Structure-Function Studies of Interleukin 15 using Site-specific Mutagenesis, Polyethylene Glycol Conjugation, and Homology Modeling*

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Interleukin (IL)-15 is a multifunctional cytokine that shares many biological activities with IL-2. This functional overlap, as well as receptor binding subunits shared by IL-15 and IL-2, suggests tertiary structural similarities between these two cytokines. In this study, recombinant human IL-15 was PEGylated via lysine-specific conjugation chemistry in order to extend the circulation half-life of this cytokine. Although PEGylation did extend the β-elimination circulation half-life of IL-15 by greater than 50-fold, the biological activity of polyethylene glycol (PEG)-IL-15 was significantly altered. Specifically, PEG-IL-15 lost its ability to stimulate the proliferation of CTLL, but took on the properties of a specific IL-15 antagonist in vitro. In comparing sequence alignments and molecular models for IL-2 and IL-15, it was noted that lysine residues resided in regions of IL-15 that may have selectively disrupted receptor subunit binding. We hypothesized that PEGylation of IL-15 interferes with β but not α receptor subunit binding, resulting in the IL-15 antagonist activity observed in vitro. The validity of this hypothesis was tested by engineering site-specific mutants of human IL-15 as suggested by the IL-15 model (IL-15D8S and IL-15Q108S block β and γ receptor subunit binding, respectively). As with PEG-IL-15, these mutants were unable to stimulate CTLL proliferation but were able to specifically inhibit the proliferation of CTLL in response to unmodified IL-15. These results support our model of IL-15 and confirmed that interference of β receptor subunit binding by adjacent PEGylation could be responsible for the altered biological activity observed for PEG-IL-15.

IL-15 and IL-2 share many biological activities, including the ability to stimulate T cells, NK cells, and activated B cells (1–5). The overlapping biological activities of IL-15 and IL-2 can be explained, at least in part, by the composition of the multisubunit receptor complexes for these two cytokines. The high affinity IL-2R complex consists of at least three chains, designated α, β, and γ, and interaction of IL-2 with both the β and γ chains is necessary for initiation of signaling events (6). The IL-15 receptor complex also consists of at least three subunits. It shares use of the common β and γ chains of the IL-2R complex but contains an α subunit that is distinct from IL-2Ra (3, 7, 8). Similar to the IL-2R complex, interaction of IL-15 with both the β and γ subunits is necessary for initiation of signaling. The IL-2R and IL-15R α subunits share significant structural homology (8); however, there is at least one distinct difference in the interaction of these subunits with their respective ligands. The IL-2Ra subunit binds IL-2 with very low affinity (Kd = 109), whereas the IL-15Ra subunit binds IL-15 with unusually high affinity (Kd = 1011). This difference suggests that IL-15 may have in vivo functions distinct from those it shares with IL-2.

Mutagenesis studies with recombinant human IL-2 have indicated several regions and key amino acid residues involved in α, β, and γ receptor subunit binding. In particular, mutation of mature IL-2 residue Lys35, Arg38, Thr42, or Lys43 reduces or blocks IL-2Ra subunit binding (9–11); mutation of residue Asp20 inhibits β receptor subunit binding and signal transduction (10, 12, 13), and mutation of residue Gln126 blocks γ receptor subunit binding and signaling (12–14). Mutagenesis studies with murine IL-2 have demonstrated analogous regions of amino acids involved in α, β, and γ receptor subunit binding (15).

Whereas protein mutagenesis allows for specific investigation of protein structure to function relationships, other protein modification techniques may also influence specific protein-ligand interactions. The process of conjugating chains of polyethylene glycol (PEG) onto proteins such as IL-2, granulocyte-macrophage colony-stimulating factor, asparaginase, immunoglobulins, hemoglobin, and others has traditionally been used to prolong circulation half-lives in vivo, enhance solubility, and reduce immunogenicity (16–18). However, PEGylation has also been found to reduce or alter biological activities, the extent of which depends on the degree of conjugation, the type of conjugation chemistry used, and the specific location on the protein where the conjugation has occurred. The independent variation of two biological activities has also been demonstrated with immunoglobulin and granulocyte-macrophage colony-stimulating factor by selectively PEGylating regions of these proteins involved with one biological function while not interfering with the other (19, 20). These examples likely demonstrate the importance of the location of lysine residues within the tertiary protein structure when using lysine-specific PEG conjugation chemistry.

In this paper we describe the PEGylation of IL-15 and the in vivo and in vitro characterization of this molecule. Whereas the
**PEGylation and Mutagenesis of IL-15**

Pharmacokinetics were enhanced following PEGylation of IL-15, the biological activity of this molecule was significantly altered. Specifically, PEG conjugation eliminated the CTLL-2 proliferative activity of IL-15 and generated a molecule that functioned as a specific antagonist in vitro. We hypothesize that lysine-specific PEGylation of IL-15 resulted in steric interference of the region of IL-15 that interacts with the β receptor subunit, while not affecting the region of IL-15 that interacts with the α receptor subunit. This hypothesis is based on sequence alignment of IL-15 with IL-2 and the construction of a molecular model of IL-15. Two mutants of IL-15, designed to disrupt either β or γ receptor subunit binding, were prepared to verify the accuracy of these regions of the IL-15 model. The in vitro characterization of these IL-15 mutants was consistent with the predictions of the molecular model and supported the hypothesis concerning PEGylated IL-15. Furthermore, when taken together, the PEGylation and mutagenesis data support the model of IL-15 in which each of the receptor subunits, α, β, and γ bind to IL-15 in a spatial orientation similar to that established for IL-2.

