Predominant expression of the long isoform of GP130-like (GPL) receptor is required for interleukin-31 signaling

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ABSTRACT. Gp130-like receptor (GPL) is a newly identified cytokine receptor. A recent study reported the involvement of GPL, together with OSMR, in the formation of the receptor complex for IL-31, a novel immune cytokine with a skin tropism. In the present work, we analyzed the signaling properties of IL-31 in glioblastoma and melanoma tumor cells. We demonstrate that in response to IL-31, its receptor complex recruits Jak1, Jak2, STAT1, -3, -5 signaling pathways, as well as the PI3 kinase / AKT cascade. SHP-2 and Shc adapter molecules are also recruited and contribute to an increased activation of the MAP kinase pathway in response to IL-31. Different responses were observed depending on the expression of short or long GPL receptor isoform within the studied cell lines. We show that the short form of GPL receptor exerts a profound inhibitory effect on the signaling of IL-31 and behaves as a dominant negative receptor.

Keywords: IL-31, cytokine, GPL, OSM, receptor, isoform

INTRODUCTION

A novel cytokine receptor displaying similarities to the gp130 signaling receptor, and called GPL for GP130-like receptor, or GLM-R, has recently been described [1, 2]. It also displays some degree of homology with LIFR, OSMR and the IL-12 receptor family members. Four different isoforms, diverging in their carboxy terminus ends, have been isolated. GPL contains a cytokine binding domain with four conserved cysteines, a WSDWS motif, and a region consisting of three fibronectin type III domain repeats. The intracellular part of the longer isoform contains a proline-rich region defining the box 1 motif implicated in the interaction with the Janus kinases. Specific GPL transcripts were observed in tissues involved in reproduction. Transcripts were also found in blood cells and in bone marrow, revealing the expression of GPL in all cells of the myelo-monocytic lineage, from hematopoietic stem cells to activated dendritic cells. In monocytes and dendritic cells, the expression of GPL was strongly up-regulated by IFN-γ, indicating the possible involvement of GPL in Th1 type immune responses.

The combination of different cytokine receptor components leads to high affinity receptor complexes. The shared use of receptor subunits has been used to classify and analyze the biological functions of cytokines. Based on this notion, IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 were gathered to define the cytokine family sharing the “c” chain in the formation of their functional receptors [3, 4]. Similarly, additional groupings of cytokines were defined based on their common use of bc (IL-3, IL-5, GM-CSF) [5], IL-4R (IL-4, IL-13) [6], IL-7R (IL-7, TSLP) [7] or IL-12Rβ1 (IL-12, IL-23) [8-10].

The latest family of cytokines is known as the IL-6 family, and encompasses in addition to IL-6, viral IL-6, IL-11, LIF, OSM, CNTF, CT-1, CLC, neuropoietin (NP) and IL-27. All share the common gp130 signaling subunit in their multimeric receptors [11, 12]. Depending on the activating ligand, gp130 can either homo-dimerize in the presence of IL-6 or IL-11 [13, 14], or associate with a related type I cytokine receptor, LIF receptor (LIFR) [15], when recruited by LIF, OSM, CNTF, CT-1, CLC or NP [16-20]. OSM and IL-27 can also activate specific receptors, implicating the OSM receptor (OSMR) or the IL-27 receptor, respectively [12, 21, 22].

Using chimeric receptors, we have previously shown that the internal part of GPL could hetero-dimerize with either LIFR or gp130 to transduce a functional signal [1].
recent study, based on the use of similar chimeric receptors, demonstrated that the internal portion of GPL could also cooperate with the equivalent part of OSMR to deliver signaling information and recruit the STAT signaling pathway [23]. More recently, the identification of IL-31 as a ligand for the GPL / OSMR receptor complex was reported. IL-31 is mainly produced by T cells and has an important immune function in skin diseases [24].

In the present work, we analyze the signaling properties of IL-31 in glioblastoma and melanoma tumor cells. We demonstrate that in response to IL-31, its receptor complex recruits Jak1, Jak2, STAT1, STAT3, STAT5 signaling pathways, as well as the Pi3-kinase / AKT cascade. Shp-2 and Shc adapter molecules are also recruited and contribute to an increased activation of the MAP kinase pathway in response to IL-31. We studied the different biological responses mediated by the short and long GPL receptor isoforms and show that a short form of GPL receptor exerts a profound inhibitory effect on the signaling of IL-31, and behaves as a dominant negative receptor.

