A fusion protein of interleukin-11 and soluble interleukin-11 receptor acts as a superagonist on cells expressing gp130

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Abstract Interleukin-11 is a hematopoietic cytokine that signals via the signal transducer gp130. Although gp130 is ubiquitously expressed, interleukine-11 responsiveness is restricted to cells that express the interleukine-11 receptor α-subunit. The interleukine-11 receptor α-subunit can be functionally replaced by its soluble form indicating that the transmembrane and cytoplasmic parts are not required for signal transduction. Here, we show that a recombinant fusion protein of a fragment of the human interleukine-11 receptor α-subunit ectodomain linked to human interleukine-11 acts as a superagonist on cells expressing gp130 but lacking the membrane-bound interleukine-11 receptor α-subunit. It induces acute phase protein synthesis in hepatoma cells and efficiently promotes proliferation of Ba/F3 cells stably, transfected with gp130. In these bioassays, the fusion protein of a fragment of the human interleukine-11 receptor α-subunit ectodomain linked to human interleukine-11 is 50 times more potent than the combination of interleukine-11 and the soluble interleukine-11 receptor α-subunit. Thus, our findings support the concept that covalent fusion of two soluble proteins required for receptor activation dramatically increases their bioactivity.

Key words: Fusion protein; Interleukin-11; Superagonist; Gp130

1. Introduction

Interleukin-11 (IL-11) is a hematopoietic cytokine that exerts various biological responses on different cells and tissues (for review see [1]). IL-11 acts synergistically with other growth factors to stimulate various stages of hematopoiesis both in vitro and in vivo [2–4]. In a phase II clinical trial, it has been shown that due to its thrombopoietic activity, recombinant human IL-11 ameliorates severe chemotherapy-induced thrombocytopenia therefore representing a valuable therapeutical protein [4]. More recently, anti-inflammatory acitivities of IL-11 have been described [5,6]. Female mice that do not respond to IL-11 due to targeted deletion of a critical receptor component are infertile as a consequence of impaired trophoblast implantation [7].

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Abbreviations: IL, interleukin; s, soluble; R, receptor; IL-11/R-FP, interleukin-11/receptor fusion protein; Jak, Janus kinase; STAT, signal transducer and activator of transcription; CBM, cytokine binding

IL-11 belongs to a subfamily of the 4-helix bundle cytokines including interleukin-6 (IL-6), leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1) that signal via the common signal transducing receptor protein gp130 (for review see [8]). IL-11 first binds to its specific α-receptor subunit (IL-11R) [9,10] and subsequently, the complex of IL-11 and IL-11R triggers the dimerization of gp130 [11] leading to activation of the Janus kinase (Jak)/signal transducer and activator of transcription (STAT) signal transduction pathway [12]. Both receptor components belong to the family of hematopoietic cytokine receptors which are characterized by the presence of at least one cytokine binding module (CBM) [13]. A CBM consists of two fibronectin type III-like domains. The N-terminal domain is characterized by four conserved cysteine residues, the C-terminal domain contains a WSXWS motif. The extracellular part of the IL-11R is proposed to consist of an Ig-like domain (D1) followed by a single CBM (D2 and D3) [9]. Whereas gp130 is expressed ubiquitously [14], IL-11R expression is more restricted [9,10]. Cells expressing gp130 but lacking membrane-bound IL-11R can be stimulated by the combination of IL-11 and soluble IL-11R (sIL-11R) indicating that the cytoplasmic and transmembrane parts of IL-11R are not required for signal transduction [15].

In this study, we show that a fusion protein of human IL-11 and a fragment of human sIL-11R acts as a superagonist to which gp130 expressing cells respond more sensitive than to the combination of IL-11 and sIL-11R. Superagonistic activity has also been described for a recombinant fusion protein consisting of a section of sIL-6R connected to IL-6 by a polypeptide linker [16]. The covalent linkage of two soluble receptor activating components exists naturally as well: the two separately encoded subunits of IL-12 (p35 and p40) undergo disulfide-stabilized heterodimerization to constitute its biologically active form [17]. Our results support the idea that the covalent linkage of two soluble components required for receptor activation potentiates their bioactivity.

