Reconstitution of two isoforms of the human interleukin-11 receptor and comparison of their functional properties

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Abstract Long-term stable Ba/F3 transfectants (B13Rα1 and B13Rα2) expressing two isoforms of the human IL-11Rα receptor (α1 full length or α2 lacking the cytoplasmic domain) in combination with human gp130 were established. IL-11Rα1 and IL-11Rα2 were each expressed and detected as three bands upon Western blot analysis, with apparent molecular masses in agreement with those of the polypeptide backbone (47 and 44 kDa, respectively) with no, one or two N-linked sugars. B13Rα1 and B13Rα2 bound IL-11–thioredoxin with similar efficiencies and proliferated with superimposable dose–response curves to IL-11, demonstrating that the intracellular domain of IL-11Rα has no significant contribution on ligand binding and signaling. Analysis of a set of anti-human gp130 mAbs confirmed the similar responsiveness of B13Rα1 and B13Rα2 transfectants.

Key words: Human interleukin-11 receptor; Isoform; Transfection (Ba/F3 cells); Proliferation; Monoclonal antibody

1. Introduction

Interleukin-11 (IL-11) is a cytokine produced by a variety of cells from mesenchymal origin such as fibroblasts, keratinocytes, chondrocytes, synoviocytes, bone marrow stromal cells and osteoblasts. It also displays multiple biological activities, many of which are similar to those exerted by interleukin-6 (IL-6) and, to a lesser extent, by other pleiotropic cytokines such as LIF (leukemia inhibitory factor), OSM (oncostatin M) and CNTF (ciliary neurotrophic factor) [1–4]. For example, IL-11 and IL-6 act in synergy with IL-3 to support the growth of pluripotent bone marrow progenitors and to stimulate megakaryogenesis [5]. They both regulate the synthesis and secretion of acute-phase proteins by hepatocytes [6], promote the differentiation of B lymphocytes into immunoglobulin-secreting cells [7], function as adipogenesis inhibitory factors [8] and participate in the development of osteoclastic cells [9]. The molecular identification of the components of the functional high-affinity receptors for these pleiotropic cytokines has provided some explanation for this redundancy of action. All cytokines of this family require the common gp130 transmembrane glycoprotein to transduce their biological activities. The gp190 glycoprotein (low-affinity LIF receptor) is also required for signal transduction through LIF, OSM (type I), CNTF and CT-1 receptors [10,11].

In the case of the CNTF, IL-6 and IL-11 receptors, additional receptor components (α chains) have been described which confer cytokine specificity. The CNTF-Rα chain is a 72 kDa glycoprotein (gp72) which is attached to the plasma membrane through a glycophosphatidyl inositol (GPI) anchor [12]. The IL-6Rα chain is a 80 kDa membrane anchored glycoprotein with a 82 amino acids intracellular domain [13]. More recently IL-11Rα chains have been described in mouse [14] and human [15,16]. We have shown the existence of two human IL-11Rα cDNAs resulting from alternative exon splicing [15,17]. The first cDNA encodes a transmembrane glycoprotein containing, like the IL-6Rα chain, a cytoplasmic domain (32 amino acids). The second cDNA isoform encodes a protein lacking, like the CNTF-Rα chain, a cytoplasmic domain. In this study, we describe the establishment and characterization of stable Ba/F3 cell lines transfected with the two human IL-11Rα isoforms and use these cells to evaluate the contribution of the intracytoplasmic domain of IL-11Rα in ligand binding and signal transduction.

2. Experimental procedures

2.1. Expression constructs

Human IL-11 cDNA was subcloned into pTrxFus (Invitrogen BV, Leek, The Netherlands) to construct a thioredoxin–IL-11 fusion cDNA. Expression was performed in *E. coli* and the fusion protein (IL-11–Trx) was prepared as a crude extract from *E. coli* osmotic shock treatment [18]. *Eco*R1 fragment of BQM15.C cDNA clone containing the human IL-11Rα1 coding sequence was inserted into the pLXSPuro (Transgen) eukaryotic expression vector to obtain pLXSP/IL-11Rα1 and selected via the puromycin-resistance gene. Human IL-11Rα2 complete coding sequence was obtained by PCR amplification with the oligonucleotides, 5′-GCGGAATTCATGAGGGACCAATGGCAGT and 5′-GGACTTTCTAGATGCTGGCAC, using BQP32.21 cDNA clone as template and ligated to the *Eco*R1–*Xba*1 fragment of BQM15.C cDNA. The sequence of the PCR fragment was confirmed by double-strand DNA sequencing. This insert was subcloned in pLXSPuro to obtain pLXSP/IL-11Rα2.

