

## A functional interleukin 12 receptor complex is composed of two $\beta$ -type cytokine receptor subunits

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Communicated by John J. Burns, Roche Institute of Molecular Biology, Nutley, NJ, September 16, 1996 (received for review July 9, 1996)

**ABSTRACT** We have identified a cDNA from a human phytohemagglutinin-activated lymphoblast library encoding a protein that binds  $^{125}\text{I}$ -labeled human interleukin 12 ( $^{125}\text{I}$ -huIL-12) with a  $K_d$  of about 5 nM when expressed in COS-7 cells. When coexpressed in COS-7 cells with the previously identified IL-12  $\beta$  receptor (IL-12R $\beta$ ) protein, two classes of  $^{125}\text{I}$ -huIL-12 binding sites were measured with  $K_d$ s of about 55 pM and 8 nM, corresponding to the high- and low-affinity binding sites seen on phytohemagglutinin-activated lymphoblasts. This newly identified huIL-12R subunit is a member of the cytokine receptor superfamily, with closest resemblance to the  $\beta$ -type cytokine receptor gp130 and the receptors for leukemia inhibitory factor and granulocyte colony-stimulating factor. Consequently, we have reclassified the previously identified IL-12R $\beta$  subunit as huIL-12R $\beta$ 1 and designated the newly identified subunit as huIL-12R $\beta$ 2. huIL-12R $\beta$ 2 is an 862-amino acid type I transmembrane protein with a 595-amino-acid-long extracellular domain and a cytoplasmic tail of 216 amino acids that contains three tyrosine residues. A cDNA encoding the mouse homolog of the huIL12R $\beta$ 2 protein has also been isolated. Human and mouse IL-12R $\beta$ 2 proteins show a 68% amino acid sequence identity. When expressed in COS-7 cells, huIL-12R $\beta$ 2 exists as a disulfide-linked oligomer with an apparent monomeric molecular weight of 130 kDa. Coexpression of the two identified IL-12R subunits in Ba/F3 cells conferred IL-12 responsiveness, and clones of these cotransfected Ba/F3 cells that grew continuously in the presence of IL-12 were isolated and designated LJM-1 cells. LJM-1 cells exhibited dose-dependent proliferation in response to huIL-12, with an  $\text{ED}_{50}$  of about 1 pM huIL-12. Interestingly, Ba/F3 cells transfected with IL-12R $\beta$ 2 alone proliferated in response to huIL-12 with an  $\text{ED}_{50}$  of about 50 pM, although a role for endogenous mouse IL-12R $\beta$ 1 in IL-12 signal transduction in these transfectants cannot be ruled out. These results demonstrate that the functional high-affinity IL-12R is composed of at least two  $\beta$ -type cytokine receptor subunits, each independently exhibiting a low affinity for IL-12.

Interleukin 12 (IL-12) is a heterodimeric cytokine produced primarily by antigen-presenting cells that has important activities in the regulation of various aspects of the immune response. IL-12 stimulates proliferation of activated T and natural killer cells (1–4), enhances lymphokine-activated killer/natural killer and cytotoxic T lymphocyte lytic activity (2, 5), and induces IFN- $\gamma$  production by T and natural killer cells (6–8). IL-12 plays a central role in promoting Th1-type immune responses and thus cell-mediated immunity (9–14). The biological activities of IL-12 are mediated via binding to specific cell surface receptors. Phytohemagglutinin (PHA)-activated lymphoblasts and IL-2-activated natural killer cells exhibit at least two classes of  $^{125}\text{I}$ -labeled human IL-12 ( $^{125}\text{I}$ -

huIL-12) binding sites with  $K_d$ 's of 5–20 pM and 2–6 nM (15, 16).

We have previously described the identification of a cDNA encoding an IL-12 receptor (R) protein, designated IL-12R $\beta$ . When expressed in COS-7 cells, IL-12R $\beta$  mediates binding of  $^{125}\text{I}$ -huIL-12 with a  $K_d$  of about 2–5 nM, corresponding to the low affinity binding sites seen on PHA-activated lymphoblasts (17). This IL-12R $\beta$  chain is a member of the cytokine receptor superfamily, and is related most closely over its entire length to the “ $\beta$  type” (18) cytokine receptor glycoprotein (gp)130 and the receptors for leukemia inhibitory factor and granulocyte colony-stimulating factor. Although this IL-12R $\beta$  protein has a short cytoplasmic tail, the box 1 and box 2 cytokine receptor motifs are present. In light of the important role of tyrosine phosphorylation in signal transduction pathways, it is interesting to note that there are no tyrosine residues present in the cytoplasmic tail of this IL-12R $\beta$  protein. Nevertheless, antibody inhibition experiments demonstrated that anti-IL-12R $\beta$  monoclonal antibodies were able to specifically inhibit IL-12-induced proliferation of PHA-activated lymphoblasts, production of interferon- $\gamma$  from resting peripheral blood mononuclear cells, and development of lymphokine-activated killer activity (19), demonstrating that this IL-12R $\beta$  protein is necessary for IL-12 signaling.

The inability to confer high affinity ( $K_d$  of 5–20 pM)  $^{125}\text{I}$ -huIL-12 binding to COS-7 cells transfected with the previously identified IL-12R $\beta$  protein, as well as the lack of cytoplasmic tyrosine residues, suggested that at least one additional IL-12R subunit exists. We now report the identification, using expression cloning techniques, of an additional  $\beta$  type IL-12 receptor protein. This receptor is designated IL-12R $\beta$ 2, and the previously identified IL-12R $\beta$  is therefore reclassified as IL-12R $\beta$ 1. IL-12R $\beta$ 2 by itself binds  $^{125}\text{I}$ -huIL-12 with low affinity. When coexpressed with IL-12R $\beta$ 1, it confers high-affinity  $^{125}\text{I}$ -huIL-12 binding and IL-12 responsiveness.