2. Materials and methods

2.1. Enzymes, antibodies and cell culture

Enzymes were purchased from Boehringer Mannheim (Mannheim, Germany). The hIL-11 polyclonal antibody used in this study was obtained from R and D systems (Wiesbaden-Nordenstadt, Germany). α[^{32}P]dATP was purchased from Amersham International (Amersham, UK) and tran[^{35}S]-label metabolic labelling reagent from ICN (Meckenheim, Germany). Other
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combinant Trx/IL-11 and sIL-11R were prepared from bacteria or insect cells, respectively, as described previously [18]. Ba/F3-gp130 cells [19] were cultured in DMEM containing 5% (v/v) conditioned medium from X63Ag8-653 BPV-mIL-3 myeloma cells (as a source of IL-3) in the presence of 1 mg/ml G418 in a water-saturated atmosphere containing 5% CO_2. Both media were supplemented with 10% (v/v) fetal calf serum, streptomycin (100 μg/ml) and penicillin (60 μg/ml).

2.2. Plasmid construction

First, a section of the hIL-11R cDNA, encoding domains 2 and 3 (amino acids (aa) 109–318), was amplified by standard PCR methods. *Eco*RI/*Sfi*I restriction sites (underlined) were introduced using the following primer pair: IL-11R-sense 5′-ACTCTCGGATCCCTGGGCTACCCTCCAGC-3′ and IL-11R-antisense 5′-TGAGAGGGCCGGCTGGGCCCCCAGTGCTCGGAGTTCC-3′. Second, a fragment of hIL-11 cDNA encoding the proposed functional part of hIL-11 (aa 29–199) was amplified. *Asp*718/*Sac*II restriction sites were introduced using the following primer pair: IL-11-sense 5′-AGACACGGTACCCCTCGAGTTTCCCCAGAC-3′ and IL-11-antisense 5′-ACTGTGCCGCGGTCACAGCCGAGTCTTCAG-3′. The plasmid pPICZαA (Invitrogen) for secreted expression of recombinant proteins in the methylotrophic yeast *P. pastoris* was digested with the restriction endonucleases *Eco*RI and *Sfi*I. Then, the first amplicon was ligated (pPICZαA/IL-11R). The resulting plasmid was digested with *Asp*718 and *Sac*II and the second amplicon was ligated, which led to a plasmid encoding both proteins but lacking a peptide linker (pPICZαA/IL-11R/IL-11). The two following oligonucleotides, encoding the linker sequence, were annealed and subsequently ligated into the *Sfi*I/*Asp*718-digested pPICZαA/IL-11R/IL-11 plasmid: linker-sense 5′-CGGCCAATCAGGAGGTGGAGGAGGCTCCGGAGGAGGTTCTGGTGGAGGATCG-3′ and linker-antisense 5′-GTACCGATCCTCCACCAGAACCTCCTCCGGAGCCTCCTCCACCTCCTGATTGGCCGGCT-3′ (pPICZαAIL-11/R-FP). All plasmids were sequenced using an ABI Prism automated sequencer (Perkin Elmer).

2.3. Transfection of P. pastoris GS115 and expression of IL-11/R-FP

Competent *P. pastoris* GS115 were transfected with pPICZαA (control cells) or with pPICZαAIL-11/R-FP applying the LiCl method according to the manufacturer's instructions. Transfected cells were selected in YPD medium supplied with 0.1 mg/ml zeocin. Before induction of protein synthesis by supplementation of methanol, cells were grown in standard BMGY medium until $A_{600} > 1.5$ was reached. The expression conditions for IL-11/R-FP were optimized in a standard BMGY medium containing 2% v/v methanol and 1% w/v casamino acids. For protein expression, the GS115 culture was incubated at 29°C for 36 h.

2.4. Immunoblotting and enhanced chemiluminescence (ECL) detection

Proteins separated by SDS-PAGE were transferred to a PVDF membrane by a semi-dry electroblotting procedure. PVDF membranes were blocked in a solution of 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Nonidet-P40 containing 10% bovine serum albumin and probed with antibody, followed by incubation with horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were detected by chemiluminescence using the ECL-kit (Amersham, UK) following the manufacturer's instructions.

