Identification and Characterization of Two Alternative Splice Variants of Human Interleukin-2*

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Our previous work showed that alternative splicing is used to make an inhibitory variant of human interleukin (IL)-4. Because of homology between IL-4 and IL-2 proteins and receptors, we tested whether alternative splicing is used to generate similar inhibitory variants of human IL-2. Messenger RNA from peripheral blood mononuclear cells was subjected to reverse transcription-polymerase chain reaction using IL-2 exon 1- and exon 4-specific primers. Two amplification products, named IL-2\textsubscript{d2} and IL-2\textsubscript{d3}, were found in addition to the native IL-2 product. The IL-2\textsubscript{d2} cDNA sequence was identical to IL-2 cDNA throughout the entire coding region, except exon 2 was omitted by alternative splicing. In IL-2\textsubscript{d3} cDNA, the third exon of IL-2 was omitted by alternative splicing. Unlike IL-2, IL-2\textsubscript{d2} and IL-2\textsubscript{d3} did not stimulate T cell proliferation. However, both inhibited IL-2 costimulation of T cell proliferation, and both inhibited cellular binding of rhIL-2 to high affinity IL-2 receptors. Thus, IL-2 is the second cytokine that uses alternative splicing to generate variants that are competitive inhibitors.

Interleukin-2 (IL-2) is a 15–18-kDa glycosylated protein produced by activated T cells (1, 2). The IL-2 molecule has a four α helix up-up-down-down configuration (3–6), making it a member of the IL-4-related cytokine family (7). Among its many functions (reviewed in Ref. 8), IL-2 is an autocrine growth factor for T cells and supports the development of cytotoxic T cells. It stimulates B cell differentiation and immunoglobulin secretion. Interleukin-2 enhances monocyte cytotoxicity, increases phagocytosis and proliferation of macrophages, and stimulates natural killer cell proliferation and cytolytic activity.

The magnitude of a cellular immune response is dependent in part upon the amount of IL-2 secreted by T cells (9, 10). Cellular responses to IL-2 depend upon expression of specific cell surface receptors. Interleukin-2 receptors of different affinities are formed by combinations of α (p55), β (p70), and γc (p64) chains (11, 12). The α chain alone is the low affinity receptor, with K\textsubscript{d} of 10\textsuperscript{-5}M (13, 14). The βγc heterodimer is an intermediate affinity receptor, with K\textsubscript{d} of 10\textsuperscript{-8}M(15). Inclusion of the α chain into an α/βγc heterotrimer makes a high affinity receptor, with K\textsubscript{d} of 10\textsuperscript{-11}M (16). The formation of high affinity IL-2 receptors is regulated primarily through induction of the α chain, which turns over rapidly (17).

Increased IL-2 activity is thought to contribute to pathology in certain infectious diseases (18), leukemias, lymphomas, and solid tumors (19–24), autoimmunity (25, 26), and graft rejection (27–29). Because of therapeutic potential, efforts have been made to inhibit IL-2 function. These efforts include creation of genetically engineered mutant IL-2 molecules (30–32) and use of monoclonal antibodies to block IL-2 binding to the α chain of high affinity receptor (29, 33).

In this study, we explore another potential mechanism of regulating IL-2 function, one that occurs naturally in vivo. This study is based on our previous observation that alternative splicing is used to generate an inhibitory variant of human IL-4, called IL-4\textsubscript{d2} (34, 35). In IL-4\textsubscript{d2}, exon 2 of IL-4 is omitted by alternative splicing. This IL-4 variant has little IL-4 agonistic effects, but inhibits IL-4 costimulation of T cell proliferation, through competitive binding to IL-4 receptors (34). Because of homology between IL-4 and IL-2 proteins and receptors (7), we tested whether alternative splicing was also used to create competitive inhibitors of IL-2. We report that alternative splicing is used to create two variants of IL-2. One variant, called IL-2\textsubscript{d2}, omits exon 2. The other variant, IL-2\textsubscript{d3}, omits exon 3. Both inhibit IL-2 binding to high affinity IL-2 receptors.