### MATERIALS AND METHODS

**Expression Cloning and Identification of IL-12R cDNA.** A plasmid expression library in the vector pEF-BOS (20) was established using mRNA isolated from lymphoblasts activated for 3 days with PHA as previously described (17). From this library, plasmid pools consisting of  $\approx$ 100 clones were coexpressed with the IL-12R $\beta$ 1 cDNA (17) and screened for high-affinity IL-12 binding by a modification of the previously

Abbreviations: IL, interleukin; R, receptor; huIL-12, recombinant human IL-12; aa, amino acid; PHA, phytohemagglutinin; gp, glycoprotein.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U64198 and U64199).

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described method (15, 21) incorporating low concentrations of  $^{125}\text{I}$ -huIL-12 (about 30 pM) and a 3-hr wash at room temperature to allow for the selective measurement of high-affinity binding. By screening 440 pools and subsequent sib-selection, one positive cDNA was isolated and sequenced using an automated Applied Biosystems DNA sequencer. The corresponding mouse cDNA was isolated by crosshybridization-screening of a cDNA library generated from concanavalin A-stimulated splenocyte mRNA. Standard recombinant DNA methods were used for additional library screening, probe labeling, and RNA blotting (22).

**Preparation of Cell Lines.** COS-7 cells were transfected by electroporation with expression constructs containing the indicated protein coding sequence in the pEF-BOS expression vector and harvested after 48–72 hr as described previously (17). Ba/F3-expressing huIL-12R $\beta$ 1 were produced by electroporation with linearized pRC-Rous sarcoma virus-IL-12R DNA as previously described for production of cytotoxic T lymphocyte lytic cells expressing huIL-12R $\beta$ 1 (17). G-418-resistant clones were subcloned by limiting dilution, screened for huIL-12R $\beta$ 1 expression by flow cytometry using the anti-huIL-12R $\beta$ 1 antibody 2–4E6 (19, 23), and the resultant cell line designated Ba/F3.huIL-12R $\beta$ 1 cells. Linearized expression constructs encoding huIL-12R $\beta$ 2 in the pEF-BOS expression vector and a puromycin resistance gene expression vector, pBSpac $\Delta$ p (24), were introduced by electroporation into Ba/F3 or Ba/F3.huIL-12R $\beta$ 1 cells, and stable transfectants were selected by growth in medium containing 10 ng/ml IL-12 (a gift of Alvin Stern, Hoffmann–La Roche) or in IL-3-containing medium supplemented with 3  $\mu\text{g}/\text{ml}$  puromycin (Sigma). The surviving cells were cloned by limiting dilution in IL-12- or puromycin-containing medium, as appropriate.

**Characterization of huIL-12R $\beta$ 2 Protein.** Expression constructs encoding huIL-12R $\beta$ 2 with the addition of a C-terminal FLAG peptide tag (25) were prepared in the pEF-BOS expression vector. COS-7 cells were transfected by electroporation with huIL-12R $\beta$ 1 and/or huIL-12R $\beta$ 2-FLAG expression constructs as described above and metabolically labeled with [ $^{35}\text{S}$ ]cysteine for 6 hr at 37°C. The cells were then solubilized in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate lysis buffer (CHAPS; 10 mM CHAPS/50 mM Tris/0.3 M NaCl/500 mM iodoacetamide/100 mM phenylmethylsulfonyl fluoride) at a density of  $1 \times 10^6$  cells per ml, and the cell lysates were immunoprecipitated by the addition of 5  $\mu\text{g}$  of the anti-FLAG M2 antibody bound to 50  $\mu\text{l}$  of GammaBind Plus Sepharose (Pharmacia). The immunoprecipitated proteins were analyzed by SDS/PAGE 4–12% gradient gels, and the labeled proteins visualized by autoradiography.

**IL-12 Binding and Proliferation Assays.**  $^{125}\text{I}$ -huIL-12 was prepared by the Iodogen method, and specific binding to cells was measured as described previously (15, 26). Specific binding was analyzed using the LIGAND nonlinear regression computer program (27) and plotted by the method of Scatchard (28). For measurement of IL-12-stimulated proliferation, Ba/F3 cells expressing the indicated proteins were washed and cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 2 mM glutamine at  $1 \times 10^4$  cells per well in flat-bottom 96-well microculture plates at 37°C for 24 hr. Various concentrations of IL-12 were added, the cells were cultured for an additional 42 hr at 37°C, and the wells were then pulsed with 0.5  $\mu\text{Ci}$  (1 Ci = 37 GBq) per well  $^3\text{H}$ -thymidine (New England Nuclear) for 6 hr at 37°C. Incorporation of  $^3\text{H}$ -thymidine into DNA was determined by harvesting the contents of each well onto glass fiber filters. All samples were assayed in triplicate.