2.2. DNA transfection experiments

Ba/F3/gp130/IL-11Rα1 (B13Rα1) and Ba/F3/gp130/IL-11Rα2 (B13Rα2) were established by electroporating (at 900 μF and 300 V) mouse pro-B-cell Ba/F3 with 20 μg of pLXSP/IL-11Rα1 or pLXSP/IL-11Rα2 and 20 μg of pRCNeo/. Transfected cells were selected in a culture medium containing: RPMI-1640, 10% fetal calf serum, 1% glutamine, 0.8 mg/ml G418 (Sigma-Aldrich, St. Quentin

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Abbreviations: IL-11Rα, interleukin-11 receptor; Trx, thioredoxin; s-IL-6Rα, soluble IL-6 receptor; mAb, monoclonal antibody

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Fallavier, France), 5 μg/ml puromycin (Sigma) and 5 ng/ml (0.25 nM) human IL-11. Ba/F3/IL-11Rα1 (BRα1), Ba/F3/IL-11Rα2 (Brα2) and Ba/F3/gp130 (B13) were obtained using the same procedure except that cells were only transfected with one construct and selected in medium containing 10% of WEHI-3-conditioned medium as a source of IL-3 instead of IL-11 and either 5 μg/ml puromycin (BRα1, BRα2) or 0.8 mg/ml G418 (B13).

2.3. Cytokines and monoclonal antibodies

Human *E. coli* recombinant IL-11 was from Peprotech Inc. (London, UK). IL-6, sIL-6Rα and the anti-IL-6Rα monoclonal antibody (mAb) PM1 were kindly given by Dr. Yasukawa (Tosoh Corp., Tokyo, Japan). The anti-human gp130 mAbs B-P8, B-S12, B-R3, B-P4 and the anti-human IL-6Rα mAb B-N12 were from Diaclone (Besançon, France). Their initial characterisation and analysis of functional properties have already been described [19–21].

2.4. Proliferation assays

Transfected Ba/F3 cells were washed twice in culture medium lacking growth factor and seeded in 96-well microtiter plates at a density of 15×10^3 cells in 50 μl per well. In a first type of assay, cells were incubated (in a final volume of 100 μl) with serial dilutions either of IL-11 (5 nM), of IL-6 (20 nM) in the presence or not of sIL-6Rα (fixed concentration of 8 nM), or of a mixture of B-P8 (6.66 nM) plus B-S12 (20 nM). In a second type of assay, cells were stimulated with IL-11 (0.25 nM) or IL-6 (0.6 nM) plus sIL-6Rα (8 nM), and serial dilutions of B-N12 (20 nM), PM-1 (20 nM), B-R3 (20 nM) or B-P4 (20 nM). Assays were performed in triplicate. After incubation at 37°C for 36 h, cellular proliferation was assessed by a (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) based assay (Sigma).

2.5. Flow cytometric analysis

Cells (1×10^5) were preincubated for 3 h in culture medium lacking growth factor and washed 3 times. For gp130 staining, the B-R3 mAb was added at a final concentration of 67 nM and incubated on ice for 1 h. After washing (3 times), cells were stained with a phycoerythrin-labeled goat anti-mouse antiserum (1:200; Immunotech, Marseille, France). For IL-11Rα staining, cells were incubated with IL-11–Trx (30 nM), washed 3 times, further incubated for 1 h with a mouse anti-Trx antibody (1:5000) (Invitrogen), washed again (3 times), and finally stained with a phycoerythrin-linked goat anti-mouse antiserum (Immunotech). In some experiments, a 42-fold molar excess of human IL-11 (1.25 μM) was added together with IL-11–Trx.

2.6. Immunoblot analysis

Cells were washed 3 times in 10 ml of phosphate-buffered saline (PBS) and pelleted by centrifugation. Pellets were frozen (−80°C) and thawed once, homogenized in a Dounce homogenizer, and centrifuged for 20 min at 2500 rpm at 4°C. The supernatant was centrifuged for 30 min at 14000 rpm at 4°C. Pellets were resuspended in 10 mM Tris buffer (pH 7.4), containing leupeptin at 0.1 mg/ml (Sigma). The protein concentration was determined by the Bicinchoninic Acid (BCA) method (Pierce). Membrane proteins (50 μg/lane) were resolved by 10% SDS-PAGE and transferred onto a PVDF membrane (Amersham, Arlington Heights, USA). After blocking, the membrane was incubated with a rabbit polyclonal antibody (1:100) directed against a 20-amino-acid peptide of murine IL-11R (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed using chemiluminescence (ECL kit, Amersham).