2.5. Induction of α1-antichymotrypsin synthesis in HepG2 cells

HepG2 cells were stimulated with Trx/IL-11 and sIL-11R or IL-11/R-FP as indicated in Fig. 3. After 18 h, cells were metabolically pulse-labelled with [^{35}S]methionine/cysteine for 4 h. Induction of α1-antichymotrypsin was measured in cell culture supernatants by immunoprecipitation. Immunocomplexes were separated on 10% SDS polyacrylamide gels, visualized by autoradiography and quantified using a Phosphorimager.

2.6. Proliferation assay

Ba/F3-gp130 cells (20 000 cells/well) were plated on 96 well plates and stimulated as indicated in Fig. 4. After 72 h of incubation, viable and metabolically active cells were quantified using a colorimetric assay based on the Cell Proliferation kit II (XTT) (Boehringer Mannheim, Germany).

2.7. Electrophoretic mobility shift assay (EMSA)

Ba/F3-gp130 cells were incubated at 37°C in the presence of 0.5 nM IL-11/R-FP or 5 nM of each Trx/IL-11 and sIL-11R for periods of

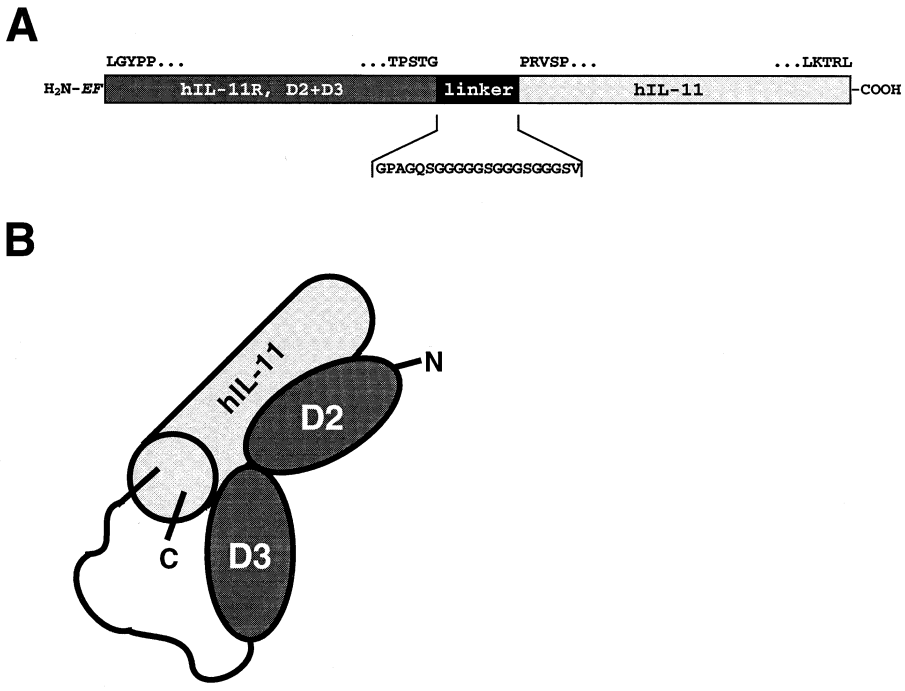

Fig. 1. Schematic representations of the fusion protein IL-11/R-FP. (A) Schematic representation of the IL-11/R-FP primary structure. The protein starts with domains 2 and 3 of human IL-11R (Leu-109–Gly-318), followed by a 21 amino acid linker and human IL-11 (Pro-29–Leu-199). Due to the cloning strategy, the fusion protein contains two additional N-terminal amino acids, shown in italic letters. (B) Schematic representation of the IL-11/R-FP tertiary structure. The linker (black line) should allow binding of IL-11 to domains 2 (D2) and 3 (D3) of IL-11R. N-

time as indicated in Fig. 5. Preparation of nuclear extracts and EMSAs were performed as described [20]. A double-stranded oligonucleotide derived from the c-fos promoter (m67SIE: 5′-GGGAGGGATTACGGGGAAATGCTG-3′) was used as ^{32}P-labelled probe [21]. The protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol. The electrophoresis was performed using 0.25-fold TBE buffer at 260 V.