## RESULTS

**Characterization of IL-12R $\beta$ 2 cDNA.** Using an expression cloning strategy that was specifically directed toward identi-

fying high-affinity IL-12R, we have isolated a novel IL-12R cDNA. This cDNA, when coexpressed with the previously described IL-12R, reconstitutes a high-affinity IL-12R complex (see below). Due to its homology to other  $\beta$ -type cytokine receptor proteins (see below), we are proposing that this novel IL-12R be termed IL-12R $\beta$ 2, and that the previously isolated IL-12R $\beta$  (17) be renamed IL-12R $\beta$ 1. The first IL-12R $\beta$ 2 cDNA that was isolated contained a very long (640 bp) and G+C-rich (73–78%) 5' noncoding region (data not shown). Analysis of four additional independent clones confirmed the presence of this unusual 5' end structure. Sequencing of the cDNAs showed the presence of one long open reading frame encoding an 862-amino acid (aa) protein (Fig. 1). This IL-12R $\beta$ 2 is a type I transmembrane protein, with a predicted molecular weight of 97,140 Da (including the signal peptide) and 94,059 Da (predicted mature protein). An extracellular domain of 595 aa is separated by a 24-aa transmembrane region from a 216-aa cytoplasmic tail that contains three tyrosine residues. Fig. 1 also shows that the corresponding mouse IL-12R $\beta$ 2 protein is very similar to the human receptor subunit, with an overall aa sequence identity of 68%.

IL-12R $\beta$ 2 is a member of the cytokine receptor superfamily. Similar to IL-12R $\beta$ 1, IL-12R $\beta$ 2 is also most closely related to the members of the gp130-type subgroup (gp130, granulocyte colony-stimulating factor-R, leukemia inhibitory factor-R). Comparison of the huIL-12R extracellular domains using the ALIGN program (29) yields scores of 20 (IL-12R $\beta$ 2/gp130), 8 (IL-12R $\beta$ 1/gp130), and 3 (IL-12R $\beta$ 1/IL-12R $\beta$ 2). Scores greater than 3 are considered to demonstrate significant evolutionary relationships. A score of 3 indicates that the similarity would have occurred by chance with a probability of only  $10^{-3}$ , with increasing scores corresponding to decreasing probabilities. In contrast to IL-12R $\beta$ 1, IL-12R $\beta$ 2 contains an N-terminal Ig motif, and is thus more similar to gp130. The cytoplasmic region of IL-12R $\beta$ 2 also contains the characteristic box 1 motif and a possible box 2 motif: thus, both cloned IL-12 receptor chains have the general makeup of a  $\beta$ -type subunit (18).

RNA blot analysis of PHA-activated human lymphoblasts indicated the presence of two major transcripts of 4.5 and 6 kb (Fig. 2). Both mRNAs are up-regulated from undetectable or very low levels when peripheral blood mononuclear cells are treated with PHA. By analyzing uncloned cDNA from PHA-activated lymphoblasts using reverse transcription-PCR, we have not been able to detect any evidence for a soluble form of huIL-12R $\beta$ 2, since variant transcripts in which the transmembrane encoding region is absent have not been found (data not shown). However, production of soluble forms of huIL-12R $\beta$ 2 could result from proteolytic cleavage of the extracellular portion of this receptor subunit from the cell surface.

**Characterization of the IL-12R Subunits in COS-7 Cells.** COS-7 cells were transfected by electroporation with cDNA expression constructs for huIL-12R $\beta$ 1 and huIL-12R $\beta$ 2, alone and in combination, to investigate the functional properties of these proteins. As previously reported (17, 30), huIL-12R $\beta$ 1 mediates low affinity binding of  $^{125}\text{I}$ -huIL-12, with a  $K_d$  of about 10 nM and about 100,000 sites per cell (Fig. 3 *Top*). Expression of huIL-12R $\beta$ 2 in COS-7 cells yields similar results with a single class of  $^{125}\text{I}$ -huIL-12 binding sites with a  $K_d$  of about 5 nM (Fig. 3 *Middle*). When both proteins are coexpressed, two classes of binding sites can be detected (Fig. 3 *Bottom*). We have measured two classes of  $^{125}\text{I}$ -huIL-12 binding sites in cotransfected COS-7 cells with  $K_d$ s of  $55 \pm 10$  pM ( $1,860 \pm 380$  sites per cell) and  $7.5 \pm 0.6$  nM ( $68,700 \pm 16,500$  sites per cell) ( $n = 8$ ). The low number of high affinity sites detected on the cotransfected cells may be due to either competition for expression between huIL-12R $\beta$ 1 and huIL-12R $\beta$ 2 at the level of mRNA or protein, or may be due to competition between homo- and hetero-association of the  $\beta$ 1