3. Results

3.1. Generation of Ba/F3 transfectants with long-term proliferation in response to IL-11

The murine Ba/F3 cell line has been widely used to study the reconstitution of cytokine receptors [22]. It is absolutely dependent on IL-3 for growth and does not proliferate in response to IL-6 or LIF. Human IL-11Rα1 or IL-11Rα2 cDNAs were transfected in Ba/F3 cells either alone or in combination with full-length human gp130 cDNA. Transfected cells were subsequently cultured in selection media con-

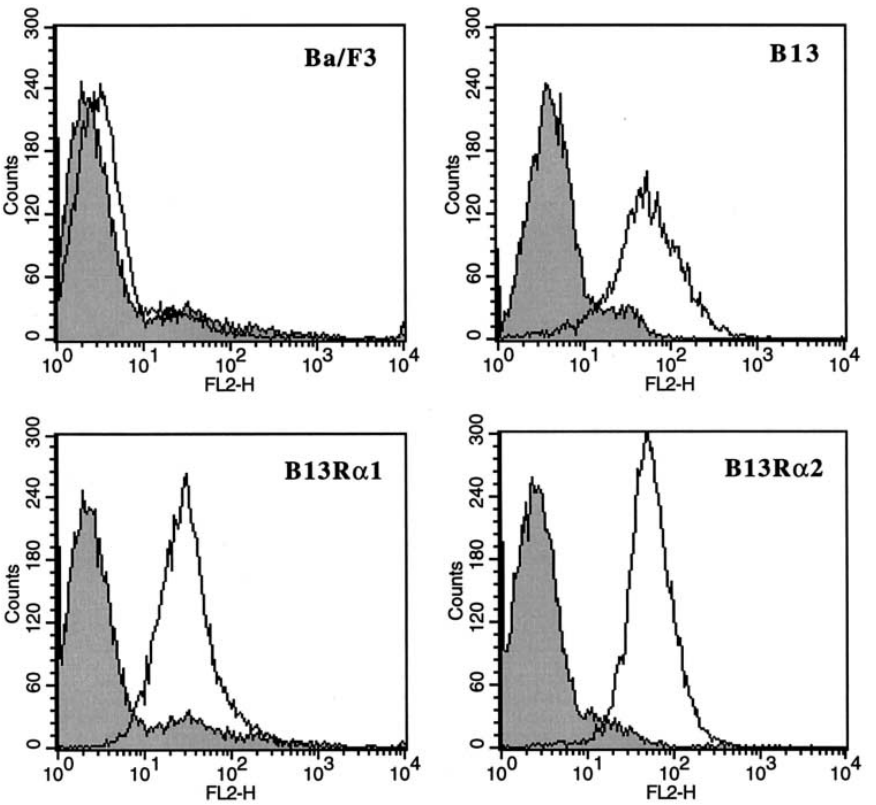

Fig. 1. Flow cytometric analysis of human gp130: B-R3 mAb and phycoerythrin-conjugated second antibody (open area). Control: second antibody alone (closed area).