EXPERIMENTAL PROCEDURES

Isolation and Activation of Peripheral Blood Mononuclear Cells (PBMC)—Human PBMC were isolated by Ficoll-Hypaque density centrifugation of heparinized blood samples from healthy adult volunteers. To activate T cells within the PBMC, the PBMC were cultured at 10⁶ cells/ml in 1-ml cultures in complete tissue culture medium containing a final concentration of 1% anti-CD3 monoclonal antibody (OKT3 hybridoma, American Type Culture Collection, Rockville, MD). Complete tissue culture medium was RPMI 1640 containing 10% heat inactivated fetal bovine serum, 10 mM HEPES, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 5 × 10⁻⁵ M 2-mercaptoethanol, and 5 mg/ml gentamicin sulfate. The cultures were incubated for the desired time (6 h to 6 days) at 37°C in a 5% CO₂ humidified air atmosphere. These activated PBMC were used for RNA isolation, assays of IL-2 costimulation of T cell proliferation, and IL-2 receptor binding studies.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) with IL-2-Specific Primers—Acid guanidinium thiocyanate/phenol/chloroform extraction (36) was used to isolate total cellular RNA from PBMC activated with OKT3 monoclonal antibody for 6 h. One μg of RNA was denatured for 5 min at 65°C with 1 μl of random primers (Life Technologies, Inc.) in sterile H₂O in 11-μl total volume. Reverse transcription of RNA into cDNA was done in a 20-μl reaction containing First Strand Buffer (Life Technologies, Inc.), 10 μM dithiothreitol, 0.5 μM each dATP, dCTP, dGTP, and dTTP, 200 units Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.), and 40 units of RNasin (Promega, Madison, WI), for 1 h at 37°C.

Three μl of this cDNA mixture were subjected to PCR amplification in a 25-μl mixture containing 0.83 units Taq polymerase, PCR Buffer II, 5 mM MgCl₂ (all from Perkin-Elmer), 0.4 μM each dATP, dCTP, dGTP, and...
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dTTP, and 0.6 mM each 3′ and 5′ PCR oligonucleotide primers. The PCR mixtures contained 200 ng of purified DNA at 95°C for 5 min, primer annealing at 55°C for 2 min, and primer extension at 72°C for 2 min, with a final extension at 72°C for 7 min. Two sets of IL-2 primers were used for this PCR amplification. IL-2 primer pair A was exon 1 forward primer 5′-ATG TAG ACC TGA CAA CTC CTG TCT T-3′ and exon 4 reverse primer 5′-GTC TGT GAA TGA GAT-3′. IL-2 primer pair B was exon 1 forward primer 5′-CTC GCC ACA ATG-3′ and exon 4 reverse primer 5′-CAG TGT GAA TGA GAT-3′. In some experiments, the forward primer was 5′ end-labeled with [γ-32P]ATP and 15 U T4 kinase (both from Amersham Life Sciences, Inc.), following the manufacturer’s protocol.

For cloning of IL-2, IL-2, and IL-23, nested PCR was done. The PCR products were first generated with IL-2 primer pair B and then subjected to a second amplification through reverse transcription with primers exon 1 forward primer 5′-GAA TTC GCA CCT ACT TCA AGT TCT ACA-3′ and IL-2 primer pair C. This IL-2 primer pair was exon 1 forward primer 5′-GAA TTC GCA CCT ACT TGA ACT TCA AGT TCT ACA-3′ and exon 4 reverse primer 5′-GGC GCC CCG TTA AGT CAG TGT GAA TGA GAT-3′. The underlined nucleotides encode an EcoRI restriction site (forward primer) and a BglII restriction site (reverse primer). The PCR products were removed from agarose gel and diluted to an amount which allowed amplification for 35 cycles, with denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and primer extension at 72°C for 3 min, with final extension at 72°C for 7 min.