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1 MAHTFRGCSLAFMPFITWLLIKAKIDACKRGDVTVKPSHVILLGSTVWNT 50
1 MAQTRECSLALLFLFWLLIKANIDVCKLGTVTQPAVPIPLGSAANIS 50
51 CSLKPRQGCFFHYSRRLKILLYKF.....DRRINFHHGHSLSNSQVT 90
51 CSLNPKQGCSSHYPSSNELILKLVNDVLENLHGKVVHDHTGHSSTFQVT 100
91 GLPLGTTTLFVCKLACINSDE...IQICGAEIFVGVAPQPNLSCIQKGE 137
101 NLSLGMTLFFVCKLNCNSNSQKKPPVPCGVEISVGVAPPEPPQNISCVQEGE 150
138 QGTVAQTWERGRDTHLYTEYTLQLSGPKNLTWOKOC...KDIYCDYLD 184
151 NCTVACSWNSGKVTYLYKTNYTLQLSGPNLTCQKQCFSDNRQNCNRDL 200
185 INLTPESPESNFTAKVTAVNSLGSSSSLPSTFTFLDIVRPLPWPDIRIF 234
201 INLSPDLAESRFIVRVTAINDLGNSSSLPHTFTFLDIVIPLPWPDIRINF 250
235 QKASVSRCTLYWRDEGLVLLNRLRYRPSNSRLWMMVNVTKAKGRHLLDL 284
251 LNAGSRGTQLQWEDGQVVLNQLRYQPLNSTSWMMVNAITNAKGYDLRDL 300
285 KPFTYEYFQISSKHLHYKGSWSDWSESRLRAQTPEEEPTGMLDVVMKRHI 334
301 RPFTEYEFQISSKHLHSGSSNSWSESRLRTRTPEEPPVGIIDWIYMKQDI 350
335 DYSRQOISLFWKNSLSEARGKILHYQVTLQELTGKAMTONITGHTSWT 384
351 DYDRQOISLFWKSLNPSRARGKILHYQVTLQEVTKKTTLQNTTRHTSWT 399
385 TVIPRTGNWAVAVSAANSKGSLLPTRINIMNLCEAGLAPRQVSANSEGM 434
400 RVIPRTGAWTASVSAANSKASAPHTINIVDLCGTGLLAPHQVSAKSENM 449
435 DNILVTWQPPRKDPQSAVQEVVVEWRELHPGGDTQVPLNWLRSRPVNSAL 484
450 DNILVTWQPPKADSAVREYVVEWRALQPGSITKFPPhwLRIPPDNMSAL 499
485 ISENIKSYICYEIRVYVALSGDQGGCSSILGNSKHKAPLSGPHINAITEEK 534
500 ISENIKPYICYEIRVHALSESGQGGCSSIRGDSKHKAPVSGPHITAITEKK 549
535 GSILISWNSIPVQEQMGCLLHYRIYWKERDSNSQPQLCEIPYRVSONSHP 584
550 ERLFISWTHIPPEQQRGCILHYRIYWKERDSTAQPELCEIQYRRSONSHP 599
585 INSLQPRVTYVLLWMTALTAAGESSHGNEREFCLQCKANWMAFVAPSICIA 634
600 ISSLQPRVTYVLLWMTAVTAAGESPQGNREFCQCKANWKAFFVSSICIA 649
635 IIMVGIFFSTHYEQQKVFVLLAALRPQWCSREIPDPANSTCAKRYPIAEK 684
650 IITVGFPSIRVFRQKAFLLSTLKPQWRSRTIDDPANSTWVKYPILEEK 699
685 TQLPLDRLLIDWPTPEDPEPLVISEVLHQVTPVFRHPPCSNWPQREKGIQ 734
700 IQLPTDNLLMAWPTPEEPEPLIHEVLYHMLIPVVRQPYFYFRGQ...GFQ 746
735 GHQASEKDMHSSASSPPPRALQAESRQLVDLYKVLERSGSDPKPENPAC 784
747 GYSTSKQDAMYIA.NPQATGTLTAETROLVNLKVLERSRDPDSKLANLTS 795
785 PWTVLPAGDLPDTHDGYLPSNIDDLPSHEAPLADSLLEELEPQHISLSVFP 834
796 PLTVTPVNYLPSHEGYLPSNIEDLSPHEADPTDSF.DLEHQHISLSIFAS 844
835 SSLHPLTFSCGDKLTLQKMRCDLML... 862
845 SSLRPLIFG.GERLTLDRKMGYDSLMSNEA. 875
    
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FIG. 1. Comparison of aa sequences of human (Upper) and mouse (Lower) IL-12Rβ2 subunits. The GAP program (Genetics Computer Group, Madison, WI) was used to construct the alignment. Signal peptides are underlined, potential N-linked glycosylation sites appear in bold italics and underlined, the hallmark sequence/structural motifs characteristic of the cytokine receptor superfamily are shaded, the putative transmembrane regions are doubly underlined, the cytoplasmic box I and II motifs are shaded, and the conserved cytoplasmic tyrosine residues are shown in bold and doubly underlined.

and β2 chains. Nevertheless, coexpression of the two IL-12R subunits creates <sup>125</sup>I-huIL-12 binding sites that correspond to the high- and low-affinity binding sites measured on activated T cells (23).

Immunoprecipitation experiments were conducted to determine the biochemical properties of huIL-12Rβ2. C-terminally FLAG-tagged huIL-12Rβ2 protein was determined to have an apparent molecular weight of 130 kDa when expressed in COS-7 cells and analyzed by SDS/PAGE under reducing conditions (Fig. 4). Under nonreducing conditions,

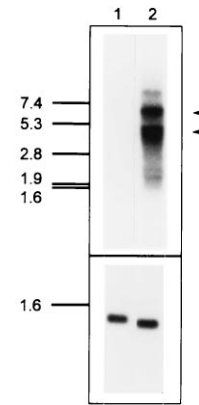


FIG. 2. RNA blot analysis of huIL-12Rβ2 expression. (Upper) A blot containing 5 μg of poly(A)<sup>+</sup> RNA from uninduced peripheral blood mononuclear cells (lane 1) or PHA-activated (3 days) peripheral blood mononuclear cells (lane 2) was probed with an IL-12Rβ2 cDNA insert. Two major RNAs at 4.5 kb and 6 kb are apparent in lane 2. Exposure time was 16 hr using an intensifying screen at -80°C. (Lower) As a loading control, the blot was rehybridized with a probe for a cell-cycle independent transcript, pHE7 (37). Exposure time was 20 min using an intensifying screen at -80°C.

huIL-12Rβ2 protein migrates as a protein with an apparent molecular weight >220 kDa, suggesting the IL-12-independent formation of disulfide-bonded dimers of huIL-12Rβ2 in the transfected COS-7 cells. In addition, the existence of higher order multimers of huIL-12Rβ2 is suggested by the appearance of high molecular weight material at the top of the electrophoresis gels. However, this material may be an artifact of

