gp130 is essential for signalling and endocytosis, the equivalent part of gp80 was found to be dispensable for both processes (Dittrich et al., 1994; Taga et al., 1989), and no function for the gp80 cytoplasmic domain has so far been proposed.

IL-6 receptors (IL-6R) are found on a number of different cells, some of which are polarized, e.g. hepatocytes and intestinal epithelial cells (Castell et al., 1988; Molmenti et al., 1993). Polarized epithelial cells have two distinct plasma membrane domains characterized by different protein and lipid compositions and morphology: an apical membrane that acts as a barrier towards the external milieu and a basolateral membrane that faces neighbouring cells and the extracellular matrix, to which the cells adhere (Eaton and Simons, 1995; Matter and Mellman, 1994; Rodriguez-Boulan and Powell, 1992). In the hepatocyte, several apical membranes surround the bile canaliculi whereas the basolateral domain faces the sinusoid (Simons and Fuller, 1985). To date there is scant information on the polarity of expression of gp80 and gp130. In intestinal cells, endogenous gp80 was found exclusively on the basolateral side of T84 human colonic adenocarcinoma cells whereas it is localized on both sides of Caco-2 human intestinal epithelial cells (Molmenti et al., 1993). In hepatocytes, localization of the components of the IL-6R complex has so far not been determined.

To learn more about the polarized expression of the interleukin-6 receptor and the signals responsible for targeting to the respective plasma membrane compartment, we expressed wild-type and mutant forms of human gp80 and gp130 in Madin-Darby canine kidney (MDCK) polarized

epithelial cells. This well-characterized cell line is a useful model for studying polarized sorting of membrane proteins and the respective signals involved in these processes (Rodriguez-Boulan and Powell, 1992). We found gp80 and gp130 to be sorted predominantly to the basolateral side of MDCK cells. Deletion of the respective cytoplasmic regions resulted in a reversed polar expression, suggesting that basolateral sorting information is localized in this domain. Two discontinuous sorting signals were identified within the cytoplasmic part of gp80. In addition, the polarity of expression of endogenous canine gp130 has been determined.

## MATERIALS AND METHODS

### Cell culture

MDCK cells, strain II, were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, streptomycin (100 mg/l) and penicillin (60 mg/l) at 37°C in a water-saturated atmosphere containing 5% $CO_2$. HepG2 cells were grown in Dulbecco's modified Eagle's medium/Nut.Mix.F12 supplemented as above. For polarity studies, MDCK cells were plated and grown for 5 days on Costar Transwell filters (van Meer et al., 1987). Confluent monolayers were treated with 10 mM sodium butyrate for 15 hours to increase the expression level of the transfected genes (Brewer and Roth, 1991).

### Preparation and characterization of MDCK cell lines

Transfection of MDCK cells was performed by a modification of the calcium-phosphate precipitation procedure as previously described (Graham and van der Eb, 1973; Rodriguez-Boulan et al., 1989). The transfected cells were selected with 500 μg/ml G418 for 10-14 days. All experiments were performed on populations of transfectants to minimize the effect of clonal variation. The populations were screened for expression by indirect immunofluorescence and western blot analysis.

### cDNA constructs

Standard cloning procedures were performed as outlined by Sambrook et al. (1989). All human gp80 constructs were cloned or subcloned into the expression vector pCB6 (kindly provided by Dr Le Bivic, Marseilles, France) (Brewer and Roth, 1991) under the control of the cytomegalovirus immediate-early promoter.

The *Xba*I restriction site was used to subclone gp80wt. The deletion mutants gp80Δ429 and gp80Δ426 were generated by exonucleaseIII digest and subcloned using the *Xba*I restriction site. The first construct ends with amino acid (aa) 428 (plus two non-authentic aa: AS) and the second construct ends with aa 425 (plus two non-authentic aa: HS). Deletion mutants gp80Δ412 and gp80Δ408 were generated by polymerase chain reaction using primers that introduce a stop codon downstream of the last authentic amino acid. Primer sequences are available upon request. The gp80ΔCD construct ends with the last amino acid of the transmembrane region (aa 386) (plus two non-authentic aa: HS). It was generated by exonucleaseIII digest and subcloned using the *Hin*dIII and *Xba*I restriction sites.

The sgp80 cDNA was subcloned into the expression vector pBMG Neo (kindly provided by Dr Müllberg, Mainz, Germany). The gp130 constructs were subcloned into the expression vector pCAGGS (kindly provided by Dr Hirano, Osaka, Japan) under the control of the chicken β-actin promoter. The gp130wt was subcloned using the *Xho*I and *Bam*HI restriction sites. The *Bam*HI site was ligated with the *Bgl*II site of the vector. The deletion mutant gp130ΔCD (Behrmann et al., 1997) was subcloned as gp130wt. All cloning products were verified by dideoxynucleotide sequencing (Sanger et al., 1977).

### Immunofluorescence staining

The transfected MDCK cells were grown for 4 days on collagen-coated coverslips (approx. 10 μg/cm$^2$ of a 25% solution) and fixed with 2% (w/v) paraformaldehyde as described before (Graeve et al., 1990). After permeabilization with 0.2% saponin the cells were incubated with a 1/200 dilution of an sIL-6R-specific rabbit antiserum and with a 1/100 dilution of a BC11 (basolateral marker protein)-specific mouse antiserum (kindly provided by Dr Le Bivic, Marseille, France). Antibodies bound to cell components were detected using a 1/300 dilution of a Cy3-conjugated anti-rabbit IgG antibody (Jackson Laboratories) and a 1/100 dilution of an FITC-conjugated anti-mouse IgG antibody (Dako). Coverslips were mounted on slides with Mowiol$^{TM}$ 4-88 (Calbiochem-Novabiochem). For negative controls, samples were incubated either with the combination of secondary antibodies alone or with each single first antibody together with both secondary antibodies. Microscopy was performed using the confocal laser scanning system TCS-NT (argon-krypton laser, Leica). Light of wavelength 488 nm or 568 nm was used for excitation and images were acquired at a wavelength of 530-580 nm for FITC or 590 nm and above for Cy3, respectively.