MATERIALS AND METHODS

Cells and reagents
Cos-7, HEK 293, glioblastoma, melanoma, myelomonocytic leukemia, multiple myeloma, hepatoma, pulmonary, prostatic and mammary cell lines were cultured in RPMI medium 1640 supplemented with 10% fetal calf serum. BA/F3 gp130/OSMR and BA/F3 gp130/OSM/GPL factor-dependent cell lines were grown in the same medium-supplemented with OSM and IL-31, respectively. Human recombinant OSM, IL-6, IL-5, soluble IL-6R, anti-OSM and anti-STAT5B antibodies were purchased from R&D Systems (Oxon, UK). Gp130-Fc purified fusion protein was kindly provided by Dr. K.J. Kallen (Kiel, Germany). The anti-OSMR antibody (XR-M70) and the OSMR-Fc construct were kindly provided by Dr. B. Mosley (Immunex, Seattle, WA, USA). Other Fc fused soluble receptors, human recombinant LIF, anti-gp130 (AN-HH1) and IgG1 isotype control antibodies were produced in the laboratory. The polyclonal anti-GPL antibody was raised by immunizing rabbits with a 15-mer peptide chosen in the AB loop of the receptor as previously described [1]. Antibodies raised against phospho-STAT1, STAT1, phospho-STAT3, phospho-MAPK, MAPK, phospho-AKT, AKT and phosphotyrosine (4G10) were bought from Upstate Biotechnology (Lake Placid, NY, USA). Anti-STAT3, SHP-2, Jak1, Jak2, PI3-kinase antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-V5 antibody coupled to peroxidase and the anti-Shc antibody were purchased from Invitrogen (Carlsbad, CA, USA) and Transduction Laboratories (Lexington, KY, USA), respectively. Goat anti-mouse and anti-rabbit peroxidase labeled immunoglobulins were from Clinisciences (Montrouge, France).

Cell transfection and proliferation assays
BA/F3 cell lines stably expressing with gp130 and OSMR were transfected with cDNA encoding the long form GPL by electroporation (960 microfarads and 230V). Cells were selected by growing in the presence of 150 µg/mL zeocin (Invitrogen, Carlsbad, CA, USA). For proliferation assays, transfected BA/F3 cells were seeded in 96-well plates at 5 x 10³ cells per well in RPMI medium 1640 containing 5% fetal calf serum. Serial dilutions of cytokines were performed in triplicates. After a 72-h incubation, 0.5 µCi of ³H thymidine was added to each well for the last four hours of the culture and the incorporated radioactivity determined (Packard Topcount luminometer, Meriden, CT, USA). Cos-7 cells were transfected using the DEAE-dextran method. Seventy-two hours after transfection, cells were deprived of serum and stimulated with the indicated cytokines. A375 melanoma and U87MG glioblastoma cell lines were transfected with the cDNA encoding the GPL long form and the GPL short form using the Exgen transfection reagent (Euromedex, Souffleuwsheim, France), respectively. Seventy-two hours after transfection, cells were deprived of serum and were stimulated with cytokines.

Cloning, expression and purification of IL-31
Total RNAs were isolated from human peripheral blood lymphocytes after a six-hour activation with 10 nM PMA, 0.5% PHA using TRIZol reagent (Invitrogen, Carlsbad, CA, USA). Full-length IL-31 cDNA was PCR amplified and cloned into the pcDNA3.1/D/V5-His-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the available sequence (accession number AY499343) [24]. The resulting cDNA was fused to two sequences encoding the V5 and histidine hexamer tags to allow protein detection and purification. For IL-31 production, the HEK 293 cell line was stably transfected with IL-31-V5-His pcDNA3.1D/TOPO plasmid using the Exgen transfection reagent (Euromedex, Souffleuwsheim, France). IL-31 was affinity purified using Ni⁺ beads. Purified fractions were next submitted to SDS/PAGE silver staining and Western blotting analysis.

RT-PCR analyses
cDNAs were synthesized from 2 µg of total RNA by random hexamer primers using MMLV reverse transcriptase (Promega, Madison, WI, USA). Reverse transcription products were subsequently amplified by 35 cycles of PCR using the following primers: TGGAGTC- CCTGAAAAACGAAAG (sense), TTAGCTTCTCCCT-TGGTGTCG (anti-sense) for long form GPL, GAC-CAAGGTGGAGAAACATTTG (sense), CTTCAAGGGTTAGCTTATCTTG (anti-sense) for short form GPL and by 25 cycles of PCR using the following primers: CCTGAGCTCCTGAAAAACGAG (sense), ACATTTGTTGACCTTCTTT (anti-sense) for OSMR, ACCACATCTGCATGC (sense), TCCACCAC-CCTGTGTGCTGTA (anti-sense) for GAPDH. Amplified products were analysed by 2% agarose gel electrophoresis.