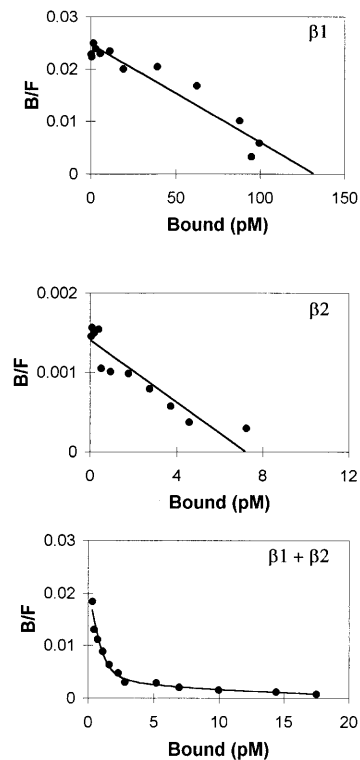


FIG. 3. Coexpression of huIL-12Rβ1 and huIL-12Rβ2 in COS-7 cells yields high affinity binding of <sup>125</sup>I-huIL-12. Specific binding of <sup>125</sup>I-huIL-12 to COS-7 cells transfected with the indicated expression vectors was determined and analyzed by the method of Scatchard. (Top) huIL-12Rβ1, 6 nM K<sub>d</sub>, 123,000 sites per cell; (Middle) huIL-12Rβ2, 5 nM K<sub>d</sub>, 6500 sites per cell; (Bottom) both huIL-12Rβ1 and huIL-12Rβ2 expression vectors, 65 pM K<sub>d</sub>, 1100 sites per cell and 10 nM K<sub>d</sub>, 20,400 sites per cell.

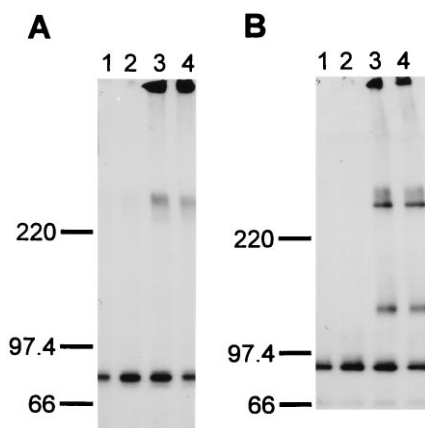


FIG. 4. Analysis of huIL-12R $\beta$ 2 protein expressed on the surface of transfected COS-7 cells. COS-7 cells were mock-transfected (lane 1) or transfected with a huIL-12R $\beta$ 1 expression construct (lane 2), a huIL-12R $\beta$ 2-FLAG expression construct (lane 3), or cotransfected with huIL-12R $\beta$ 1 and huIL-12R $\beta$ 2-FLAG expression constructs (lane 4) as described in *Materials and Methods*. The cells were metabolically labeled with [ $^{35}$ S]cysteine, cell lysates were prepared, and proteins were immunoprecipitated using the M2 anti-FLAG antibody. The precipitated proteins were analyzed on SDS/4-12% polyacrylamide gels under nonreducing (A) and reducing (B; 700 mM 2-mercaptoethanol) conditions. The migration of protein standards electrophoresed in parallel lanes is shown. Exposure time was 6 hr using an intensifying screen at  $-80^{\circ}\text{C}$ .

solubilization. It is interesting that this dimer/oligomer is still present when the samples are prepared in the presence of 700 mM 2-mercaptoethanol, demonstrating resistance of dimer/oligomer breakdown upon disulfide bond reduction. The protein migrating with an apparent molecular weight of 90 kDa is unrelated to the IL-12 receptor subunits as it appears in mock transfected COS-7 cells. Coimmunoprecipitation of the two IL-12 receptor subunits was not observed, as evidenced by the absence of the 100-kDa huIL-12R $\beta$ 1 protein from the pattern of proteins immunoprecipitated by anti-FLAG antibody from cell lysates of COS-7 cells cotransfected with huIL-12R $\beta$ 1 and huIL-12R $\beta$ 2-FLAG (Fig. 4). Additional experiments conducted in the presence of huIL-12 and chemical crosslinking agents also did not detect coimmunoprecipitation of heterodimeric  $\beta$ 1/ $\beta$ 2 protein complexes (data not shown).

#### Analysis of the Signaling Properties of the IL-12R Subunits.

To investigate the role the various IL-12R subunits play in mediating IL-12 signal transduction, the Ba/F3 IL-3-dependent mouse pro B cell line (31) was stably transfected with huIL-12R $\beta$ 1 and huIL-12R $\beta$ 2 alone and in combination. Stable Ba/F3 clones expressing huIL-12R $\beta$ 1, Ba/F3.huIL-12R $\beta$ 1 cells, were isolated on the basis of G-418 resistance. These cells express about 30,000 huIL-12R $\beta$ 1 molecules on the cell surface (data not shown), as determined using  $^{125}\text{I}$ -labeled Fab fragments of the anti-huIL-12R $\beta$ 1 monoclonal antibody 2-4E6 (23). As previously reported for the CTLL mouse IL-2-dependent T cell line (17),  $^{125}\text{I}$ -huIL-12 binds very inefficiently to the Ba/F3.huIL-12R $\beta$ 1 cells, with a  $K_d > 50$  nM (data not shown). In contrast, Ba/F3 cells expressing huIL-12R $\beta$ 2 and selected in puromycin-containing medium exhibited  $^{125}\text{I}$ -huIL-12 binding with a  $K_d$  of about 5 nM (Fig. 5 *Top*). Similar results with Ba/F3.huIL-12R $\beta$ 2 cells selected for growth in IL-12-containing medium were seen. However, these IL-12-selected cells expressed about 5-fold fewer  $^{125}\text{I}$ -huIL-12 binding sites than Ba/F3.huIL-12R $\beta$ 2 cells selected for growth in puromycin-containing medium, and the combination of low affinity and small site number made Scatchard analysis of binding to the IL-12-selected cells difficult (data not shown). Cotransfected Ba/F3 cells, designated LJM-1 cells, exhibited two classes of  $^{125}\text{I}$ -huIL-12 binding sites with  $K_d$ s of