### Immunoprecipitation

Cells were washed twice with PBS and solubilized in 1 ml of lysis buffer (1% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 60 mM EDTA, 0.4% deoxycholic acid) in the presence of protease inhibitors (0.25 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin) for 30 minutes at 4°C. Insoluble material was removed by centrifugation and the supernatants were pretreated with *S. aureus* Prot A$^+$ (ImmunoPrep, Strathmann Biotech.). SDS was added to a final concentration of 0.3%. After incubation with the appropriate antisera for at least 2 hours at 4°C (for gp80: an sIL-6R-specific rabbit antiserum; for gp130: mAb BR-3; Wijdenes et al., 1995) the immune complexes were precipitated with protein A sepharose (5 mg/ml in washing buffer: 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4). For precipitating immune complexes of mouse antisera, the lysates were first incubated with a rabbit anti-mouse IgG antibody. After centrifugation of the immunocomplexes, the Sepharose beads were washed three times with washing buffer and boiled in reducing sample buffer. The eluted proteins were separated on a 7.5% SDS-polyacrylamide gel.

### $^{125}$I-IL-6 binding assay

Recombinant human IL-6 was kindly provided by Dr T. Kishimoto (Osaka, Japan). IL-6 was iodinated with the Bolton-Hunter reagent to a specific radioactivity of 3MBq/μg protein (Bolton and Hunter, 1972). Parental and transfected MDCK cell lines were grown on Transwell filters for 5 days and the polarized monolayers were washed twice with cold binding medium (DMEM with 0.2% BSA and 20 mM Hepes, pH 7.2). Either the basolateral or the apical membranes were incubated with $^{125}$I-IL-6 (20 ng/ml binding medium) at 4°C overnight. The opposite side was incubated with an excess of unlabelled IL-6 (200 ng/ml). After extensive washing, filters were excised and incubated with lysis buffer before an immunoprecipitation of gp80 was performed. The amount of $^{125}$I-IL-6 bound was determined by measuring the protein A-sepharose conjugated immunocomplexes in a γ-counter. Data were analysed by one-way ANOVA. Comparison of all pairs was performed using the Tukey-Kramer test. $P<0.05$ was considered to be significant.

### Biotinylation assay

Sulfo-NHS-biotin (Pierce) was used to label cell surface proteins (Graeve et al., 1989). MDCK cells were grown on Transwell filters for 5 days, then washed three times with PBS$^{2+}$ (PBS with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$) and once with biotin buffer (120 mM NaCl, 20 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 8.5) at 4°C for 15 minutes. Sulfo-NHS-biotin labelling (0.5 mg/ml in biotin buffer, freshly diluted from a frozen stock of 200 mg/ml in DMSO) was performed twice for 20 minutes at 4°C, either basolaterally or apically.

Afterwards the cells were washed once with binding medium and three times with $PBS^{2+}$ for 5 minutes at 4°C each.

### Western blotting and immunodetection

The electrophoretically separated proteins were transferred to polyvinylidene difluoride membranes by the semi-dry western blotting method. Non-specific binding was blocked with 5% bovine serum albumin in TBS-N (20 mM Tris/HCl, pH 7.4, 137 mM NaCl, 0.1% Nonidet P-40) for 1 hour. To detect gp80, the immunoblot was first incubated with a mixture of two sgp80-specific mAbs BR6 and BN12 and then incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody, each for 1 hour. To detect biotinylated gp130, the immunoblot was incubated with horseradish peroxidase-conjugated streptavidin (Pierce; 1 mg/ml in TBS-N, 1 M D-glucose, 10% glycerol) for 30 minutes. The immunoblots were developed using the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

### Electrophoretic mobility shift assay (EMSA)

Parental and transfected MDCK cells were grown for 5 days on Transwell filters. The cells were stimulated with IL-6 (100 U/ml) and sIL-6R for 15 minutes. Nuclear extracts were prepared as described (Andrews and Faller, 1991). The protein concentration was determined by a Bio-Rad® protein assay. EMSAs were performed using a double-stranded $^{32}$P-labelled mutated m67SIE-oligonucleotide from the c-fos promoter (m67SIE: 5′-GAT CCG GGA GGG ATT TAC GGG GAA ATG CTG-3′) (Wagner et al., 1990; Wegenka et al., 1993). The protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE (20 mM Tris, 20 mM boric acid, 0.5 mM EDTA, pH 8) at 20 V/cm for 4 hours. Gels were fixed in 10% methanol, 10% acetic acid and 80% water for 1 hour, dried and autoradiographed.

## RESULTS

### The cytoplasmic domain is necessary for basolateral sorting of gp80 in MDCK cells

We have previously generated stably transfected MDCK cells expressing human gp80 in order to determine the receptor half-life (Gerhartz et al., 1994). When these cells were cultured on a permeable support that allowed establishment of a polarized monolayer, gp80 was found to be predominantly expressed at the basolateral side, as assessed by a surface biotinylation protocol (data not shown).

Since basolateral sorting signals are typically confined to the cytoplasmic domain of transmembrane proteins (Matter and Mellman, 1994) and since no function has so far been found for the respective domain of gp80, we constructed the mutant gp80ΔCD, which lacks the cytoplasmic 82 aa of wild-type gp80 (Fig. 1). MDCK cells were transfected with expression vector pCB6 (Brewer and Roth, 1991) containing either the gp80wt or gp80ΔCD cDNA and stable clones were generated using G418 resistance as a selection marker. Expression was verified using indirect immunofluorescence (data not shown) and western blot analysis. Proteins of the expected size (about 85 kDa for gp80wt and 75 kDa for gp80ΔCD) were expressed and detected in the stable MDCK clones (Fig. 2, second and last lane).