Oligonucleotide Hybridization—RT-PCR amplification products generated with IL-2 primer pair A were size-separated by gel electrophoresis in 2.5% agarose. The gels were soaked sequentially in denaturation solution (1.5 M NaCl, 0.5 M NaOH) and neutralization solution (1.5 M NaCl, 1 M Tris-HCl, pH 7.4) for 30 min each. The RT-PCR amplification products were next transferred to nylon membranes by blotting overnight in 20 × standard saline citrate (SSC) buffer. The DNA samples were cross-linked to the membrane by ultraviolet light irradiation. Membranes were pre-hybridized in 6 × SSC, 10 × Denhardt’s solution, 0.1% SDS, and 50 μg/ml salmon sperm DNA for 1 h at 42°C. The membrane was hybridized overnight with 0.2 μg of 32P-labeled oligonucleotide probe at 49°C in 6 × SSC and 1% SDS. The oligonucleotide probe for the IL-2 exon 1-exon 3 junction was 5′-GGA ATT AAT GCC ACA GAA C-3′. This probe was 5′ end-labeled with [γ-32P]ATP, using a random primer labeling system (Life Technologies, Inc.). The membrane was washed three times in 6 × SSC, 1% SDS for 10 min at room temperature, followed by a final wash at 49°C for 20 min. The membrane was subjected to autoradiography.

Cloning of IL-2, IL-2, and IL-23 cDNAs—IL-2, IL-2, and IL-23 RT-PCR amplification products were generated by nested PCR with IL-2 primer pairs B and C. These products were cloned individually into the pCR® II vector (Invitrogen Corp.), according to the manufacturer’s directions. The IL-2, IL-2, and IL-23 inserts were separated from the pCR® II vector by digestion of the plasmid DNA with EcoRI and NotI restriction enzymes (Life Technologies, Inc.). The digested DNA was subjected to gel electrophoresis through 1.2% low melting point agarose gel, and cDNAs were isolated from gel slices using Glastrac/GS QuickKit. After ethanol precipitation, the IL-2, IL-2, and IL-23 cDNAs were ligated overnight with T4 DNA ligase into the pPIC9 plasmid (Invitrogen Corp., San Diego, CA), according to the manufacturer’s directions. The IL-2, IL-2, and IL-23 inserts were ligated into the Gibco (Life Technologies, Inc.) vector using the pPIC9 cloning kit (Amersham Life Sciences, Inc.), according to the manufacturer’s directions.

Expression, Detection, and Partial Purification of Recombinant Human (rh) IL-2, IL-2, and IL-23—The pPIC9 plasmids containing IL-2, IL-2, and IL-23 were used individually to transform the pro tease-deficient strain SMD1168 of Pichia pastoris yeast (Invitrogen Corp.), according to the manufacturer’s directions. The resulting Mut′ or Mut+ transformed P. pastoris clones were identified and selected. To confirm that IL-2, IL-2, or IL-23 cDNAs had integrated into the Pichia genome, DNA was isolated from transformed clones using Easy-DNATM kit (Invitrogen Corp.). Amplification by PCR of IL-2, IL-2, and IL-23 cDNAs was done with the 5′ and 3′ AOX1 primers included in the Easy-DNATM kit, according to the manufacturer’s directions. Amplification of DNA of the expected size confirmed integration of the IL-2, IL-2, and IL-23 cDNAs into the genome.

Protein expression was induced by culturing the transformed yeast in medium containing 1% methanol for 24 h at 30°C, according to the manufacturer’s instructions. Protein expression by several transformed Mut′ and Mut+ P. pastoris clones was compared to identify clones producing high levels of IL-2, IL-2, or IL-23 protein. For SDS-polyacrylamide gel electrophoresis, 50 μl of yeast supernatant were denatured by heating at 100°C for 10 min, loaded onto 10–20% gradient gel (Bio-Rad), and subjected to electrophoresis in Laemmlibuffer. These gels were stained using a silver stain kit (Sigma) or transblotted onto nitrocellulose membrane (Amersham Life Sciences, Inc.) at 90 V and 0.25 A for 4 h. Membranes were subjected to Western blotting with polyclonal rabbit anti-human IL-2 antibody (Genzyme Corporation, Cambridge, MA) at 1:10,000 dilution. Western blotting detection reagents from Amersham Life Sciences were used for membrane blocking, washing, and development.