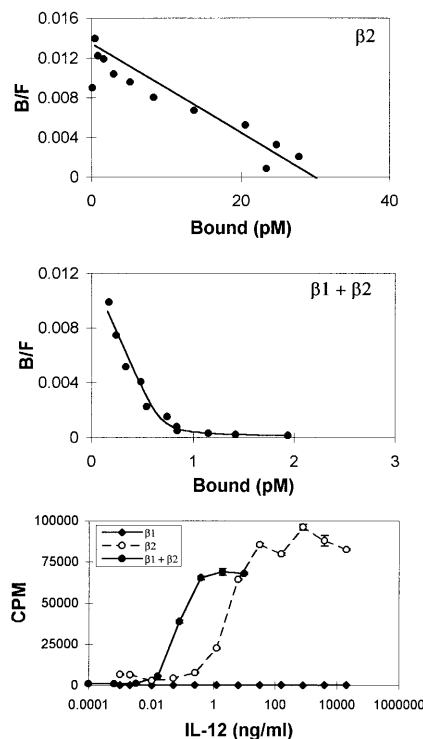


FIG. 5. Coexpression of huIL-12R $\beta$ 1 and huIL-12R $\beta$ 2 in Ba/F3 cells yields high affinity  $^{125}\text{I}$ -huIL-12 binding and confers IL-12 responsiveness. Specific binding of  $^{125}\text{I}$ -huIL-12 to Ba/F3 cells expressing the indicated proteins was determined and analyzed by the method of Scatchard. (*Top*) huIL-12R $\beta$ 2, 3 nM  $K_d$ , 5600 sites per cell; (*Middle*) huIL-12R $\beta$ 1 and huIL-12R $\beta$ 2, 60 pM  $K_d$ , 120 sites per cell and 17 nM  $K_d$ , 500 sites per cell. (*Bottom*) IL-12-stimulated proliferation of Ba/F3 transfectants expressing huIL-12R $\beta$ 1 ( $\blacklozenge$ ), huIL-12R $\beta$ 2 ( $\circ$ ), or both huIL-12R $\beta$ 1 and huIL-12R $\beta$ 2 ( $\bullet$ ) was determined as described.

about 50 pM and 10 nM (Fig. 5 *Middle*), similar to the results seen with cotransfected COS-7 cells.

We next examined the ability of huIL-12 to stimulate proliferation of the transfected Ba/F3 cells. In keeping with the ability to select these cells by long-term growth in huIL-12-containing medium, LJM-1 (cotransfected Ba/F3) cells proliferated in response to huIL-12 in a dose-dependent fashion (Fig. 5 *Bottom*). Ba/F3.huIL-12R $\beta$ 2 cells that grew slowly in huIL-12-containing medium were also identified, and subsequent cloning of these huIL-12R $\beta$ 2 transfectants in the presence of huIL-12 yielded clones that exhibited high levels of huIL-12-stimulated proliferation (Fig. 5 *Bottom*). In contrast, we were unable to isolate any Ba/F3.huIL-12R $\beta$ 1 cells by long-term growth in huIL-12-containing medium, and Ba/F3.huIL-12R $\beta$ 1 cells selected on the basis of G-418 resistance did not proliferate in response to huIL-12 (Fig. 5 *Bottom*). In parallel with the observed lack of high affinity  $^{125}\text{I}$ -huIL-12 binding to the Ba/F3.huIL-12R $\beta$ 2 cells, these cells exhibited an ED $_{50}$  for IL-12-induced proliferation (50 pM) that was 50-fold greater than that observed with the LJM-1 cells (1 pM).

## DISCUSSION

The identification of an IL-12 binding protein that is a  $\beta$ -type cytokine receptor subunit and that mediates low-affinity  $^{125}\text{I}$ -huIL-12 binding when expressed in COS-7 cells has been described (17). This IL-12 receptor protein was originally designated IL-12R $\beta$  and has now been renamed IL-12R $\beta$ 1. By analogy to other cytokine receptor systems, the inability to confer high-affinity  $^{125}\text{I}$ -huIL-12 binding through expression of IL-12R $\beta$ 1 suggested that additional receptor protein(s) re-

mained to be identified. An expression cloning strategy specifically directed toward the isolation of cDNAs that, upon coexpression with IL-12R $\beta$ 1 protein, would yield high-affinity IL-12 binding sites was therefore employed. The key features of this strategy were the use of picomolar concentrations of  $^{125}\text{I}$ -huIL-12 and a 3-hr wash period during which  $^{125}\text{I}$ -huIL-12 bound with low affinity was allowed to preferentially dissociate (21). A cDNA encoding a novel IL-12 receptor protein, designated IL-12R $\beta$ 2, was thus isolated. This IL-12R $\beta$ 2 protein, similar to the previously isolated IL-12R $\beta$ 1 subunit, is a member of the gp130-type subgroup of the cytokine receptor superfamily. Alignment scores for a sequence comparison between the  $\beta$ 1 and  $\beta$ 2 subunits of the IL-12R are significant. However, each of the two IL-12R subunits itself is more closely related to gp130 than to each other. In contrast to IL-12R $\beta$ 1, which does not contain any cytoplasmic tyrosine residues, the cytoplasmic region of IL-12R $\beta$ 2 contains three tyrosine residues, suggesting an important role for the  $\beta$ 2 subunit in IL-12 signal transduction. Comparison of the human and mouse IL-12R $\beta$ 2 proteins reveals a high level of conservation, with a 68% aa sequence identity. This is in contrast to the situation for the IL-12R $\beta$ 1 subunit, which exhibits only a 54% sequence identity between the human and mouse proteins.