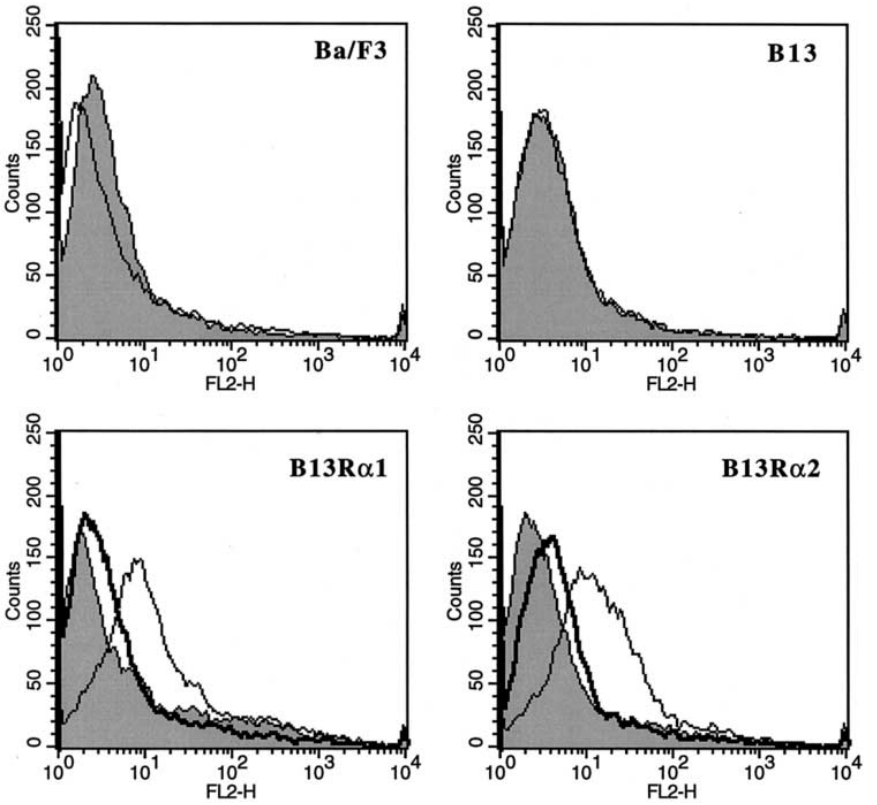

Fig. 2. Flow cytometric analysis of human IL-11Rα: IL-11–Trx/anti-Trx antibody and phycoerythrin-conjugated second antibody in the absence (thin lane, open area) or presence (thick lane, open area) of a 42-fold molar excess of human IL-11. Control: anti-Trx plus second antibody (closed area)

taining appropriate antibiotic(s) and IL-3 conditioned medium or 5 ng/ml of human recombinant IL-11. In the presence of IL-3, cellular expansion was observed in all transfected cultures. In IL-11 containing medium, cellular expansion was only observed from Ba/F3 cells co-transfected with IL-11Rα1 and gp130 (B13Rα1) or IL-11Rα2 and gp130 (B13Rα2). Furthermore, when cells selected in IL-3 conditioned medium were shifted to IL-11 containing medium, only cells co-transfected with gp130 and IL-11Rα1 or IL-11Rα2 continued to grow, whereas cells transfected without insert, with IL-11Rα1 alone (BRα1), IL-11Rα2 alone (BRα2) or gp130 alone (B13) rapidly died. These results were consistently obtained in several independent transfection experiments. Long-term proliferation of B13Rα1 and B13Rα2 were observed. These cell lines are now routinely grown (for more than 6 months) in IL-11 containing medium and retain their IL-11 growth-dependency (see Fig. 4A).

3.2. *Analysis of the expression of the transfected receptor chains*

Ba/F3 cells expressing different combinations of the transfected cDNAs were established as stable cell lines. The level of expression at the cell surface of the transfection products was evaluated by flow cytometry and Western blots. Fig. 1 shows the flow cytometric histograms obtained with an anti-gp130 mAb (B-R3). In contrast to the parental BaF3 cell line which was negative, B13, B13Rα1 and B13Rα2 expressed gp130 with similar intensities. Due to the lack of a suitable anti-IL-11Rα for flow cytometric analysis, the expression of IL-11Rα was measured with the combination of an IL-11–thioredoxin fusion protein (IL-11–Trx) and an anti-Trx antibody. As shown in Fig. 2, both B13Rα1 and B13Rα2 transfectants were similarly labeled with IL-11–Trx, whereas Ba/F3 and B13 cells were negative. The specificity of IL-11–Trx binding was assessed by the observation that a 42-fold molar excess of unlabeled recombinant IL-11 almost completely inhibited this binding.

Western blots were performed on membrane preparations from these different cell lines, using a polyclonal antibody raised against a N-terminal peptide of murine IL-11Rα (Fig. 3). This antibody was negative on parental Ba/F3 cell lines and B13 cells and very clearly identified specific bands on IL-11Rα/gp130 transfected cells. Three bands with molecular masses of 47, 50 and 54 kDa were detected on cells transfected with the IL-11Rα1 isoform, whereas on IL-11Rα2 transfectants, these three bands had lower masses of 44, 47 and 51 kDa, respectively.