Concentration and partial purification of rhIL-2, rhIL-2, and rhIL-23 was done. For each protein, 1 liter of yeast supernatant was concentrated to 50 ml by filtration through UltraTrapeze 3K filter (Filtron Technology Corp., Northborough, MA). The concentrated IL-2, IL-2, and IL-23 preparations were diluted with 1 liter of 0.15 M phosphate-buffered saline, pH 7.4. These preparations were again concentrated 20-fold through UltraTrapeze 3K filters to exchange buffers, followed by filtration through 200 K OMEGA membranes (Filtron Technology Corp.) to remove unbound protein. The concentrated rhIL-2, rhIL-2, and rhIL-23 protein was then used for membrane binding assays. The concentration of rhIL-2, rhIL-2, and rhIL-23 was determined using the Sequenase v.2.0 DNA sequencing kit (Amersham Life Sciences). The concentrated IL-2, IL-2, and IL-23 was measured with a 1205 Betaplate liquid scintillation counter (LKB-Wallac). Nonspecific binding, defined as residual cpm of 125I-IL-2 (DuPont NEN) was added to cultures alone or in combination with rhIL-2, rhIL-2, or rhIL-23 alone, or with rhIL-2 in combination with rhIL-2 or rhIL-23. Cells were incubated at 37°C in a 5% CO2 humidified air atmosphere. After 3 days, the cultures were pulsed with 1 μCi of [3H]thymidine (DuPont NEN), incubated overnight, and then harvested with a 1295 Cell Harvester (LKB-Wallac, Turku, Finland). Tritiated thymidine incorporation was measured with a 1205 Betaplate liquid scintillation counter (LKB-Wallac). The mean ± standard deviation (S.D.) of cpm of quadruplicate cultures was determined. Cell viability was assessed by trypan blue dye exclusion.

For any cell proliferation assay, preparations were dialyzed extensively against RPMI 1640 tissue culture medium.

T Cell Costimulation Assay—PBMC were isolated and stimulated with OKT3 monoclonal antibody, as described above. After 6 days, the mononuclear cells were washed twice and resuspended in complete tissue culture medium without OKT3 monoclonal antibody. The cells were cultured at 105 cells per 100-μl culture in 96-well microtiter plates (Falcon/Becton-Dickinson Labware, Oxnard, CA) in complete tissue culture medium alone, or with rhIL-2, rhIL-2, or rhIL-23 alone, or with rhIL-2 in combination with rhIL-2 or rhIL-23. Cultures were incubated at 37°C in a 5% CO2 humidified air atmosphere. After 3 days, the cultures were pulsed with 1 μCi of [3H]thymidine (DuPont NEN), incubated overnight, and then harvested with a 1295 Cell Harvester (LKB-Wallac, Turku, Finland). Tritiated thymidine incorporation was measured with a 1205 Betaplate liquid scintillation counter (LKB-Wallac). The mean ± standard deviation (S.D.) of cpm of quadruplicate cultures was determined. Cell viability was assessed by trypan blue dye exclusion. Cell numbers were determined using a hemocytometer.

Binding Studies—PBMC were induced to express high affinity IL-2 receptors by stimulation with OKT3 monoclonal antibody for 6 days (37). The activated mononuclear cells were washed twice with RPMI 1640, pH 3, at 4°C to remove endogenously produced IL-2 that was bound to IL-2 receptors on the cell surface (37). The cells were then washed and resuspended at 105 cells/ml in 1-ml cultures in complete tissue culture medium. To measure IL-2 binding to these cells, 10 pg 125I-IL-2 (DuPont NEN) was added to cultures alone or in combination with serial dilutions of unlabeled rhIL-2, rhIL-2, or rhIL-23. The unlabeled rhIL-2, rhIL-2, and rhIL-23 were always added 5 min before the 125I-IL-2. The cells were incubated for 1 h at 4°C with gentle shaking on a platform shaker. The cells were collected by centrifugation, washed twice in 5 μl of Hanks’ balanced salt solution containing 2% bovine serum albumin at 4°C, and transferred to a clean tube. Bound 125I-IL-2 was measured using a 1272 CliniGamma automatic γ counter (LKB-Wallac). Nonspecific binding, defined as residual cpm of cells incubated with 125I-IL-2 in the presence of 200-fold molar excess of unlabeled rhIL-2, was subtracted from all data points to determine the amount of specific binding. The results were presented as the pmol of specifically bound 125I-IL-2 in quadruplicate cultures.