Site-directed mutagenesis
The pcDNA3.1 vector containing the cDNA encoding long form GPL was subjected to site-directed mutagenesis using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer’s instructions. The three intracellular tyrosines in positions 652, 683 and 721 were alanine substituted. Intro-
duced mutations were verified by DNA automatic sequencing. The receptor-Fc expression constructs contained cDNAs encoding the extracellular portions of receptors cleaved, by site-directed mutagenesis, before their transmembrane domains and fused to the amino acid sequence of the Fc portion of human IgG1. Fc fusion proteins were expressed in Cos-7 cells, and the culture supernatants were used for the precipitation experiments.

**Tyrosine phosphorylation, immunoprecipitation and Western blot analyses**

After a thirty-six-hour serum starvation, cells were stimulated for 10 min in the presence of the indicated cytokines. Cells were lysed in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromophenol blue), sonicated and then submitted to SDS-PAGE and transferred onto an Immobilon membrane. The membranes were subsequently incubated overnight with the primary antibody before being incubated with the appropriate peroxidase-labelled secondary antibody for 60 min. The reaction was visualized by chemiluminescence according to the manufacturer’s instructions (Amerham Biosciences, Les Ulis, France). Membranes were stripped in 0.1 M glycine, pH 2.8 for two hours and neutralized in 1 M Tris-HCl, pH 7.6 before reblotting. For immunoprecipitation experiments, cells were lysed after activation in 10 mM Tris HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, protease inhibitors and 1% Brij 96 detergent. After lysis and centrifugation to remove cell debris, the supernatants were immunoprecipitated overnight. Complexes were then isolated using beads coupled to protein A and treated as described above. For co-precipitation experiments, the soluble receptors fused to an immunoglobulin Fc portion were incubated in the presence of indicated cytokines for 16 h at 4°C, before being immunoprecipitated with protein A beads. The samples were then treated as described above.

**Reporter gene assay**

Transient transfection of GO-G-UVM cells was carried out in 24-well culture plates using the Exgen transfection reagent (Euromedex, Souffelweyersheim, France). The cells were transfected with 300 ng of reporter gene as described previously [25]. Forty-eight hours after transfection, the cells were starved before being incubated with 50 ng/mL of the different cytokines for an additional sixteen hours. Transfected cells were washed twice with cold PBS, and 100 μL of lysis buffer (0.1 M KH2PO4, pH 7.8, 0.1% Triton X-100) were added to the wells. The extracts were then used directly to measure the luciferase activity using a Packard Topcount luminometer (Meriden, CT, USA).

**RESULTS**

**IL-31 expression and receptor subunit recruitment**

IL-31 cDNA was PCR amplified from human peripheral blood cells treated with a phorbol ester and PHA for six hours, and the resulting cDNA was inserted into the pcDNA3.1D/V5-His-topo vector. The cytokine was expressed as a tagged protein and purified from culture supernatants of HEK 293 transfected cells. SDS-PAGE gels and Western blot analyses showed that the polypeptide had a MW of 29 kDa, corresponding, after subtracting the tag molecular weight, to a mature protein of 24 kDa (figure 1A). We controlled the functionality of the expressed protein by using derivatives of the IL-3-dependent Ba/F3 cell lines rendered responsive to cytokines of the IL-6 family by transfection with the appropriate receptor chains (figure 1B). Ba/F3 cells expressing gp130 and OSMR proliferated in response to OSM, but not to IL-31. In contrast, the cells co-expressing GPL together with gp130 and OSMR were able to proliferate in response to both OSM and IL-31. The results obtained were in agreement with recently published data characterizing IL-31 and its receptor complex [24]. Furthermore, the addition of an anti-OSMR antibody to the cultures abrogated the proliferation of the Ba/F3 cell line in response to IL-31, whereas no inhibition was observed upon addition of an anti-gp130 antibody in control experiments. These results confirm the involvement of the OSMR as a signaling subunit in the IL-31 receptor (figure 1C).

To identify subunit(s) directly contacting IL-31, we expressed external portions of gp130, OSMR and GPL as Fc...
fusion proteins in Cos-7 cells. After adding IL-31 or OSM, receptors were immunoprecipitated with protein A beads. The purified fractions were assayed by Western blotting using an anti-OSM antibody or an anti-V5 tag antibody to respectively detect OSM and IL-31 receptor associations (figure 2). As previously reported, OSM directly bound gp130 but not OSMR [16, 26]. In contrast, GPL behaved as a binding subunit for IL-31, whereas OSMR alone failed to recognize the cytokine. Increased IL-31 binding was noted when OSMR was combined with GPL. CD40-Fc fusion protein was used as an irrelevant receptor.