In order to analyze the polarized expression of gp80wt and gp80ΔCD, cells were grown for 4 days on collagen-coated coverslips, fixed, permeabilized and processed for indirect immunofluorescence staining using a polyclonal antibody against the human gp80 extracellular domain and a Cy3-conjugated secondary antibody. As a control, cells were also incubated with antibody BC11, which is directed against a basolateral marker protein (Beau et al., 1998), and an FITC-conjugated secondary antibody. Stained cells were analyzed by confocal laser scanning microscopy (Fig. 3). When cells were analyzed by *xy* scan, a typical rim-like staining was observed for the basolateral marker, which indicates that this protein is mainly concentrated at the lateral membranes of adjacent MDCK cells (Fig. 3A,E). An almost identical staining was obtained for gp80wt, especially as depicted in the overlay (Fig. 3B,C). In the *xz* scan, lateral localization of gp80wt and the basolateral marker BC11 is seen as a bar-like structure separating adjacent cells (Fig. 3D). These data support our

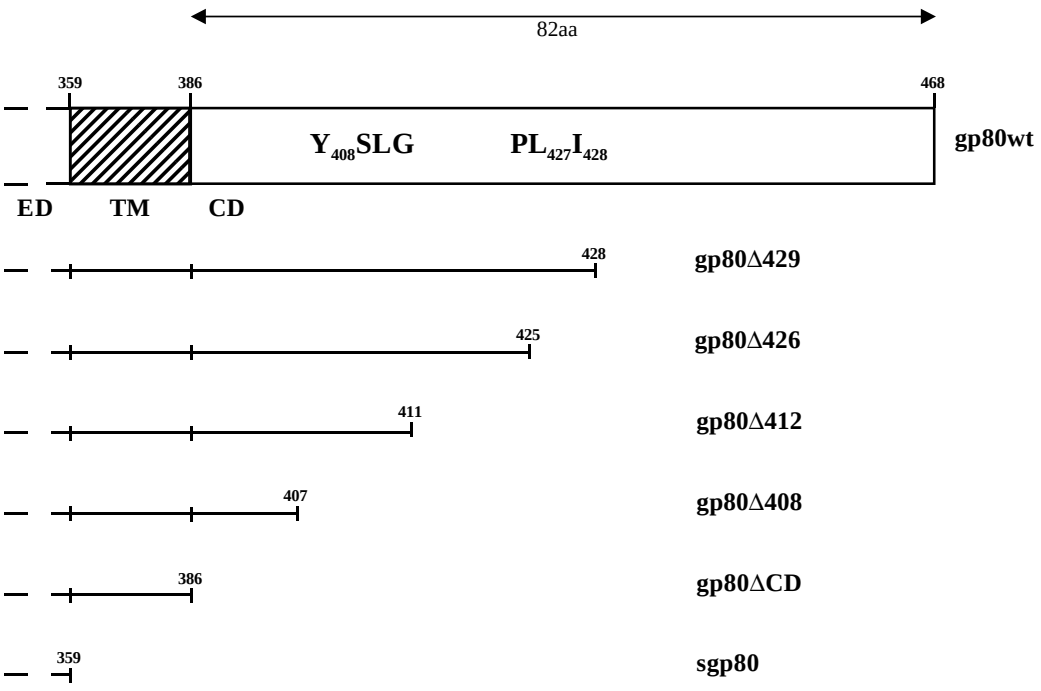


**Fig. 1.** Scheme of the structure of gp80wt and the different deletion mutants. Intact IL-6R consists of 468 aa; the extracellular domain (ED) ends at aa 359 and the putative transmembrane region (TM) at aa 386. The cytoplasmic domain (CD) of gp80wt consists of 82 aa, which contain two putative basolateral sorting signals: a membrane-proximal tyrosine-based ($Y_{408}$SLG) and a dileucine-type ($PL_{427}I_{428}$) signal. The different deletion mutants are shown below, and in each case the last aa encoded by the C-terminally deleted cDNA constructs is indicated.

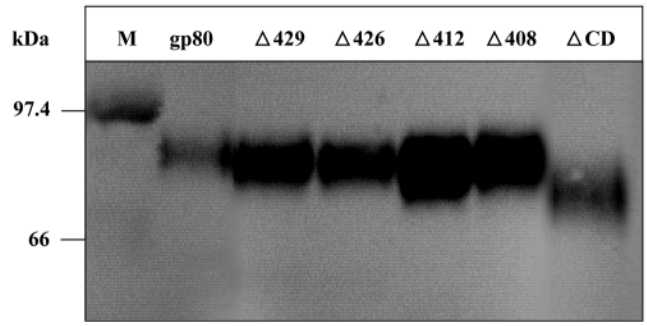


**Fig. 2.** Expression of different gp80 cDNA constructs in stably transfected MDCK cells. MDCK cells stably transfected with different gp80 cDNA constructs were lysed and immunoprecipitated with a polyclonal anti-sIL-6R antibody. The proteins were analyzed by 7.5% SDS/PAGE and western blotting. The membrane-bound proteins were detected with a mixture of two monoclonal anti-gp80 antibodies and a secondary horseradish peroxidase-conjugated antibody and visualized by enhanced chemiluminescence (ECL). The positions of molecular size markers are indicated (Lane M).

finding that gp80wt is preferentially localized at the basolateral domain of MDCK cells. In contrast, gp80ΔCD gave an even surface staining of the apical membrane (Fig. 3F) that did not colocalize with BC11 (Fig. 3E,G). Also the *xz* scan clearly demonstrates an opposite localization of gp80ΔCD and BC11 (Fig. 3H). Thus, deletion of the cytoplasmic domain of gp80 results in expression of the truncated protein at the apical plasma membrane of MDCK cells.

In order to obtain more quantitative data, binding studies using $^{125}$I-IL-6 were performed. Parental MDCK cells do not bind appreciable amounts of human IL-6, suggesting that they do not express the IL-6R or that the canine IL-6R is not able to interact with human IL-6 (data not shown). Stable transfectants and parental cells were cultured for 5 days on Transwell filters in order to allow complete polarization of the monolayer. Filters were incubated with 1 nM $^{125}$I-IL-6, added either to the apical or to the basolateral chamber of the Transwell filter, for at least 10 hours at 4°C. After cell lysis $^{125}$I-IL-6/gp80 complexes were immunoprecipitated and quantitated in a γ-counter. An immunoprecipitation step was included to minimize experimental error by unspecific binding of $^{125}$I-IL-6 to the filter membrane and the cell surface. As shown in Fig. 4A, 95% of IL-6 binding sites are confined to the basolateral surface in cells expressing gp80wt. In contrast, almost 80% of the truncated IL-6R gp80ΔCD is localized at the apical membrane, supporting the results of the confocal microscopy studies (Fig. 4A).