RESULTS

Alternative Splicing Is Observed for Human IL-2 mRNA—We had previously identified an inhibitory variant of IL-4 in which exon 2 is omitted by alternative splicing (34, 35).
Fig. 1. Detection of two IL-2 mRNA species. Total cellular RNA was extracted from human PBMC stimulated for 6 h with OKT3 monoclonal antibody. This RNA was subjected to RT-PCR using IL-2 primer pair A. A. The 5’ PCR primer was end-labeled with 32P, and the RT-PCR amplification products were subjected to gel electrophoresis in a 6% polyacrylamide gel. Two PCR products were identified, a major band of 458 bp and a minor band of 398 bp. B. The same RT-PCR products were size-separated by polyacrylamide gel electrophoresis, transferred to a nylon membrane by blotting, and hybridized with an IL-2 exon 1-exon 3 junction probe. Lane 1 contains molecular weight markers, and lane 2 contains RT-PCR products. The smaller 398-bp band (IL-2-2) preferentially hybridizes with the probe.

Because IL-4 and IL-2 are members of the same multigene family, we examined IL-2 mRNA to determine whether alternative splicing was also used to produce a variant that is missing exon 2. Total RNA was isolated from human PBMC that were stimulated for 6 h with OKT3 monoclonal antibody. The RNA was subjected to RT-PCR amplification using IL-2 PCR primer pair A. Two RT-PCR amplification products were identified for IL-2 (Fig. 1A). The size of the larger amplification product was estimated at 458 bp, which corresponded to the size of native IL-2 cDNA (38, 39). The size of the smaller amplification product was estimated at 398 bp, the predicted size of an alternatively spliced variant of IL-2 lacking exon 2. This product was named IL-2-2.

To further test for the presence of an alternative splice variant that omitted exon 2, IL-2 RT-PCR products were size-separated by gel electrophoresis, transferred to a nylon membrane, and hybridized with an IL-2 exon 1-exon 3 junction probe (Fig. 1B). Two bands were detected with this probe. The 458-bp product represents native IL-2 cDNA, whereas the 398-bp band represents IL-2-2 cDNA. The probe preferentially hybridized to IL-2-2 rather than complete IL-2 cDNA (Fig. 1B).

Identification of IL-2-2 and IL-2-3 as Two Alternative Splice Variants of IL-2—To further identify alternative splice variants of IL-2, total cellular RNA was extracted from human PBMC stimulated for 6 h with OKT3 monoclonal antibody. The RNA was subjected to RT-PCR using IL-2 primer pair B. The amplification products were subjected to gel electrophoresis in low melting point agarose. An amplification product of the predicted size of IL-2 mRNA was visualized using ethidium bromide staining. The agarose gel containing the IL-2 amplification product and the gel underneath were sliced into four 0.5-mm horizontal slices. DNA was extracted from each slice and subjected to a second round of PCR with IL-2 primer pair C. The amplification products were again size-separated by agarose gel electrophoresis and visualized with ethidium bromide staining (Fig. 2). In addition to the expected IL-2 amplification product of 416 bp (Fig. 2, lanes 3–6), an amplification product of 356 bp was seen (Fig. 2, lanes 5 and 6), corresponding to the IL-2-2 PCR product identified in Fig. 1. Unexpectedly, a third PCR product of 272 bp was seen (Fig. 2, lane 4). This product, which had the expected size of an alternative splice variant that was missing exon 3 (38), was named IL-2-3.

Sequence Analysis of IL-2, IL-2-2, and IL-2-3—The IL-2, IL-2-2, and IL-2-3 cDNAs were cloned into pPIC9 plasmid and their DNA sequence determined. The IL-2-2 cDNA sequence consisted of IL-2 exons 1, 3, and 4, with exon 1 spliced directly to exon 3 without frameshift or nucleotide errors (Fig. 3, first panel). Sequence analysis of IL-2-3 cDNA showed IL-2 exons 1, 2, and 4, with exon 2 spliced directly to exon 4 without frameshift or nucleotide error (Fig. 3, third panel). Sequence analysis of IL-2 cDNA isolated, cloned, and sequenced in parallel with IL-2-2 and IL-2-3 cDNAs demonstrated the expected presence of exons 1, 2, 3, and 4 (Fig. 3, 2nd and 4th panels).