3. Results

3.1. Expression of IL-11/R-FP in the methylotrophic yeast *P. pastoris*

Using a PCR-based methodology, we constructed an expression vector encoding a fusion protein of the human IL-11R CBM (domains 2 and 3) and human IL-11. In order to allow binding of IL-11 to the binding epitope of IL-11R, both proteins were connected by a flexible polypeptide linker (Fig. 1A). In Fig. 1B, the anticipated ternary structure of the fusion protein IL-11/R-FP is schematically represented in which the CBM of IL-11R accommodates IL-11. This arrangement of the two fusion partners is based on the analogy to the structure of the growth hormone/growth hormone receptor complex [22]. Similarly, fusion proteins of the two IL-12 subunits p35 and p40 [23] as well as IL-6 and sIL-6R [16] were constructed and shown to be biologically active. The methylotrophic yeast *P. pastoris* was transfected with the vector pPICZαAIL-11/R-FP allowing the expression and secretion of the fusion protein (for details see Section 2). Supernatants of mock- or pPICZαAIL-11/R-FP-transfected cells were analyzed by SDS-PAGE, followed by electroblotting and immunostaining with a hIL-11 polyclonal antibody (Fig. 2). A prominent band corresponding to a protein of approximately 50 kDa was detected in the IL-11/R-FP supernatants but not in the supernatants of mock-transfected cells. The electrophoretic mobility of the 50 kDa protein was increased after pretreatment with endoglycosidase H resulting in a 44 kDa protein. This is in good agreement with the calculated molecular

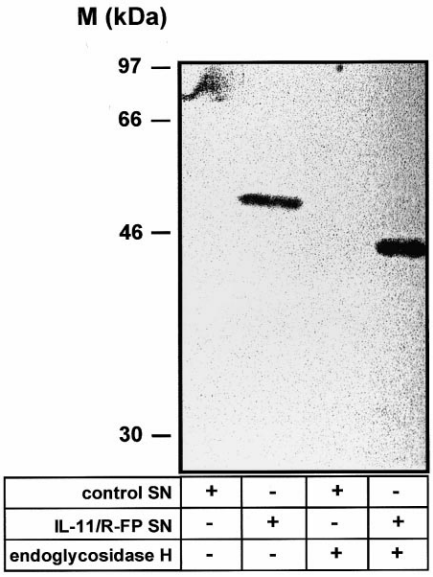

Fig. 2. Detection of IL-11/R-FP in the supernatants of transfected yeasts. Supernatants of mock-transfected (control SN) and pPICZαAIL-11/R-FP-transfected yeasts (IL-11/R-FP SN) were concentrated, treated with endoglycosidase H as indicated and, after separation of proteins on a 10% polyacrylamide SDS gel, analyzed by immunoblotting using a human IL-11 polyclonal antibody. Electrophoretic mobilities of molecular mass marker proteins are indicated on the left.