To investigate whether the transmembrane region of gp80 has an influence on the sorting of gp80ΔCD, we generated stably transfected MDCK cells expressing a soluble form of gp80 (sgp80; Fig. 2). The amount of sgp80 secreted into the apical or basolateral chamber was analyzed using an sgp80-specific ELISA (Müller-Newen et al., 1996). 78.5±3.5% ($n$=2) of the total sgp80 secreted by filter-grown MDCK cells was released into the apical chamber. This shows that the transmembrane domain of gp80 does not have an impact on the sorting of gp80ΔCD, since the cytoplasmically deleted mutant is also preferentially localized at the apical plasma membrane.

## The cytoplasmic domain of gp80 contains two discontinuous basolateral sorting motifs

Since the cytoplasmic domain of gp80 was necessary for basolateral targeting in MDCK cells, additional deletion mutants of the IL-6R were created in order to localize the sorting signal within this domain. A number of identified basolateral targeting motifs either contain a tyrosine residue or are of the dileucine-type, both of which are usually localized in proximity to the membrane (Matter and Mellman, 1994). The gp80 cytoplasmic domain contains a membrane-proximal putative tyrosine motif $Y_{408}$SLG as well as a putative dileucine-type motif $PL_{427}I_{428}$. Therefore, deletion mutants were constructed that either included or excluded one or both putative targeting motifs (Fig. 1). MDCK cells stably expressing these deletion constructs were generated and expression was verified by western blot analysis (Fig. 2) and indirect immunofluorescence (data not shown). The polarized expression of the different receptor proteins was studied by binding assays with $^{125}$I-IL-6. In MDCK cells that stably express a receptor mutant which lacks the 40 carboxyterminal amino acids of the cytoplasmic domain (gp80Δ429) 84% of the IL-6 binding sites are still localized at the basolateral membrane (Fig. 4B). Thus the 42 membrane-proximal aa of gp80 must contain the basolateral sorting information. When three additional amino acids were deleted (gp80Δ426), namely $PL_{427}I_{428}$, the preferential basolateral localization of IL-6 binding sites was lost ($P$<0.01), resulting in an approximately equal distribution on both membrane domains (Fig. 4B). Thus, the amino acids $PL_{427}I_{428}$ clearly contribute to the basolateral targeting of gp80. However, since the gp80ΔCD mutant was almost 80% apical, additional sequences upstream of $PL_{427}I_{428}$ must also contribute to the basolateral sorting.

Mutant gp80Δ412 lacking an additional 15 amino acids similarly showed an equal distribution between the two plasma membranes as gp80Δ426 (Fig. 4C). When another four amino acids, namely $Y_{408}$SLG, were removed the resulting receptor mutant gp80Δ408 was preferentially (67%) sorted to the apical side of MDCK cells (Fig. 4C). Thus, the $Y_{408}$SLG motif also contributes to the basolateral sorting of the IL-6R.

## STAT factors are only activated via IL-6 induced signal transduction at the basolateral membrane of MDCK cells

Binding of IL-6 to its receptor complex composed of gp80 and the signal transducer gp130 leads to the activation of tyrosine kinases of the Janus family and subsequently to the tyrosine-phosphorylation of gp130 and transcription factor STAT3 (Heinrich et al., 1998). We thus asked whether activation of the Jak/STAT pathway by IL-6 is also restricted to the basolateral surface in MDCK cells. In order to study this, parental MDCK, MDCKgp80wt and MDCKgp80ΔCD cells were grown on Transwell filters and stimulated with IL-6 added from either the apical or the basolateral side for 15 minutes at 37°C. For comparison, HepG2 cells grown on plastic dishes were stimulated in the same way. After stimulation, nuclear extracts were prepared and analyzed by an electrophoretic mobility-shift assay using a STAT3-specific DNA oligonucleotide (Gerhartz et al., 1996). Stimulation of HepG2 cells resulted in a prominent band that was previously identified as a STAT3 homodimer (Fig. 5A, lane 2); the weak faster migrating band corresponds to a

STAT3/STAT1 heterodimer (Gerhartz et al., 1996). Stimulation of parental MDCK cells with IL-6 added to both sides did not result in any significant formation of a STAT dimer (Fig. 5A, lane 3), supporting our previous notion that MDCK cells do not express an IL-6R capable of binding human IL-6 (Graeve et al., 1996). When MDCKgp80wt cells were stimulated from either side, only basolaterally added IL-6 led to STAT activation (Fig. 5A, lanes 4+5); similar to HepG2 cells, almost exclusively STAT3 was activated. These data indicate that the complex of human IL-6/gp80 can interact with endogenous canine gp130 and demonstrate that ligation of canine gp130 results in an activation of the Jak/STAT pathway in MDCK cells. In addition they are consistent with the results of the immunofluorescence and binding studies showing that gp80 is restricted to the basolateral domain. It is well documented that cells expressing only gp130 can be stimulated by complexes of IL-6 and the recombinant soluble IL-6R (Mackiewicz et al., 1992). When parental cells that do not express the IL-6R endogenously were costimulated with a combination of IL-6 and recombinant soluble IL-6R, STAT3 activation was again only observed when the cells were stimulated from the basolateral side (Fig. 5B, lanes 1+2). This also suggests that the endogenous gp130 and/or other crucial components of the Jak/STAT signalling pathway are restricted to the basolateral side. This also became apparent in MDCK gp80ΔCD cells in which 80% of the truncated gp80 are expressed at the apical domain and only IL-6 added to the basolateral side led to STAT activation (Fig. 5B, lanes 5+6). In conclusion, activation of the Jak/STAT pathway in MDCK cells via the endogenous gp130 and a complex of IL-6 and the membrane-bound or soluble IL-6R exclusively occurs at the basolateral plasma membrane.