Expression and Characterization of rhIL-2, rhIL-2-2, rhIL-2-3 Proteins—Recombinant human IL-2, rhIL-2-2, and rhIL-2-3 in pPIC9 plasmids were used individually to transform P. pastoris yeast strain SMD1168. Expression of each protein was induced by culturing a transformed P. pastoris clone in 1% methanol. Yeast supernatants were prepared as described above and subjected to SDS-polyacrylamide gel electrophoresis through a 10–20% gradient gel (Fig. 4A). Silver staining of the gel showed single bands of proteins of the expected sizes, with IL-2 approximately 15 kDa, IL-2-2 approximately 13 kDa, and IL-2-3 approximately 10 kDa (Fig. 4A, lanes 1, 2, and 3, respectively). Few other proteins were in these yeast supernatants, because P. pastoris secretes few proteins of its own (40). The yeast supernatants were also subjected to Western immunoblotting analysis with polyclonal rabbit anti-human IL-2 antibody. The IL-2, IL-2-2, and IL-2-3 proteins were all recognized by this antibody (Fig. 4B).

Ability of rhIL-2, rhIL-2-2, and rhIL-2-3 to Costimulate T Cell Proliferation—IL-2 is a potent costimulator of T cell proliferation (1, 2, 8). Experiments were designed to determine whether IL-2-2 and IL-2-3 had similar functional effects. Commercial rhIL-2 made in Escherichia coli (Life Technologies, Inc.) and our rhIL-2, rhIL-2-2, and rhIL-2-3 preparations were tested for ability to costimulate proliferation of activated human PBMC. The PBMC were stimulated for 6 days with 1% OKT3 monoclonal antibody to activate T cells and then washed twice. These cells were cultured for 3 days in complete tissue culture medium alone, with commercial rhIL-2, or with rhIL-2, rhIL-2-2, or rhIL-2-3. [3H]Thymidine was added during the last 12 h of culture. Commercial rhIL-2 and our rhIL-2 stimulated similar degrees of proliferation (Fig. 5). In contrast, rhIL-2-2 and rhIL-2-3 did not stimulate proliferation at similar concentrations (Fig. 5) or even at higher concentrations up to 500 pM (data not shown).

Ability of rhIL-2 and rhIL-2-3 to Inhibit IL-2 Costimulation of T Cell Proliferation—Next, studies were done to test whether rhIL-2-2 or rhIL-2-3 could inhibit IL-2 costimulation of T cell proliferation. Again, PBMC were stimulated for 6 days with 1% OKT3 monoclonal antibody to activate T cells and washed twice. The cells were cultured in complete tissue cul-
Sequence analysis of IL-2 exon 3, in frame (the presence of IL-2 exons 1, 3, and 4, with exon 1 spliced directly to exon 3, in frame (Fig. 6). Similar inhibitory effects of rhIL-2 serum albumin prepared in an identical manner had no effect of IL-2 to cause T cell proliferation (Fig. 6). Human PBMC were incubated in complete tissue culture medium with titrated doses of rhIL-2 and rhIL-23 (up to 500 pM), in quadruplicate 1-ml cultures. Cell viability and numbers were determined after 3 days. There was no increase in percent dead cells nor any reduction in total numbers of cells in cultures containing rhIL-2 or rhIL-23, compared with PBMC incubated with media alone (data not shown). Thus, the inhibitory effects of rhIL-422 and IL-23 do not appear to be from nonspecific toxicity.