Expression of human IL-12R $\beta$ 2 in COS-7 cells yielded one class of  $^{125}\text{I}$ -huIL-12 binding sites with a  $K_d$  of about 5 nM. Although the binding affinity for  $^{125}\text{I}$ -huIL-12 of IL-12R $\beta$ 1 and IL-12R $\beta$ 2 expressed in COS-7 cells are similar, we have consistently observed the number of  $^{125}\text{I}$ -huIL-12 binding sites seen with IL-12R $\beta$ 2-transfected COS-7 cells to be about 10-fold lower than that seen with IL-12R $\beta$ 1-transfected cells. The reasons for this difference in expression efficiency are not known. In keeping with the fact that IL-12R $\beta$ 2 was identified using a screen that employed coexpression with IL-12R $\beta$ 1 and that was optimized for detecting high-affinity  $^{125}\text{I}$ -huIL-12 binding, coexpression of the  $\beta$ 1 and  $\beta$ 2 proteins in COS-7 cells yielded high-affinity  $^{125}\text{I}$ -huIL-12 binding. Two classes of binding sites for  $^{125}\text{I}$ -huIL-12, with  $K_d$ s of about 50 pM and 10 nM, were measured in cotransfected COS-7 cells. These binding affinities are similar to the high- and low-affinity binding sites, with  $K_d$ s of about 5–20 pM and 2–6 nM, detected on PHA-activated lymphoblasts and the IL-12 responsive Kit225/K6 T cell line (17, 23). The observed affinity difference between high-affinity binding in the cotransfected COS-7 cells vs. PHA-activated lymphoblasts is small but very reproducible. Besides this quantitative difference there are also qualitative differences. Certain classes of inhibitors, such as the anti-huIL-12R $\beta$ 1 mAb 2B10, are able to inhibit IL-12 bioactivity in huIL-12R $\beta$ 1/ $\beta$ 2 cotransfected Ba/F3 cells but not in PHA-activated lymphoblasts (data not shown). However, it is clear that coexpression of both the  $\beta$ 1 and  $\beta$ 2 IL-12R chains in COS-7 cells is both necessary and sufficient for high-affinity  $^{125}\text{I}$ -huIL-12 binding. It is unclear whether the observed differences between cotransfected COS-7 or Ba/F3 cells and PHA-activated lymphoblasts reflect the involvement of additional IL-12R subunits in PHA-activated lymphoblasts or simply differences in posttranslational processing of the two known IL-12R subunits.

The low number of high-affinity binding sites measured on cotransfected cells may be due to the large excess of huIL-12R $\beta$ 1 expressed in transfected cells. Combined with the propensity for huIL-12R $\beta$ 2 to form homomultimers (as seen in Fig. 4), a small number of  $\beta$ 1/ $\beta$ 2-heterodimers may actually form in the cotransfected cells. Recent experiments have demonstrated that multiple interactions between huIL-12 and huIL-12R $\beta$ 1/ $\beta$ 2 heterodimers are important for high-affinity  $^{125}\text{I}$ -huIL-12 binding. Experiments with homodimers of the mouse IL-12 p40 subunit, a known IL-12 antagonist, have shown that the p40 chain of IL-12 interacts primarily with the huIL-12R $\beta$ 1 protein (32), whereas other epitopes exist on the IL-12 heterodimer that primarily interact with huIL-12R $\beta$ 2

(D.H.P., L.J.M., S. Gillessen, U.G., R. Chizzonite, A. S. Stern, and M.K.G., unpublished work).

The apparent molecular weight of the IL-12R $\beta$ 2 protein monomer when expressed in COS-7 cells was 130 kDa, suggesting that about 36 kDa of carbohydrate are present on the predicted 94-kDa protein backbone. Similar to huIL-12R $\beta$ 1, huIL-12R $\beta$ 2 appears to exist at the cell surface as a disulfide-bonded dimer/oligomer. Formation of this huIL-12R $\beta$ 2 oligomer is not dependent on IL-12 binding, since it was observed in cells that were never exposed to IL-12. Coimmunoprecipitation experiments designed to examine the formation of  $\beta$ 1/ $\beta$ 2-containing heterodimers, conducted both in the absence or presence of huIL-12 and chemical crosslinking agents, have not yet been successful. This could be due to disruption of the  $\beta$ 1/ $\beta$ 2 heterodimers by the solubilization procedures, or simply due to insufficient sensitivity of the methods employed since only about 2000 high-affinity  $^{125}\text{I}$ -huIL-12 binding sites per cell are reconstituted on cotransfected COS-7 cells, in the presence of a large excess of  $\beta$ 1 and  $\beta$ 2 homomultimers that will interfere with the immunoprecipitation of the heterodimeric complexes.