## Basolateral expression of gp130 is also mediated by its cytoplasmic domain

To analyze the polarity and the putative basolateral sorting signal of gp130 directly, stable MDCK transfectants were generated that either express human gp130wt or gp130ΔCD. The polarity of the signal transducer was assessed by selective surface-labelling of either the apical or basolateral plasma membrane of filter-grown cells with sulfo-NHS-biotin, followed by immunoprecipitation, western blot analysis and detection with horseradish peroxidase-conjugated streptavidin employing ECL. Human gp130 was preferentially labelled at the basolateral membrane of transfected MDCK cells (Fig. 6, upper panel), supporting the notion that endogenous gp130 is also confined to the basolateral side of polarized epithelial cells. In cells that express a cytoplasmically deleted gp130 mutant – as with gp80 – a complete reversal of polarity was observed since the truncated gp130ΔCD was almost exclusively labelled at the apical surface (Fig. 6, lower panel). Thus, the gp130 cytoplasmic domain must also contain at least one basolateral sorting motif.

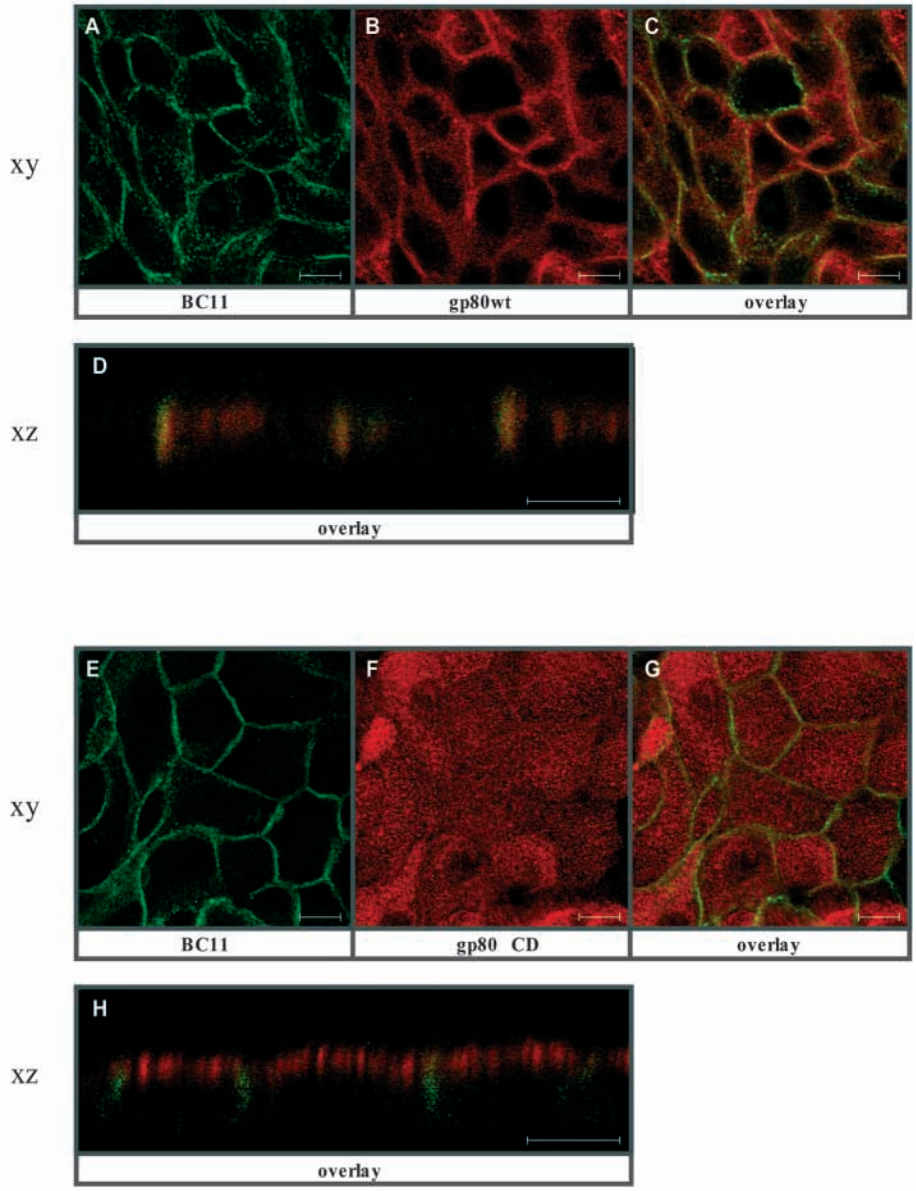


**Fig. 3.** Localization of gp80wt and gp80ΔCD in stably transfected MDCK cells. MDCK cells stably transfected with gp80wt (A-D) or gp80ΔCD (E-H) were grown on collagen-coated coverslips for 4 days prior to the experiment. Indirect immunofluorescence staining was performed as described in Materials and Methods using a monoclonal mouse antibody against a basolateral marker protein (BC11) and a polyclonal rabbit antiserum against sgp80 followed by FITC-conjugated anti-mouse and Cy3-conjugated anti-rabbit antibody. Microscopy was performed using the confocal laser scanning system TCS-NT. *xy* scans: basolateral marker, BC11 (A,E); gp80wt or gp80ΔCD (B,F), respectively; overlay for gp80wt/BC11 (C) and gp80ΔCD/BC11 (G); *xz* scan for gp80wt/BC11 (D), for gp80ΔCD/BC11 (H). Bars, 10 μm.