3.3. *Proliferative responses*

We next examined the proliferative response of the various transfectants to different cytokines. All cell lines proliferated in response to IL-3-conditioned medium with comparable dose responses (data not shown). Only IL-11Rα/gp130 double-transfected cells did proliferate in response to IL-11 (Fig. 4A), showing that both IL-11Rα and gp130 are required and sufficient for IL-11 signaling in Ba/F3 cells. The dose-response curves relative to the effects of IL-11 on the B13Rα1 and B13Rα2 proliferative responses were almost superimposable,

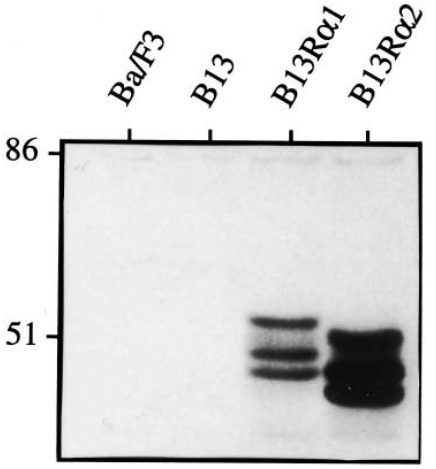

Fig. 3. Western blot analysis of human IL-11Rα. Molecular weight markers (left) are in kDa.

thereby indicating that the two isoforms have comparable efficiencies in complementing gp130 for signaling. The concentration of IL-11 inducing half maximal proliferative responses (IC_{50}) were 97 and 118 pM, respectively, values compatible with a high-affinity interaction.

The responses of these two double transfectants to the combination of IL-6 plus sIL-6Rα were also comparable (Fig. 4B), a result further indicating that the two cell lines express equivalent amounts of functional human gp130. The IC_{50}s for IL-6 in the presence of a fixed concentration of sIL-6Rα (8 nM) were 66 and 75 pM, respectively, values in agreement with its high-affinity equilibrium binding constant [23]. We also tested two anti-human gp130 mAbs (B-P8 and B-S12) which have been described to display synergistic and agonistic functions [19]. In agreement with these findings, B-P8 plus B-S12 induced the proliferation of B13Rα1 and B13Rα2 transfectants (Fig. 4C) with efficiencies comparable to those induced by IL-11 or IL-6/sIL-6Rα. Again, the responses of the two transfectants were superimposable, with IC_{50}s of 0.38 and 0.41 nM.

IL-11 versus IL-6/sIL-6Rα proliferative responses of the two transfectants were further assessed by the use of additional monoclonal antibodies (Fig. 5). B-R3 is an anti-human gp130 mAb which interferes with the biological effects of all known cytokines using gp130 as transducing element, whereas B-P4 is an anti-gp130 mAb which has been proposed to interfere specifically with the IL-11 response [19–21]. PM1 and B-N12 are anti-human IL-6Rα mAbs. PM1 is a blocking antibody [24], whereas B-N12 is not (J. Wijdenes, unpublished result). For each mAb tested, the responses of B13Rα1 and B13Rα2 were always almost superimposable. The B-N12 antibody had no effect in either system (Fig. 5A,B). The PM1 mAb inhibited the IL-6/sIL-6Rα responses (IC_{50}s: 6.3 and 7.5 nM) (Fig. 5D) while only slightly diminishing the IL-11 driven proliferation (Fig. 5C). The proliferation induced by either IL-11 or IL-6/sIL-6Rα was totally abbrogated by mAb B-R3 (Fig. 5E,F). The IC_{50}s describing the inhibitory effects on IL-11 were 0.30 and 0.24 nM, and those on the IL-6 response were 0.44 and 0.41 nM. These values are in full agreement with the binding affinity of B-R3 on these cells as measured by Scatchard analysis (K_d = 0.43 and 0.49 nM on B13Rα1 and B13Rα2 cells respectively; data not shown). The B-P4 antibody abbrogated the IL-11 response in B13Rα1 and B13Rα2 cell lines with a similar efficacy as B-R3 (IC_{50}s = 0.24 and 0.18 nM) (Fig. 5G). Unexpectedly, the B-P4 antibody also abbrogated the IL-6/sIL-6Rα proliferative effect on the two cell lines (IC_{50}s = 0.43 and 0.47 nM) (Fig. 5H).