**IL-31 activates the Jak / STAT pathway**

We next analyzed the implication of Jak kinases in receptor activation using the GO-G-UVM glioblastoma cell line that expresses GPL receptor as well as OSMR and gp130 on its surface [1]. A ten-min treatment of cells with IL-31 or OSM led to an activation of both Jak1 and Jak2 signaling molecules (figure 3). We failed to detect any activation of Jak3 in the GO-G-UVM cell line, which is in agreement with the specific recruitment of Jak3 in cells of immune origin. In contrast, a strong auto-active Tyk2 signal was observed (data not shown), even after a long starvation culture period, precluding the study of Tyk2 activation by IL-31 in the GO-G-UVM cell background.

Once activated, Jakks are known to stimulate the phosphorylation of downstream signaling molecules. Therefore, STAT signaling pathways were analyzed in response to IL-31 in the GO-G-UVM cell line. As shown in figure 4A-C, IL-31 induced activation of STAT1, STAT3 and STAT5B. Similar responses were observed for OSM as previously reported [27, 28]. The signaling capacity of the OSMR subunit was further underlined by showing that addition of an anti-OSMR monoclonal antibody to the cultures led to a complete neutralization of the STAT3 response (figure 4D). The reciprocal experiment using an anti-GPL antibody could not be performed since neutralizing mAb for this receptor subunit are not yet available.

The transcriptional activity of STAT3 in response to IL-31 was then studied. For this, GO-G-UVM cells were transfected with a reporter construct containing three STAT3 consensus binding sites located upstream of a thymidine kinase minimal promoter [25]. Forty-eight hours post-transfection, the cells were serum-starved and stimulated for an additional 16 h with saturating concentrations of IL-31 or OSM. A 3–4-fold increase in luciferase expression was induced by IL-31, as well as by OSM (figure 4E). Altogether, these data indicate that IL-31 recruits STAT3 for both signaling and transcriptional activation of target genes.

**Involvement of SHP-2 and Pi3-kinase / AKT pathway in IL-31 signaling**

The tyrosine phosphatase SHP-2 is known to be recruited into the tyrosine kinase signaling pathway via its binding to phosphotyrosine motifs expressed by signaling receptor chains [29, 30]. IL-31 was found to induce the tyrosine phosphorylation of SHP-2, as previously reported for OSM and LIF [27] (figure 5A). It is also known that SHP-2 can regulate the gp130 signaling cascade by recruiting the Pi3-kinase / AKT pathway [31]. We therefore investigated the possibility that the OSMR / GPL receptor complex could similarly recruit Pi3-kinase (figure 5B). Whereas OSMR / gp130 induced a robust phosphorylation of Pi3-kinase upon stimulation with OSM, a slight but significant increase in the tyrosine phosphorylation level of the kinase was observed after activation of the OSMR / GPL complex by IL-31. Figure 5C shows that, in contrast, IL-31 led to a
marked increase in the tyrosine phosphorylation level of AKT, similar to that observed when treating the cells with OSM.

**IL-31 induces activation of the MAP kinase pathway**

In addition to the Pi3-kinase / AKT activation pathway, SHP-2 is also known to associate with the GRB2 / Sos adapters and regulate the MAP kinase pathway [32-34]. The ERK1 / ERK2 MAP kinases have been shown to play important roles in mediating the mitogenic effects of the IL-6 family members [35]. ERK1/2 activation was determined by measuring their tyrosine phosphorylation levels. Stimulation of the glioblastoma cell lines with IL-31 quickly increased the MAP kinase phosphorylation after a 10-min contact with the cytokine (figure 6A), before returning to basal levels after a 30-40-min period (data not shown). These results demonstrate activation of the MAP-kinase signaling pathway in functional responses to IL-31.

**Figure 4**

IL-31 induces tyrosine phosphorylation of STAT1, STAT3 and STAT5 transducing proteins. Following exposure to 50 ng/mL OSM, IL-31 or IL-5 (negative control) for 10 min, the GO-G-UVM cell line was lysed, and the (A.) STAT1, (B.) STAT3, (C.) STAT5B tyrosine phosphorylation levels were determined. D. Before stimulation with IL-31, cells were incubated for two hours in the presence of neutralizing anti-gp130 (AN-HH1) or anti-OSMR (XR-M70) mAbs, or with an isotype control mAb (final antibody concentration, 15 ng/mL). E. GO-G-UVM was transiently transfected with a reporter plasmid gene. Forty-eight hours later, cells were stimulated with 50 ng/mL IL-5 (negative control), OSM or IL-31 for 16 hours. Cellular extracts were prepared and used to measure luciferase activity. IP, immunoprecipitation; WB, Western blot; Ptyr, phosphotyrosine.