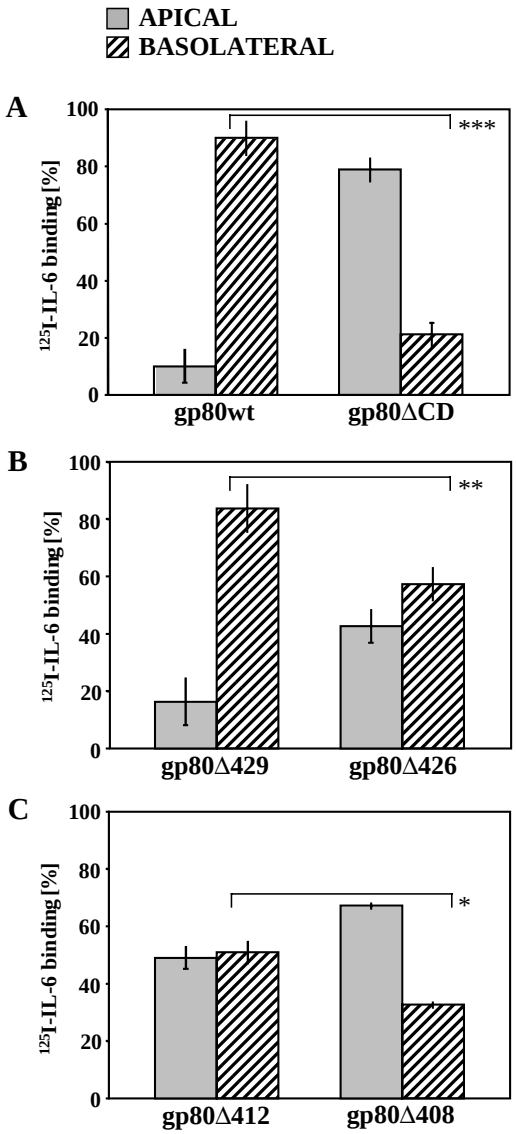


**Fig. 4.** Binding of $^{125}$I-IL-6 to polarized MDCK cells stably expressing gp80wt or gp80 deletion mutants. Polarized parental and stably transfected MDCK cells grown on Transwell filters were incubated with $^{125}$I-IL-6 added either to the basolateral or to the apical side. The cells were lysed and the $^{125}$I-IL-6/IL-6R complexes were immunoprecipitated with a polyclonal anti-sgp80 antibody. The ligand/receptor/antibody complexes were precipitated using protein A-sepharose and detected in the γ-counter. Specific binding to the transfected cell lines was determined by subtracting unspecific binding to the parental cells (usually less than 10%) from the total binding. The total specific binding of $^{125}$I-IL-6 to both plasma membranes was set as 100% for every cell line. Values are expressed as means ± s.d. ($n$=3). *$P$<0.05; **$P$<0.01; ***$P$<0.001. In addition to the significant differences indicated, gp80Δ426 and gp80Δ408 were significantly different ($P$<0.01).

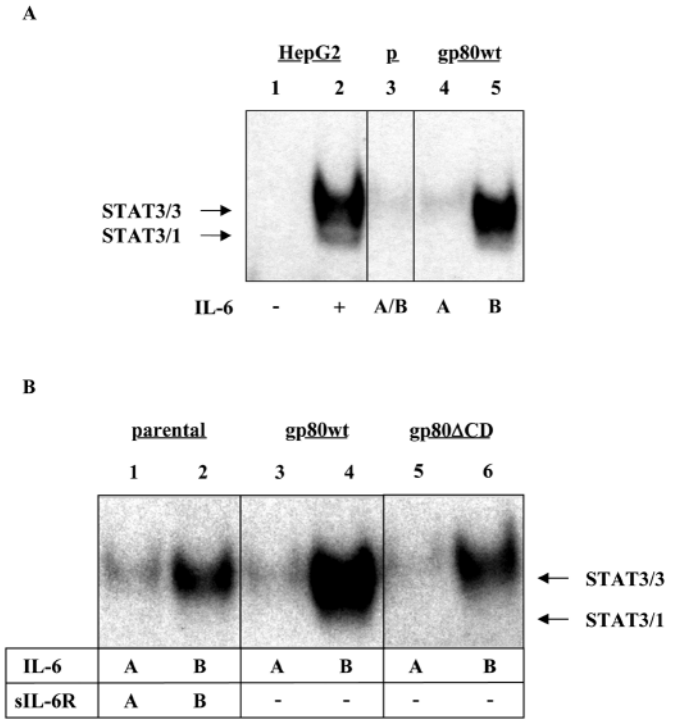


**Fig. 5.** STAT activation in stably transfected polarized MDCK cells. Non-polarized HepG2 cells were grown on plastic dishes subconfluently and either mock-treated or stimulated with IL-6 (100 U/ml) for 15 minutes (A, lanes 1+2). Parental (p) MDCK cells grown for 5 days on Transwell filters were either stimulated with IL-6 added to both sides for 15 minutes (A, lane 3) or with IL-6 plus recombinant soluble IL-6R (0.5 μg/ml) added either apically (A) or basolaterally (B) (B, lanes 1+2). MDCKgp80wt (B, lanes3+4) or MDCKgp80ΔCD (B, lanes 5+6) were stimulated with IL-6 added from the apical or basolateral side. Nuclear extracts were prepared and an EMSA was performed. The identity of the shifted bands (detected by fluorography) in nuclear extracts from HepG2 cells as STAT3 homodimers and STAT3/1 heterodimers has previously been established (Gerhartz et al., 1996).