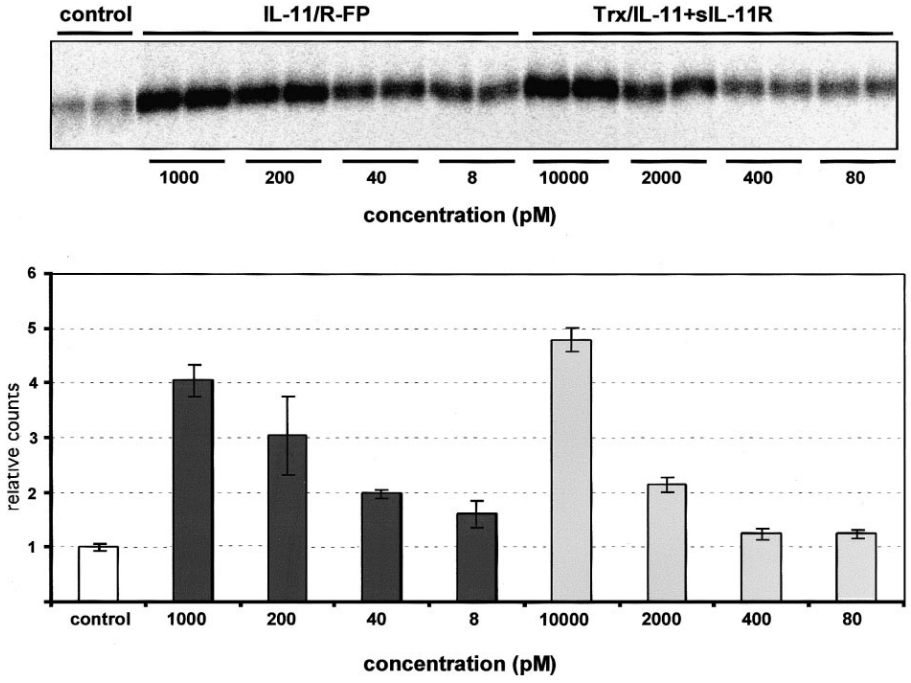

Fig. 3. Induction of the α1-ACT synthesis in HepG2 cells. HepG2 cells were stimulated with equilibrated supernatants containing IL-11/R-FP or the combination of Trx/IL-11 and sIL-11R, each protein at molar concentrations as indicated. Cells incubated with supernatants of mock-transfected yeast cells served as a control. Newly synthesized proteins were metabolically labelled using [^{35}S]methionine/cysteine. After immunoprecipitation from cell supernatants and separation by SDS-PAGE, radioactively-labelled α1-ACT was visualized by autoradiography (upper panel). Quantification of radioactivity was performed using a Phosphorimager (lower panel). The S.D.s were calculated from two independent

mass of 43.5 kDa. Thus, the glycoprotein detected by the antibody can be envisaged as IL-11/R-FP. Band intensities of IL-11/R-FP and serial dilutions of an IL-11 preparation of a known concentration were quantified using a Lumi-Imager (not shown). From these data, the concentration of IL-11/R-FP in the yeast supernatant was calculated to be 200 ng/ml. Yeast supernatants were equilibrated with PBS previous to application in the following bioassays.

3.2. *IL-11/R-FP efficiently induces acute phase protein synthesis in hepatoma cells*

The combination of IL-11 and sIL-11R induces the acute phase protein synthesis in hepatoma cells [24]. In order to evaluate the bioactivity of IL-11/R-FP, HepG2 cells were stimulated with either the combination of Trx/IL-11 and sIL-11R or IL-11/R-FP. Supernatants from mock-transfected cells served as a control. Trx/IL-11 is a well-characterized recombinant fusion protein of thioredoxin and IL-11 which has been shown to have biological properties indistinguishable from IL-11 wild-type [18,25]. After metabolic labelling of the proteins with [^{35}S]methionine and [^{35}S]cysteine, secreted α1-antichymotrypsin (α1-ACT) was precipitated from supernatants, transferred to SDS-PAGE and quantified using a Phosphorimager (Fig. 3). Whereas no stimulation of the α1-ACT synthesis was evident at a concentration of 400 pM Trx/IL-11 in the presence of equimolar amounts of sIL-11R, the hepatoma cells responded significantly to 8 pM IL-11/R-FP. Obviously, IL-11/R-FP is a more potent agonist than the combination of Trx/IL-11 and sIL-11R.