Inhibition of Binding of 125I-IL-2 to High Affinity IL-2 Receptors by IL-23 and IL-23—Experiments were done to determine if IL-23 and IL-23 could inhibit the binding of radiolabeled rhIL-2 to cells, indicating these proteins bind similar receptors. Human PBMC were activated by incubation for 6 days with 1% OKT3 monoclonal antibody, to induce high affinity IL-2 receptors. Then the PBMC were washed with RPMI 1640, pH 3, at 4 °C to remove endogenously produced IL-2 that bound to IL-2 receptors during the culture period (37). The activated cells were washed and incubated with medium alone, with 10 pM 125I-IL-2 alone, or with 10 pM 125I-IL-2 plus rhIL-22 or rhIL-23 in concentrations from 10 pM to 10 nM. Residual nonspecific binding of 125I-IL-2 in the presence of 2 nM unlabelled IL-2 was subtracted from data points to determine specific binding. Titrated doses of rhIL-2 and rhIL-23 inhibited specific binding of 125I-IL-422, in a dose-dependent manner (Fig. 7). In contrast, the human serum albumin preparation had no effect.

**DISCUSSION**

This report shows that alternative splicing is used to produce splice variants of human IL-2, called IL-2222 and IL-233. The nucleotide sequence of IL-2222 is otherwise identical to IL-2 (38, 39) throughout the entire protein encoding region, with IL-2 exons 1, 3, and 4 spliced in an open reading frame. In parallel, the nucleotide sequence of IL-233 is identical to IL-2 throughout the entire protein encoding region, with IL-2 exons 1, 2, and 4 spliced in an open reading frame. We report that PBMC activated with anti-CD3 monoclonal antibody produce RNA for IL-2, IL-222, and IL-233. Unlike rhIL-2, neither rhIL-22 nor rhIL-23 is otherwise identical to IL-2 (38, 39) throughout the entire protein encoding region, with IL-2 exons 1, 3, and 4 spliced in an open reading frame. In parallel, the nucleotide sequence of IL-233 is identical to IL-2 throughout the entire protein encoding region, with IL-2 exons 1, 2, and 4 spliced in an open reading frame. We report that PBMC activated with anti-CD3 monoclonal antibody produce RNA for IL-2, IL-222, and IL-233. Unlike rhIL-2, neither rhIL-22 nor
rhIL-2 are effective costimulators of T cell proliferation. In contrast, both show dose-dependent inhibition of IL-2 costimulation of T cell proliferation. Both inhibit binding of radiolabeled rhIL-2 to cells expressing high affinity IL-2 receptors. These results indicate that IL-282 and IL-283 are competitive inhibitors of IL-2.

This report adds to our previous finding that alternative splicing is used to generate an inhibitory variant of human IL-4, called IL-482 (34, 35). Thus, both IL-4 and IL-2, which share protein structure, receptor structure, and even the γC chain of their high affinity receptors (7, 41, 42), use the same mechanism to create natural competitive inhibitors. Our previous experiments suggest that other members of the IL-4-related cytokine family, human IL-3, IL-5, IL-13, and granulocyte macrophage-colony stimulating factor, do not use alternative splicing to delete exon 2 (35). At this time, it is possible to distinguish between complete IL-2, IL-282, and IL-283 RNAs with RT-PCR techniques that use primer pairs specific for exon 1 and exon 4, combined with size separation of the amplified products. Discrimination among the three proteins may require antibodies specific for epitopes that include exon 2 (complete IL-2), the exon 1-exon 3 junction (IL-282), and the exon 2-exon 4 junction (IL-283).

Information about the intron-exon structure of the IL-2 gene (38), the structure of the IL-2 molecule (3–6), and areas of interaction with the α, β, and γC receptor chains (43–51) is invaluable when trying to predict how IL-282 and IL-283 might interact with IL-2 receptors. The protein structure of IL-2 is four left-handed α helices in an up-up-down-down configuration (4–6). Exon 1 of IL-2 encodes Ala1–Asn29, which forms a short strand plus helix A. Exon 2 encodes Asn30–Lys49, which forms a connecting strand with short α helix and β-pleated sheet. Exon 3 encodes Ala50–Lys97, which forms helix B and connecting strand, and helix C. Helix B + B’ is flexible because of proline at position 65 (3). Exon 4 encodes Gly98–133. This makes a short connecting strand, a β-pleated sheet, and helix D. Voss et al. (51) have articulated the following model of IL-2 interactions with the high affinity IL-2 receptor. Helix A contacts the β chain of the IL-2 receptor. The minor α helix and connecting strand encoded by exon 2 contact the α chain and then loop around the γC chain of the IL-2 receptor. Helix B + B’, the short connecting strand, and helix C encoded by exon 3 do not contact the IL-2 receptor directly but lie on top of the contact residues. Helix D lies in a groove between the β and γC chains and is important in engagement of the γC chain.