## DISCUSSION

This study demonstrates that both transmembranous subunits of the interleukin-6 receptor complex, the IL-6 receptor gp80 and the signal transducer gp130, are sorted to the basolateral plasma membrane of polarized MDCK cells. As a typical inflammatory cytokine, IL-6 is released by activated monocytes and endothelial cells into the blood stream where appreciable amounts of this cytokine can easily be detected (Heinrich et al., 1990). Since the basolateral domain of epithelial cells is facing the internal milieu, hormone and cytokine receptors as well as nutrient receptors are usually located at this domain. In MDCK cells a basolateral expression was demonstrated for e.g. the epidermal growth factor receptor (Maratos-Flier et al., 1987), the thyrotropin-stimulating hormone receptor (Beau et al., 1997), the follicle-stimulating hormone receptor (Beau et al., 1997) and a truncated form of the nerve growth factor receptor (Monlauzeur et al., 1995). Also the α2-adrenergic receptors (Wozniak and Limbird, 1996), the cation-dependent (Bresciani et al., 1997) and the cation-independent (Prydz et al., 1990) mannose-6-phosphate receptor, the polymeric immunoglobuline receptor (Casanova et al., 1991), the low-density lipoprotein receptor (LDL-R) (Matter et al., 1992), the transferrin receptor (Fuller and Simons, 1986), and both the human and the rat asialoglycoprotein receptor (Graeve et al., 1990; Wessels et al., 1989), were targeted to the basolateral plasma membrane in

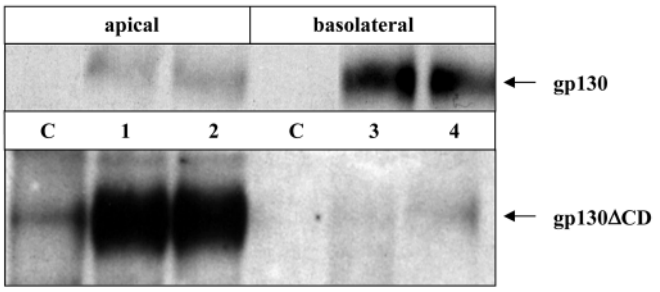


**Fig. 6.** Expression of gp130wt and gp130ΔCD in stably transfected polarized MDCK cells. Parental (C) and stably transfected MDCK cells (gp130wt: upper panel, lanes 1-4 or gp130ΔCD: lower panel, lanes 1-4) were grown on Transwell filters for 5 days. Sulfo-NHS-biotin was employed to selectively label the apical (both panels, lanes C, 1-2) or basolateral (both panels, lanes C, 3-4) surfaces. The cells were extracted with lysis buffer and the supernatants were immunoprecipitated with a monoclonal gp130-specific antibody. Immunoprecipitates were analyzed by SDS-PAGE and western blot. The biotinylated proteins were detected using horseradish peroxidase-conjugated streptavidin and visualized by ECL.

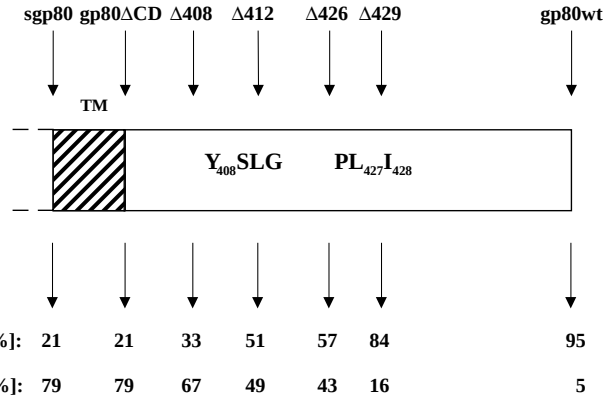


**Fig. 7.** Summary of the expression results of different gp80 constructs in stably transfected MDCK cells. The mean percentages of basolaterally (B) and apically (A) localized gp80wt and gp80 deletion mutants in stably transfected polarized MDCK cells are depicted. The results for sgp80 were obtained by an ELISA specific for sgp80, the other data were obtained by binding studies with $^{125}$I-IL-6 (details are described in Materials and Methods). TM, transmembrane region.

these cells. In hepatocytes, the basolateral domain directly faces the blood stream and a basolateral localization of the IL-6 receptor in these cells would be expected. We tried to directly localize the IL-6R in cryosections from human liver. However, due to the low receptor expression the results obtained were not conclusive. Although no costaining with an apical marker was observed, a clear basolateral staining could not be demonstrated (data not shown). Nevertheless, a number of observations make a basolateral localization of the IL-6R likely. Thus, within minutes after injection into the tail vein of rats, recombinant $^{125}$I-IL-6 accumulates in the liver and large amounts are metabolized there (Castell et al., 1988; Sonne et al., 1990). In addition, we could not detect transcytosis of $^{125}$I-IL-6 internalized from the basolateral side to the bile canaliculi in perfused rat liver (R. Fuchs and L. Graeve, unpublished observation). Furthermore, injection of IL-6 into rats also results within minutes in an activation of STAT factors in the hepatocytes of the liver (Wegenka et al., 1993). These observations suggest that the IL-6R is likely to be found only at the basolateral plasma membrane of hepatocytes, making them susceptible to IL-6 circulating in the blood stream and allowing the induction of the acute-phase response in the course of an inflammatory reaction.

Most basolateral sorting signals identified so far were found to be predominantly localized in the cytoplasmic parts of the respective transmembranous proteins. In line with this, the cytoplasmic domain of both gp80 and gp130 was found to be necessary for basolateral expression in MDCK cells. Truncated receptors were instead delivered predominantly to the apical domain. Thus, for the first time we can attribute a function to the cytoplasmic domain of gp80. These 82 amino acids were found to be dispensable for two other important processes, IL-6 signalling and endocytosis (Dittrich et al., 1994; Taga et al., 1989). The cytoplasmic domain is dispensable for signalling since gp80 is only important for binding of IL-6, whereas the activation of cytoplasmically associated Jak tyrosine kinases requires association of the IL-6/IL-6R complex with a gp130 dimer (Taga et al., 1989). Even the transmembrane domain of gp80 is not required for signalling. Therefore, soluble forms of the IL-6R act agonistically on cells only expressing the signal transducer gp130 (Mackiewicz et al., 1992). Furthermore the cytoplasmic domain of gp80 is dispensable for endocytosis of IL-6 since we found that internalization is also mediated by gp130 and depends on a cytoplasmic dileucine internalization motif of the signal transducer (Dittrich et al., 1994, 1996). Thus, the only function described so far for the gp80 cytoplasmic part is its role in basolateral sorting.