4. Discussion

This study shows that co-expression in the Ba/F3 cell line of the human IL-11Rα with human gp130 is necessary and sufficient for the acquisition of human IL-11 mediated proliferative response and for the establishment of Ba/F3 cell transfectants which can be grown on a long-term basis under the growth promoting effect of recombinant human IL-11. These transfectants are now grown in our laboratory for more than 6 months without any significant changes of their IL-11 growth response sensitivities. These results extend recent ob-

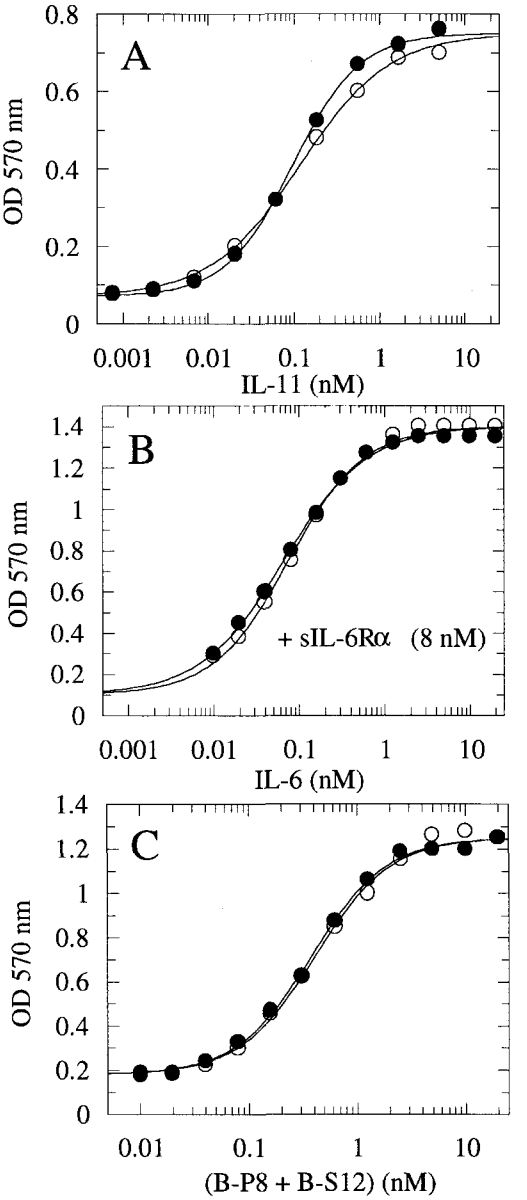

Fig. 4. Proliferative responses of B13Rα1 (●) and B13Rα2 (○) cells.