**Figure 5**

IL-31 induces the recruitment of SHP-2 adapter protein and AKT pathway. GO-G-UVM was incubated with 50 ng/mL IL-5 (negative control), OSM or IL-31 for 10 min. A. After lysis, proteins were immunoprecipitated with an anti-SHP-2 polyclonal antibody, and the tyrosine phosphorylation level was determined. B. After cell lysis, proteins were immunoprecipitated with an anti-Pi3-K polyclonal antibody, and the tyrosine phosphorylation levels were determined. C. Cell lysates were directly subjected to immunoblot analysis using an antibody directed to the activated form of AKT. IP, immunoprecipitation; WB, Western blot; Ptyr, phosphotyrosine.

Involvement of the MAP kinase pathway was previously studied in detail for cytokines of the IL-6 family by comparing the response mediated by the LIF receptor complex (gp130/LIFR) versus the OSM receptor complex (gp130/OSMR) [36-38]. It turned out that the OSM receptor complex was a much more potent activator of the Shc adapter and of the ERK1/2 cascade [29, 30]. Since OSMR
GO-G-UVM lysates and ERK2 proteins could not be separated by electrophoresis using an antibody directed to the activated form of MAPK (ERK-1 lysed and the lysates were directly subjected to immunoblot analysis).

IL-5 (negative control), OSM or IL-31 for 10 min.

The short form of GPL receptor is a dominant negative receptor.

We further assessed the functionality of the IL-31 receptor mostly contributes to the cross-activation of OSMR, differing in their intracytoplasmic region. A long isoform GPL receptor containing 745 residues and a short form of 560 amino-acids, that only contains three intracellular residues were predominantly expressed in cell lines and tissues [1]. Therefore, we decided to analyze in detail the involvement of short and long GPL isoforms in IL-31 signaling. Cos-7 cells expressing endogenous gp130 and OSMR, but not GPL, were used as recipient cells to reconstitute functional IL-31 receptor. After transfection with the long or short isoforms of GPL, cells were incubated in the presence of IL-31, or OSM used as a positive control. STAT1 and STAT3 activation levels were analyzed by Western blot. As shown in figure 7, stimulation of Cos-7 expressing endogenous gp130 and OSMR with OSM, elicited STAT1 and STAT3 activation, which was not the case with IL-31 treatment. Introduction of the full length GPL receptor isoform into Cos-7 cells allowed the reconstitution of a functional receptor for IL-31. In contrast, expression of short form GPL did not lead to STAT1 or STAT3 recruitment. This indicates that, at least, the Jak binding box 1 deleted in the short form of receptor, and possibly the GPL tyrosine residues, are required to recruit STAT signaling pathway in response to IL-31.

To further analyze the information transduced by the GPL receptor, each of the three tyrosine residues contained in the intracellular portion of long isoform GPL were mutated. Tyrosine residues were substituted by alanines, individually, by pairs or all three together. Mutated receptors were then expressed in Cos-7 cells and activated using IL-31, or OSM as control cytokine (figure 8). Each individual mutation led to only marginal decreases in STAT1 and STAT3 protein activation. When the three tyrosine residues of GPL were simultaneously mutated, a slight decrease in the STAT1 and STAT3 recruitment was observed.

Collectively, these data suggest that the GPL receptor mostly contributes to the cross-activation of OSMR, but is only poorly able to recruit the STAT pathway on its own.

We further assessed the functionality of the IL-31 receptor complex by studying the distribution of the short and long isoforms of GPL in different human cell lines (figure 9 and table 1). For this purpose, we set up RT-PCRs specifically amplifying the two receptor forms, although we have so far