To address the question of signaling through the IL-12R complex, we used the IL-3-dependent murine pro B cell line Ba/F3 as the host cell for expression of the various IL-12R proteins. The Ba/F3 cell line has previously been used to investigate signaling mediated by a number of different cytokine receptors, including erythropoietin (33), granulocyte/macrophage colony-stimulating factor (34), and IL-10 (35). Cotransfected Ba/F3 cells (designated LJM-1 cells) bound  $^{125}\text{I}$ -huIL-12 with two classes of binding sites with  $K_d$ s of about 50 pM and 10 nM, similar to the binding sites observed in cotransfected COS-7 cells. However, the number of binding sites measured on LJM-1 cells (about 150 high-affinity sites per cell and 1,000 low-affinity sites per cell) was significantly lower than that detected in cotransfected COS-7 cells. It is possible that there may be negative selective effects against high level expression of high-affinity IL-12R complexes on the Ba/F3 cell surface, i.e., supranormal levels of IL-12R signaling may inhibit cell growth. Such selective pressure would influence the number of high-affinity sites on the stably transfected Ba/F3 cells but not the transiently transfected COS-7 cells. In support of this hypothesis, huIL-12R $\beta$ 2-transfected Ba/F3 cells isolated by growth in puromycin exhibited about 5-fold more  $^{125}\text{I}$ -huIL-12 binding sites per cell than similar cells isolated by growth in high concentrations of huIL-12.

LJM-1 cells exhibited dose-dependent proliferation in response to IL-12, with an ED<sub>50</sub> of about 1 pM. This ED<sub>50</sub> is  $\approx$ 50-fold lower than the observed  $K_d$  of about 50 pM for high-affinity  $^{125}\text{I}$ -huIL-12 binding to these cells, and suggests that a very low level of receptor occupancy is sufficient for stimulation of proliferation by the IL-12R complex.

Ba/F3 cells transfected with huIL-12R $\beta$ 1 expressed about 30,000 huIL-12R $\beta$ 1 molecules per cell but bound  $^{125}\text{I}$ -huIL-12 very inefficiently ( $K_d > 50$  nM) and did not respond to IL-12. In contrast, IL-12R $\beta$ 2-transfected Ba/F3 cells bound  $^{125}\text{I}$ -huIL-12 with a  $K_d$  of about 5 nM. Surprisingly, although the huIL-12R $\beta$ 2-transfected cells did not exhibit high-affinity  $^{125}\text{I}$ -huIL-12 binding, transfectants exhibiting long-term growth in IL-12-containing medium were isolated. Therefore, expression of IL-12R $\beta$ 2 alone was sufficient to confer IL-12-responsiveness to Ba/F3 cells. It should be noted that reverse transcription-PCR analysis of Ba/F3 cells has demonstrated the presence of detectable levels of mouse IL-12R $\beta$ 1 mRNA (U.G., unpublished data), and this mouse homolog of IL-12R $\beta$ 1 may be necessary for signal transduction in the IL-12R $\beta$ 2-transfected Ba/F3 cells. Although the requirement for endogenous mouse IL-12R $\beta$ 1 in huIL-12R $\beta$ 2-mediated signaling in the transfected Ba/F3 cells has not been formally ruled out, additional evidence suggests that the homomultimers are mediating the IL-12 signaling. Ba/F3 transfectants expressing

chimeric receptor proteins consisting of the epidermal growth factor-R extracellular domain fused to the huIL-12R $\beta$ 2 intracellular domain proliferate in response to epidermal growth factor treatment (J. Zou, D. H. Presky, C.-Y. Wu, and U. Gubler, unpublished work). In addition, Ba/F3 cells transfected with mouse IL-12R $\beta$ 2 alone did not exhibit long-term growth in medium containing mouse IL-12, whereas mouse IL-12R $\beta$ 1/mouse IL-12R $\beta$ 2 cotransfected Ba/F3 cells do (V. L. Wilkinson, D. Carvajal, L.J.M., A.O.C., U.G., M.K.G., and D.H.P., unpublished work). If mouse IL-12R $\beta$ 1 expression was significant in the host Ba/F3 cell line, the mouse IL-12R $\beta$ 2-transfected Ba/F3 cells should have been able to grow in mouse IL-12. These observations suggest that endogenous mouse IL-12R $\beta$ 1 is not playing a role in the observed IL-12 signaling in huIL-12R $\beta$ 2 Ba/F3 transfectants. It is intriguing that the IL-12R $\beta$ 2 protein, in contrast to the IL-12R $\beta$ 1 protein, contains cytoplasmic tyrosine residues. By analogy to many other cytokine receptors, these IL-12R $\beta$ 2 cytoplasmic tyrosine residues are likely to play an important role in IL-12 signal transduction. Within the heterodimeric IL-12R complex, IL-12R $\beta$ 2 could therefore be considered the signal specificity determining “driver” subunit based on the concept outlined by Lai *et al.* (36). By bringing Janus kinase to the receptor complex, the  $\beta$ 1 subunit would then serve as the more generic “trigger” chain. Experiments are underway to determine if this is indeed the case, or if the  $\beta$ 1 subunit primarily acts as an affinity converter.

In conclusion, we have identified IL-12R $\beta$ 2, an additional protein subunit of the functional, high-affinity IL-12R complex. IL-12R $\beta$ 2 appears to have an important function in the IL-12 signal transduction process, and in concert with the previously identified IL-12R $\beta$ 1 subunit, can confer high-affinity binding and IL-12 responsiveness to cells expressing these proteins. We have produced a cotransfected Ba/F3 cell line, LJM-1 cells, that binds <sup>125</sup>I-huIL-12 with high affinity, and that proliferates in response to IL-12 with an ED<sub>50</sub> of 1 pM. This IL-12-responsive cell line should be useful for further investigation into IL-12 signal transduction mechanisms.

We thank Lucy Foppiani, John Duker, and Doug Larigan (Hoffmann-La Roche, Bioinformatics Department) for oligonucleotide synthesis and DNA sequencing, and Frank Podlaski and Alvin Stern (Hoffmann-La Roche, Department of Inflammation/Autoimmune Diseases) for providing purified huIL-12.

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