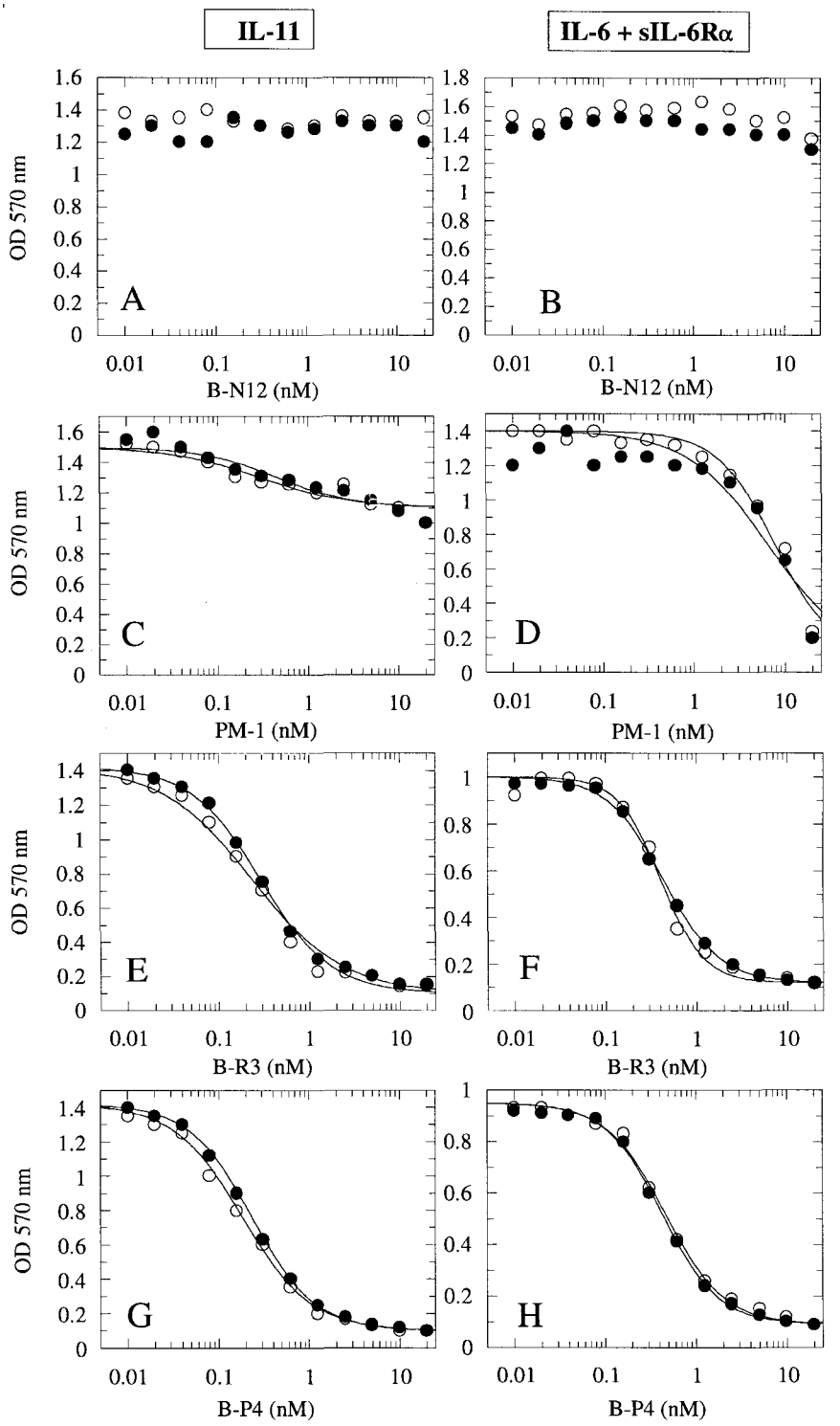

Fig. 5. Effects of anti-gp130 and anti-IL-6R mAbs on B13Rα1 (●) and B13Rα2 (○) proliferative responses induced either by IL-11 (5 nM) (left) or by IL-6 (0.6 nM) plus sIL-6Rα (8 nM) (right).

servations on similarly co-transfected Ba/F3 cells showing that IL-11 was able to maintain cell viability on a short (48 h) time period [14,16] and provide for the first time the description of stable human IL-11Rα/gp130 Ba/F3 cell transfectants proliferating in response to human IL-11.

We have previously shown that the human IL-11Rα chain exists in two different isoforms resulting from alternative splicing of a single gene. These two naturally occurring IL-11Rα isoforms differ only by the presence of the 32-amino-acids cytoplasmic domain [15]. We took advantage of this situation to analyze the functional implication of this cytoplasmic domain in the Ba/F3 system.

A polyclonal antibody raised against the N-terminal peptide (residues 25–44) of mouse IL-11Rα was shown to react with

human IL-11Rα in Western blot. This could be expected from the high degree of amino-acid sequence homology (95%) found in this region between mouse and human receptors [15]. This antibody enabled us to document for the first time the biochemical features of the human IL-11Rα chain. It reacted with three bands of molecular masses of 47, 50 and 54 kDa on the B13Rα1 cells and with three bands of molecular masses of 44, 47 and 51 kDa on the B13Rα2 cells. This pattern is consistent both with the predicted molecular masses of the mature proteins encoded by the human IL-11Rα1 and IL-11Rα2 cDNA isoforms, which are respectively 43 100 and 39 800 Da, and with the existence within the extracellular domain of the human IL-11Rα of two potential N-glycosylation sites [15]. Thus, for each receptor isoform, the lower band likely corresponds to the unglycosylated species, whereas the upper bands would correspond to glycosylated species containing one or two N-linked sugars, respectively. Indeed, the increments in molecular mass from the lower to the upper band are in the range of 3–4 kDa, values consistent with the average molecular mass of 2800 Da calculated for a complex N-linked carbohydrate [25]. When comparing the two receptor isoforms, the difference in molecular masses between two paired bands is about 3 kDa, a value compatible with that predicted from the difference in amino acids (32 amino acids; 3500 Da).