<p>| Table 1 | Expression of short and long isoforms of GPL receptor in tumor cell lines. OSMR, short and long form of GPL were studied by RT-PCR. Serial dilutions of cDNA were amplified to have a semi-quantitative analysis of transcript expression level. -, no detectable expression; +, low expression level; ++, predominant expression level. Induction of STAT3 tyrosine phosphorylation in response to IL-31 was determined by Western blot analysis. |
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Figure 6
IL-31 induces the recruitment of MAPK and tyrosine phosphorylation of SHC, GO-G-UVM cell line was stimulated with 50 ng/mL IL-5 (negative control), OSM or IL-31 for 10 min. A. Cells were lysed and the lysates were directly subjected to immunoblot analysis using an antibody directed to the activated form of MAPK (ERK-1 and ERK2 proteins could not be separated by electrophoresis of GO-G-UVM lysates) B. Cell lysates were immunoprecipitated with an anti-phosphotyrosine monoclonal antibody before blotting with an anti-SHC antibody. IP, immunoprecipitation; WB, Western blot; P: Tyr, phosphotyrosine.
not been able to develop a real time quantitative amplification. We therefore submitted RT products to serial dilutions before amplification, to determine a comparative estimate of short and long GPL isoforms (table 1). Results revealed a differential distribution of GPL isoforms depending on the cell line tested. Some of the cell lines, such as A375 melanoma, displayed co-expression of both isoforms, whereas the U87MG glioblastoma cell line only expressed the long form of the receptor. In contrast, the SK-BR-3 mammary tumor cell line only expressed the short form GPL receptor.

As illustrated in figure 10A, IL-31 was a strong inducer of STAT3 tyrosine phosphorylation in the U87MG glioblastoma cell line, whereas no recruitment of the signaling protein could be demonstrated in the A375 melanoma cell line. Increasing contact time and cytokine concentrations did not modify the response observed in the A375 cells (figure 10B and C). Despite a co-expression of OSMR and GPL receptor in a number of cell lines tested, only those expressing predominantly the long isofrom of GPL together with OSMR were able to elicit STAT3 tyrosine phosphorylation in response to IL-31 (table 1).

At this point, we hypothesized that the short GPL isoform could behave as a dominant negative receptor by inhibiting the signaling machinery mediated by the long form receptor. To test this hypothesis, we co-expressed different ratios of the short and long isoforms in Cos-7 cells and activated the cells with IL-31 (figure 11). Less than a two-fold ratio in favor of the short form of the protein was sufficient to strongly antagonize the long form signaling receptor.

We next over-expressed the 745 amino acid long form of the receptor in A375 cells and analyzed the tyrosine phosphorylation level of STAT3 in response to OSM and IL-31 (figure 12B). Whereas the response to OSM or to a combination of IL-6 and soluble IL-6 receptor remained unchanged, a clear induction of STAT3 phosphorylation was detected in the transfected cells. Reverse experiments were carried out using the U87MG glioblastoma cell line, which spontaneously expressed the long GPL isoform, but not the short subtype, as shown above in figure 9. In this mirror experiment, the 560 residue short form of receptor was transfected in U87MG cell line and the STAT3 recruitment analyzed (figure 12D). A decrease in the ability of IL-31 to recruit the STAT3 signaling pathway in cells transfected with the short form receptor was clearly shown. Collectively these results demonstrate that the 560 amino acid short isoform of GPL receptor can counteract the signaling machinery mediated by the long form of receptor. It also indicates that the relative ratio of both receptor forms is an important point to consider for analyzing the functional response to IL-31.

**DISCUSSION**

We and others previously reported the identification of an orphan cytokine receptor named GPL, co-localizing with gp130 and displaying a 28% identity rate with it [1, 2]. A recent paper has shown its implication, together with OSM, in the formation of the functional receptor complex for a newly identified cytokine, IL-31 [24]. IL-31 is a cytokine preferentially produced by T helper type 2 cells. Mice modified to over-express IL-31 developed severe pruritis, alopecia and skin lesions, indicating important immune functions of IL-31 in skin diseases.

In the present study, we have analyzed the cell signaling mechanisms triggered by IL-31 and compared the pathways recruited with those activated by OSM. The present work underscores some overlaps between the signaling cascades recruited by both IL-31 and OSM. The function of the short form GPL receptor indicates a fine tuning of the IL-31 response.

Usually, a cytokine binds preferentially to one of the subunits of a multimeric receptor [39]. For the cytokines of the IL-6 family, it has been established that LIFR preferentially binds LIF [16], whereas gp130 behaves as a converter subunit to allow receptor dimerization and the subsequent signaling events. With respect to OSM, this cytokine directly associates with gp130, whereas in this case LIFR and OSMR play the function of converter receptors for type I and type II OSM receptor complexes, respectively [16, 26]. In the present study, we observed that IL-31 directly bound to GPL. No direct association of IL-31 with OSMR could be demonstrated, suggesting that this latest subunit mainly plays a role of converter in the IL-31 receptor complex. The observation that a neutralizing anti-OSMR antibody could entirely block the functional response of IL-31 further pointed out the importance of the OSMR in cell signaling. Similarly, the substitution of all tyrosine residues in the GPL intracellular region only slightly affected the STAT signaling capacities of the IL-31 receptor complex, indicating that the OSMR plays major role in transmembrane signal transduction in response to IL-31. This was recently confirmed by expressing synthetic receptor subunits made of the external portions of the IL-5 receptor fused to the intracellular portions of GPL.
and OSMR [23]. These receptor chimeras can be artificially recruited by IL-5 and reveal some information about the signaling potential of GPL / OSMR heterochimeric complex. Dreuw et al. have shown that STAT3 and STAT5 bind to tyrosine residues 721 and 652 of GPL, respectively. They also report a marginal recruitment of STAT1 by the chimeric receptor, and that GPL by itself was insufficient to recruit ERK1/2, SHP-2 and Shc pathways.