Basolateral targeting signals can diverge considerably. Nevertheless, there are certain amino acids that are often involved. Many of the signals contain a tyrosine and are located in proximity to the putative transmembrane regions. Tyrosine-based basolateral sorting signals often resemble and actually overlap with clathrin-coated pit localization signals, necessary for endocytosis of the respective receptor. However, the amino acid requirements around the crucial tyrosine residue are often different for basolateral targeting and endocytosis, suggesting that the two sorting mechanisms are distinct. Tyrosine residues were found to be essential components of basolateral targeting sequences in the LDL (Matter et al., 1993; Yokode et al., 1992), the polymeric immunoglobulin (Casanova et al., 1991), the truncated nerve growth factor (Monlauzeur et al., 1995) and the human asialoglycoprotein (Geffen et al., 1993) receptor. For the basolateral targeting of some transmembranous proteins the dependence on a dileucine motif was reported, e.g. for the macrophage immunoglobulin Fc receptor (Hunziker and Fumey, 1994), for a member of the immunoglobulin superfamily, the cell adhesion molecule lutheran glycoprotein (El Nemer et al., 1999) and for the major histocompatibility complex class II-associated invariant chain (Simonsen et al., 1998). We found that within the cytoplasmic domain of gp80 two sequence motifs are responsible for efficient basolateral targeting, a tyrosine-based one and a dileucine-type one (Fig. 7). Deletion of the more C-terminally located $PL_{427}I_{428}$ resulted in a partial loss of polarity. The respective mutant was distributed equally between the apical and the basolateral domain. Only after additional truncation of the membrane-proximal $Y_{408}$SLG motif was a preferential apical localization

observed. Yet, even this mutant was still less apically polarized than gp80ΔCD (although the difference was not significant), suggesting that the 21 amino acids between the transmembrane domain and $Y_{408}$ might also contribute to a small extent to basolateral targeting of gp80. However, no tyrosine or dileucine is found within this 21-aa sequence.

The preferential apical localization of gp80ΔCD indicates the existence of a recessive apical targeting signal that only becomes functional when the cytoplasmic part of gp80 is deleted. We can rule out any involvement of the transmembrane domain of gp80 in this putative apical targeting signal since a soluble form of the IL-6R shows the same polarity of expression in MDCK cells as gp80ΔCD (Fig. 7). Therefore, a recessive apical sorting signal must reside within the extracellular domain. A recent study suggested that N-linked carbohydrates might constitute an apical targeting signal in proteins devoid of basolateral sorting information (Gut et al., 1998). However, this obviously is not the case for a number of soluble secretory proteins (Rodriguez-Boulan and Gonzalez, 1999). In addition, some nonglycosylated membrane-bound proteins are still targeted apically (Rodriguez-Boulan and Gonzalez, 1999). The gp80 contains five putative N-glycosylation sites and is clearly glycosylated (Gerhartz et al., 1994). Whether these carbohydrate chains constitute a recessive apical targeting signal has to be elucidated in the future.

Soluble IL-6 receptors are formed in vivo and can be detected in body fluids. However, it is currently unknown whether polarized cells such as hepatocytes or non-polarized blood cells such as monocytes are the major source of soluble IL-6 receptors in vivo. Two mechanisms have been proposed for sIL-6R formation: alternative splicing and shedding of the membrane-bound receptor (Müller-Newen et al., 1996). Since hepatocytes usually use the indirect route for delivery of apical proteins, i.e. first transport newly synthesized proteins to the basolateral side from which they are then transcytosed to the apical compartment, either mechanism of sIL-6R formation would result in basolateral secretion. As mentioned above, transcytosis of IL-6 was not observed, making it unlikely that the sIL-6R is transcytosed into the bile.

The basolateral sorting motifs of gp80 are not sufficient to mediate endocytosis of gp80 in the absence of gp130. When gp80 was overexpressed in COS-7 cells to an extent that endocytosis via endogenous gp130 is negligible, both gp80wt and gp80ΔCD were only marginally internalized (10% and 7%, respectively, of total surface receptors within 30 minutes; Dittrich et al., 1994 and data not shown). This suggests that basolateral sorting of gp80 in MDCK cells is independent of recognition by the clathrin-coated pit machinery. Very recently, an adapter subunit μ1B specific for basolateral sorting was identified (Fölsch et al., 1999; Ohno et al., 1999). This adapter subunit is closely related to μ1A of the general trans-Golgi adapter AP-1 and is only expressed in polarized epithelial cells (e.g. MDCK, HT-29 and Caco-2) (Ohno et al., 1999). Lack of μ1B, as in LLC-PK1 epithelial cells, leads to the missorting of basolateral proteins to the apical side (Fölsch et al., 1999). μ1B was found to interact with several tyrosine-based sorting signals in the yeast two-hybrid system. Whether it also interacts with dileucine-type sorting motifs such as μ1A and μ2 (Rodionov and Bakke, 1998) or β1 (Rapoport et al., 1998) is currently unknown. The role of μ1B in basolateral sorting of the IL-6R complex has yet to be addressed.

The basolateral sorting motif within the intracellular part of the signal transducer gp130 has yet to be identified. The gp130 cytoplasmic domain contains six tyrosine residues and the dileucine motif essential for internalization (Dittrich et al., 1996). However, only one tyrosine residue is embedded in a classical tyrosine-based sorting motif and this tyrosine is phosphorylated after IL-6 stimulation, upon which it binds the tyrosine-phosphatase SHP-2 (Stahl et al., 1995). This makes it rather unlikely that this tyrosine residue is part of a basolateral sorting motif.

Mistargeting of receptors can be part of the pathophysiology of diseases. Gene defects associated with polycystic kidney disease somehow arrest differentiation of the basolateral sorting pathway and result in epidermal growth factor receptor mislocalization (Sweeney and Avner, 1998). Also the ubiquitous transport enzyme Na,K-ATPase is apically mislocated in polycystic kidney disease (Ogborn et al., 1995). In this context the reversed polarity may be a factor in cyst formation. Another example of a receptor mistargeting in connection with a disease is the apical targeting of the LDL-R in familial hypercholesterolemia. In this case a point mutation directly affects the basolateral sorting motif of the LDL-R (Koivisto et al., 1995). Cytokine receptor mislocalization has to our knowledge not been described yet.

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