The two B13Rα1 and B13Rα2 cell lines expressed similar amounts of the human gp130 signal transducer as revealed by flow cytometry. They were also similarly able to bind the fusion protein IL-11–Trx. When the proliferative responses to human IL-11 were compared, the two transfectants were found to display nearly superimposable dose responses with high affinity (IC_{50} around 100 pM). Taken together, these data strongly suggest that, at least in the Ba/F3 transfection system, both IL-11Rα isoforms similarly conjugate with gp130 to bind IL-11 and transduce a proliferative signal. The intracellular domain of the human IL-11Rα chain seems therefore dispensable for binding to and signaling through the receptor complex in this cellular system.

In the case of the IL-6 receptor, homodimerisation of the gp130 subunit has been shown to be the key event in signal transduction and the IL-6Rα cytoplasmic domain is considered to play no role in the signaling process. This concept is based on the observations that soluble forms of the extracellular domain of IL-6Rα can combine with IL-6 and gp130 to trigger the signaling event, and that the intracellular domain of IL-6Rα lacks characteristic sequence motifs required for signaling [26]. However, analysis of the function of the IL-6Rα in hepatoma cells has indicated that, whereas its cytoplasmic domain is not absolutely required for signal transduction (C-reactive protein promoter activation), the last 40 amino acids of this domain contribute to maximal IL-6 response [27]. Similarly, soluble form of the mouse IL-11Rα chain were found to be less effective than the membrane form in hepatoma cells, embryonal carcinoma cells and T lymphocytes [28], suggesting an auxiliary function for the membrane and/or cytoplasmic domains. Our present results with the human IL-11 receptor system in Ba/F3 cells clearly demonstrate that the intracellular domain has no influence on the efficiency of signaling.

Various anti-gp130 mAbs have already been described and analysed for their ability to interfere with the biological activities mediated by cytokines using the gp130 transducing subunit. Among them are the B-P8 and B-S12 mAbs which have been shown to display agonistic and synergistic activities on XG1 myeloma and TF1 erythroleukemia cell lines and on stem cells [19]. Our data show that this agonistic activity is also observed in Ba/F3 cells transfected with gp130, confirming these previous findings. The B-R3 mAb was shown to inhibit the growth promoting activity of IL-6, CNTF, LIF and OSM on the XG4-CNTF myeloma cell line as well as the growth inducing effect of IL-11 on the XG-6–IL-11 myeloma cell line [19,21]. This mAb also inhibited the growth promoting effects of all these cytokines, also including CT-1, on the TF1 cell line and haptoglobin secretion induced by IL-6 and OSM on hepatoma HepG2 cells [20]. In agreement with these observations, B-R3 was found in this study to inhibit the proliferation of the two IL-11Rα transfectants induced either by IL-11 or the combination of IL-6 plus sIL-6Rα. The inhibitory capacities of B-R3 on the IL-11 or IL-6 signals were similar (IC_{50} around 0.3 nM and 0.4 nM, respectively) and in agreement with its binding constant (K_d around 0.45 nM) on both cell types. This observation supports the notion that B-R3 is not a competitive inhibitor of cytokine binding but rather interferes with a region of gp130 important for its dimerisation. B-P4 is another anti-human gp130 mAb which has been described to specifically inhibit (with respect to other cytokines) the IL-11 induced proliferative response of myeloma cell lines [19] and TF1 cells [20]. This antibody was indeed found in this study to be as efficient as B-R3 in inhibiting the IL-11 responses of B13Rα1 and B13Rα2 transfectants (IC_{50} around 0.2 nM). However, it also inhibited the proliferation of these transfectants in response to IL-6 plus sIL-6Rα with an efficiency again comparable to that of B-R3 (IC_{50} around 0.45 nM). As a control, the anti-IL6Rα mAb PM1 only affected the IL-6 response. These results therefore suggest that on transfected Ba/F3 cells, the epitope defined by B-P4 might be used in common by the IL-11/IL-11Rα and IL-6/IL-6Rα complexes to activate signal transduction. The discrepancy of our findings with previous observations [19–21] might be linked to the fact that we used a soluble form of IL-6Rα, whereas the other reports were dealing with membrane-anchored IL-6Rα. It could also reflect differences in sensitivity among various IL-6-induced biological responses to the blocking effect of B-P4. This discrepancy might also indicate that the conformations of the reconstituted receptors in Ba/F3 cells differ in some way with those of natural receptors constitutively expressed by cell lines. However it may be, elucidation of this apparent discrepancy deserves further investigations.

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