To better understand the physiological responses of the GPL / OSMR heterodimer to IL-31, native forms of receptors were analyzed in the present study. A majority of the cell signaling experiments was carried out using different glioblastoma cell lines that express the 745 residue GPL long form [1]. We could show that IL-31-activated receptor was able to recruit Jak1 and Jak2 kinases. A spontaneous, high activation level of Tyk2 kinase in the cells that we used hid the detection of a potential signal in response to IL-31. The possibility of Tyk2 contributing to IL-31 signaling remains open.

Following cell stimulation by IL-31 and JAK activation, information is relayed to the nucleus by a number of signaling molecules including the STATs [40]. Similarly to the data reported very recently by Dillon et al. [24], we observed a consistent activation of STAT1, STAT3 and STAT5B upon stimulation by IL-31 in a number of cell lines tested as measured by analyses of STAT phosphorylation levels and transcriptional activity of STAT3. We

Figure 8
Role of GPL intracellular tyrosine on STAT1 and STAT3 activation. Cos-7 cells were transfected with the cDNAs encoding the wild type long form GPL (WT), mutated long forms of receptor (Y1A, Y2A, Y3A, Y12A, Y13A, Y23A, Y123A) or with the empty vector. Numbering of mutants was as follow Y1A: Y652A; Y2A: Y683A; Y3A: Y721A. A. Transfection efficiency was determined by immunoblot analysis using a polyclonal anti-GPL antibody. B. Transfected cells were stimulated with 50 ng/mL OSM, IL-31, or IL-5 used as control cytokine. The lysates were subjected to immunoblot analysis using antibodies directed to the tyrosine phosphorylated forms of STAT1 or STAT3. Membranes were then reblotted with antibodies recognising all forms of STAT1 or STAT3 for a loading control. WB, Western blot.

Figure 9
Short and long form expression of GPL receptor. Short form and long form GPL receptors were detected in tumor cell lines by RT-PCR using specific primers as indicated in materials and methods. GAPDH was used as control.
notice that compared to STAT1, a preferential recruitment of STAT3 and STAT5 was reported by Dreuw et al. [23], likely reflecting differences associated with the cell lines used and the expression levels of intracellular signaling molecules.

The tyrosine phosphatase SHP-2 is a widely expressed protein, which becomes tyrosine phosphorylated after cell stimulation with a number of cytokines [31]. The gp130 family cytokines can recruit SHP-2 phosphatase through phosphorylated gp130Y759 to deliver proliferative signals [29, 30]. SHP2 acts as a positive effector by associating with the GRB2 adapter, leading to the activation of the MAP kinase pathway [32]. Contact between PI3-kinase and SHP-2 has also been reported after activation by some IL-6 family cytokines, or by thrombopoietin [33, 41]. In this work, we show that the IL-31 receptor complex is able to recruit SHP-2, which in turn leads to activation of the MAPK pathway [29, 30]. In the present work, we show that similarly to OSM, but not to LIF, IL-31 can also elicit a strong recruitment of the Shc adapter, which in turn contributes to the activation of the MAP kinase pathway. Shared, as well as distinct, biological properties have been ascribed to OSM, LIF and IL-6. The fact that IL-31, like OSM, has a much stronger potential in activating Shc, further underlines the functional similarity between the two cytokines. Subtractive strategies or micro-array analyses should contribute to delineate similarities and differences between the functional responses to OSM and IL-31.

In the experiments that were carried out initially, we observed functional differences in the A375 melanoma cell line responses to OSM and IL-31. The inhibition of A375 melanoma cell line growth by OSM was used historically to ascribe to OSM, LIF and IL-6. The fact that IL-31, like OSM, has a much stronger potential in activating Shc, further underlines the functional similarity between the two cytokines. Subtractive strategies or micro-array analyses should contribute to delineate similarities and differences between the functional responses to OSM and IL-31.