Given the above information, at a minimum, omission of exon 2 in IL-282 should alter binding to the α chain of the IL-2 receptor. Engagement of the α chain of the high affinity receptor is needed for both high affinity binding and optimal cell triggering (49, 52). Thus, IL-282 may serve as a competitive inhibitor of IL-2 binding to high affinity receptors by engaging the β and/or γC chains but not the α chain.

Exon 3 encodes the face of the IL-2 molecule away from the putative contact residues (51). Point mutations in human exon 3 at Glu282 lose ability to bind the α chain (50). Thus, loss of exon 3 may allow binding to the IL-2 receptor but may interfere with binding to the α chain. Exon 3 also encodes Cys348. A disulfide bond between Cys358 and Cys104 stabilizes the IL-2 structure and is necessary for IL-2 activity but is unnecessary for IL-2 binding to the receptor (43). Loss of Cys358 may lead to mismatched disulfide binding (43).

The prediction that IL-282 and IL-283 will serve as competitive inhibitors because of failure to engage the α chain of the high affinity IL-2 receptor is compatible with the data presented in this report. Neither rhIL-282 nor rhIL-283 had sig-
nificant agonist activity in a T cell costimulation assay, which involves the high affinity IL-2 receptor (16) and requires engagement of the α chain for optimal activity (49, 52). Both alternative splice variants inhibited IL-2 costimulation of T cell proliferation and IL-2 binding to high affinity receptors. Interaction of IL-2 with the high affinity IL-2 receptor on PBMC or peripheral blood lymphocytes is also necessary for maximal secretion of IL-1β, tumor necrosis factor-α, tumor necrosis factor-β, and interferon-γ (30). Therefore, if both IL-2β and IL-2γ fail to engage the α chain, they then also would be expected to have little agonist effect and serve as competitive inhibitors of IL-2 stimulation of cytokine secretion from activated PBMC. Of importance, failure of IL-2β and IL-2γ to engage the α chain should not reduce the ability of the α chain to form the high affinity receptor. The α chain functions of engagement of IL-2 and formation of high affinity receptors are distinct (53, 54).

Engagement of the high affinity IL-2 receptor is not always required for every biologic effect of IL-2 (31, 52–55). In humans, the intermediate affinity βγ2 IL-2 receptor is responsible for induction of natural killer and lymphokine-activated killer cells’ proliferation and cytolytic activity (31, 32). Similarly, binding of IL-2 to intermediate affinity receptors is sufficient to stimulate proliferation of γδ T cells (56). Engagement of the intermediate affinity IL-2 receptor is also sufficient to stimulate lymphocyte locomotion and expression of CD69, the IL-2 receptor β chain, and HLA-DR (57). These examples lead us to speculate that a potential role for these IL-2 splice variants in vivo is to selectively inhibit cellular responses triggered through the high affinity IL-2 receptor, while leaving intact immunity provided by activated natural killer cells and γδ T cells.

Because IL-2β and IL-2γ are naturally occurring IL-2 antagonists, they may be useful therapeutically to inhibit cell growth or functions stimulated through high affinity IL-2 receptors or to target IL-2 receptor-expressing cells. They would provide alternatives to use of synthetic IL-2 mutants and anti-IL-2 receptor antibodies. Because IL-2β or IL-2γ are naturally occurring, immune responses against them should not develop. This would be an advantage over the use of synthetic mutants or nonhumanized anti-IL-2 receptor antibodies. When conjugated to toxins, they could be used as a nonactivating means of directing toxins to cells expressing high affinity IL-2 receptors. Induction of cytokine secretion by IL-2, which requires engagement of the α chain of the high affinity receptor (30), mediates the toxicity associated with IL-2-based immunotherapies. Thus, these naturally occurring mutants might have the added advantage of less toxicity.

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