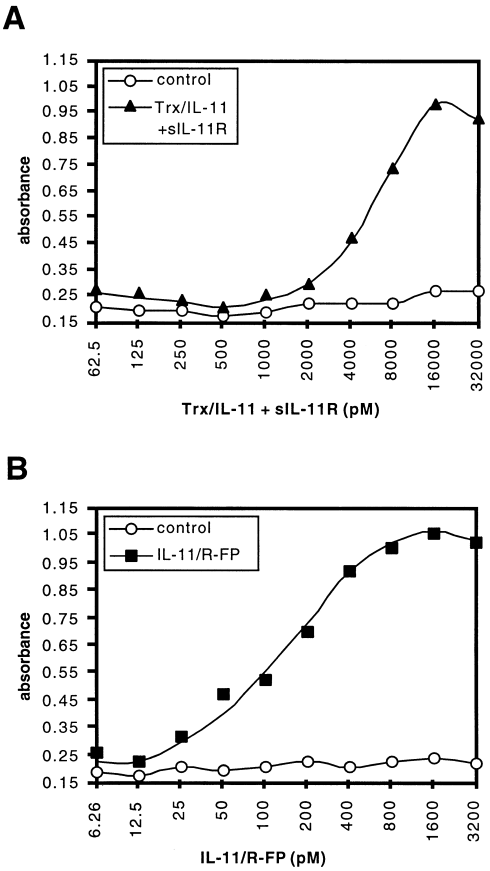

Fig. 4. Proliferation of Ba/F3-gp130 cells. Ba/F3 cells stably transfected with gp130 were seeded in a 96 well plate (20 000 cells/well) and incubated with increasing amounts of (A) Trx/IL-11 in the presence of sIL-11R or (B) IL-11/R-FP as indicated in the diagram. Open circles represent the response of cells stimulated with control supernatants. After 72 h, a tetrazolium compound was added as a substrate and incubated for 5 h at 37°C. Subsequently, the absorbance at 450 and 690 nm was measured. The difference of absorbances corresponds to the number of metabolically active cells (XTT

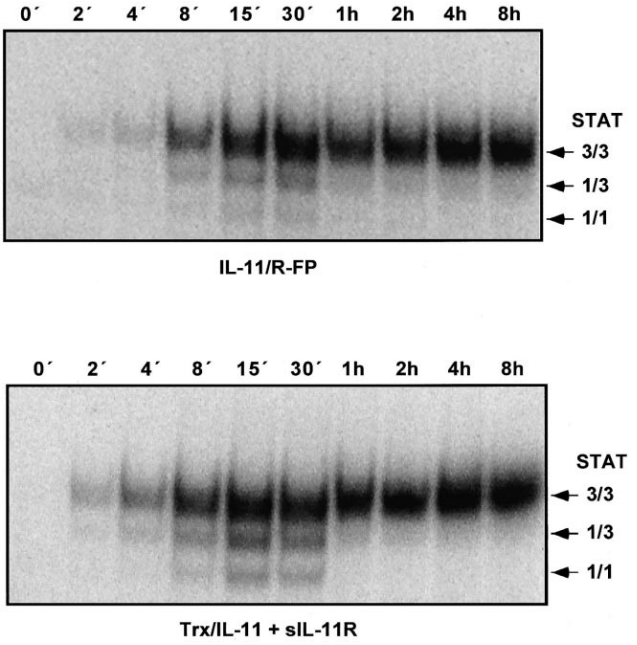

Fig. 5. STAT activation in response to IL-11/R-FP and the combination of Trx/IL-11 and sIL-11R. Ba/F3-gp130 cells were stimulated either with IL-11/R-FP (upper panel) at a concentration of 0.5 nM or the combination of Trx/IL-11 and sIL-11R (lower panel), each at a concentration of 5 nM. After different time points (as indicated), nuclear extracts were prepared and the protein concentrations were quantified. 10 μg of nuclear protein was analyzed by EMSA using a ^{32}P-labelled m67SIE probe derived from the c-fos promotor providing a binding site for STAT1 and STAT3. Protein-DNA complexes were separated by PAGE and visualized by autoradiography. STAT3 and STAT1 homodimers as well as STAT1/3 heterodimers are indicated by arrows.

3.3. *IL-11/R-FP acts as a superagonist on Ba/F3-gp130 cells*

In order to quantify the agonistic activity of IL-11/R-FP more precisely, proliferation assays were performed. Ba/F3 cells, which normally grow IL-3-dependently, are known to proliferate in response to various cytokines after transfection of the corresponding receptor chains. Ba/F3 cells transfected with gp130 (Ba/F3-gp130) proliferate after stimulation with Trx/IL-11 and sIL-11R [18]. Half-maximal proliferation was achieved with 6–7 nM Trx/IL-11 in the presence of equimolar amounts of sIL-11R (Fig. 4A). When Ba/F3-gp130 cells were stimulated with IL-11/R-FP, a concentration of 130–140 pM was sufficient for half-maximal proliferation (Fig. 4B). Equilibrated supernatants from mock-transfected yeast cells did not induce proliferation. Thus, IL-11/R-FP is a 50-fold more potent agonist compared to the combination of Trx/IL-11 and sIL-11R. Moreover, an increased sensitivity of Ba/F3-gp130 cells to IL-11/R-FP cells is obvious from the response to low concentrations of agonist. Significant proliferation of Ba/F3-gp130 cells upon incubation with IL-11/R-

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