Pi3-kinase / AKT pathway as well as to activation of the ERK1/2 cascade.

The MAPK pathway is particularly favoured in the OSM response compared to that observed for LIF or IL-6 [36-38]. This is due to the fact that the OSMR subunit can strongly associate with the Shc adapter through its Y861 residue and further reinforce the recruitment of the whole MAP kinase cascade [29, 30]. In the present work, we show that similarly to OSM, but not to LIF, IL-31 can also elicit a strong recruitment of the Shc adapter, which in turn contributes to the activation of the MAP kinase pathway. Shared, as well as distinct, biological properties have been ascribed to OSM, LIF and IL-6. The fact that IL-31, like OSM, has a much stronger potential in activating Shc, further underlines the functional similarity between the two cytokines. Subtractive strategies or micro-array analyses should contribute to delineate similarities and differences between the functional responses to OSM and IL-31.

In the experiments that were carried out initially, we observed functional differences in the A375 melanoma cell line responses to OSM and IL-31. The inhibition of A375 melanoma cell line growth by OSM was used historically to identify and clone the gene encoding OSM [42, 43]. Despite co-expression of GPL and OSMR subunits in this cell line, we failed to detect any functional response to IL-31, suggesting that additional mechanisms must regulate the IL-31 response. Since we previously identified at least four different GPL isoforms, with a predominant expression of a long (745 residues) and a short (560 residues) subtype, we tested the possibility that the short form receptor could behave as a dominant negative receptor. Short form dominant negative receptors have already been reported for several members of the cytokine receptor family. This has been particularly well studied for a subset of hormone receptors comprising this family of proteins, i.e. prolactin, growth hormone and leptin. The short form of the leptin receptor is marginally able to display a dominant negative role [44]. In contrast, short forms of growth hormone and prolactin receptors profoundly silenced their respective long form receptors [45-48]. In the present work, we were able to show that the GPL receptor long / short form ratio strongly conditioned the IL-31 functional response. Indeed, even a slight over-expression of the short form of receptor (two-fold) can strongly antagonize the IL-31 signaling cascades. Consequently, a number of the

Figure 10

Lack of IL-31 responsiveness in the A375 melanoma cell line. Following exposure to the indicated concentrations of OSM, IL-31, or IL-5 used as control cytokine for 10 min, U87MG glioblastoma (A.) and A375 melanoma (B.) cell lines were lysed. The lysates were then directly subjected to immunoblot analysis using an antibody directed to the activated form of STAT3 (P-STAT3), before reblotting using an antibody recognising all STAT3 forms. WB, Western blot.

Figure 11

Short form GPL receptor neutralizes the IL-31 response. Cos-7 cells were co-transfected with indicated concentrations of cDNAs encoding the long form GPL and/or the short form GPL. The cells were stimulated with 50 ng/mL IL-31, or IL-5 used as control cytokine. The lysates were subjected to immunoblot analysis using antibodies specifically recognising the activated forms of STAT3 (P-STAT3), before reblotting using an antibody recognising all STAT3 forms. WB, Western blot.
cell lines tested were insensitive to IL-31 treatment. Several hypotheses may explain the neutralizing activity of the short subtype of receptors. For the prolactin and growth hormone receptors, different interference levels have been reported. Slight variations in the affinity of the ligands for their cognate receptors have been observed [45]. These also included the formation of inactive hetero-dimers that failed to activate the JAK kinases [45-48]. An accumulation of non-functional receptors on the cell surface due to the non-internalization of short form receptors has also been reported [46, 47]. Similarly, truncated or short form cytokine receptors are usually more abundantly expressed at the cell surface [48]. This was also noticed for OSMR the expression of which is improved after a box1/2 region deletion [49]. For the thrombopoietin receptor, it was recently demonstrated that a truncated subtype of receptor was able to associate intracellularly with the signaling form of the receptor and counteract its expression by increasing its lysosomal degradation [50]. For the thrombopoietin receptor, it was recently demonstrated that a truncated subtype of receptor was able to associate intracellularly with the signaling form of the receptor and counteract its expression by increasing its lysosomal degradation [50].

Primary observations reported the highest expression levels of GPL in tissues involved in reproduction, in the myelo-monocytic lineage, in spleen, thymus, lung, skin and trachea [1, 2, 24]. Most of these tissues also express the OSMR suggesting they are potentially IL-31 responsive. In the light of the present work, a careful re-evaluation of GPL isoform tissue distribution should lead to a better understanding of IL-31-mediated biological functions.

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REFERENCES