**EXPERIMENTAL PROCEDURES**

Preparation of Proteins and PEG Conjugates—The cloning of simian IL-15 has been described (1) and the sequences for both human and simian IL-15 have been recently reported (21). All of the PEGylation studies described below were carried out with simian IL-15, and all of the mutagenesis studies were carried out with human IL-15. Human and simian IL-15 are 96% identical at the amino acid level, and the number and position of lysine residues are identical. Also, the binding characteristics and biological activity of human and simian IL-15 as measured by CTLL-2 cell proliferation are identical. Human IL-15 was used in all studies as the control material.

Briefly, for the expression of either human or simian IL-15, cDNA encoding IL-15 from either species was ligated into a yeast expression vector that directs secretion of the recombinant protein into the yeast medium (22). Supernatants were collected following ultracentrifugation of the yeast broth, and IL-15 was purified as described previously (21).

Site-specific mutants of hIL-15 (IL-15D8S and IL-15Q108S) were generated by two separate PCR amplifications. In the primary PCR reaction, amplification was with primer pairs that either introduced the appropriate mutation or amplified the wild-type sequence. In the secondary PCR reaction, material from the first round was reamplified with a primer set that introduced restriction sites necessary for cloning into the yeast expression vector. The mutagenic oligonucleotides had the sequence 5′-AATGTAATAAGTTCTTTGAAAAAAATT-3′ (Asp99→Ser, 5′ sense oligo), and 5′-GTTGATGACATAGACAATATG-3′ (Gln108→Ser, 3′ antisense oligo). PCR amplification was performed under standard conditions using Taq polymerase (Boehringer Mannheim) and cycling conditions of denaturation at 94 °C for 45 s, annealing at 45 °C for 45 s, and extension at 72 °C for 1 min for a total of 30 cycles, using approximately 10 ng of wild-type human IL-15 cDNA as template.

 Approximately 20 ng of gel purified protein from the primary amplification was used as the template for the secondary PCR amplification, which used nonmutagenic, flanking oligonucleotides that appended the necessary restriction enzyme sites. Cycling conditions were denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 1 min for a total of 20 cycles.

Amplification products were gel purified and digested with Asp718 (Boehringer Mannheim) and Ncol (New England Biolabs) and ligated into the 2-μm yeast expression vector pIXY456, which contains the ADH2 promoter and the α-factor leader. Ligations were transformed into Escherichia coli DH10b cells by electroporation. Plasmid DNA from single transformants was sequenced to confirm sequence integrity and used to transform S. cerevisiae X2181. Purification of the mutant proteins was carried out by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B (Pharmacia Biotech, Inc.). Recombinant human IL-2 (des-αα-1, Ser125 human interleukin-2, Aldesleukin, Proleukin®) was obtained from Chiron Corp. (Emeryville, CA).

PEG conjugation reactions were carried out with 5-kDa PEG, which was obtained in the activated form of succinimidyl carbonate PEG from Shearwater Polymers (Huntsville, AL). PEGylation of simian IL-15 was carried out in 50 mM NaH2PO4, pH 8.5. For PEGylation of IL-2, the addition of 0.1% SDS improved the solubility of IL-2 and the extent of PEGylation. Reactions with both IL-15 and IL-2 were carried out in 0.5-mL volumes at 100 μg/mL for in vitro characterization studies. Large volumes were prepared for in vivo pharmacokinetic experiments. Succinimidyl carbonate PEG was added to the reaction mixtures at molar ratios of PEG to lysine of 1:1, 3:1, 10:1, and 100:1 (7 lysine residues + 1 N terminus per IL-15 molecule and 11 lysine residues + 1 N terminus per IL-2 molecule). All reactions were allowed to proceed overnight at 4 °C. For subsequent in vivo pharmacokinetic experiments, PEGylated IL-15 was dialyzed against PBS to remove residual N-hydroxysuccinimide, a byproduct of succinimidyl carbonate PEG hydrolysis.

Protein concentrations of IL-15, PEG-IL-15, and PEG-IL-2 samples were determined by amino acid analysis.

Physical Characterization of PEG-IL-15 and PEG-IL-2—PEG-IL-15 and PEG-IL-2 were characterized by SDS-polyacrylamide gel electrophoresis, SE-HPLC, and MALDI-TOF mass spectrometry. In the chromatography experiments, a Waters HPLC system (Millipore Corp., Milford, MA) was equipped with two 300 × 8 mm SEC-250 Biosil columns (Bio-Rad), which were run in series in order to resolve the various PEG-modified species. For the mass spectrometry experiments, PEG-IL-15 (PEG to lysine ratio of 100 to 1) was first purified by SE-HPLC in order to reduce residual levels of unmodified IL-15 and other reaction byproducts. PEG-IL-15 samples were further prepared by buffer exchange with water and concentrated to approximately 1 mg/mL. PEG-IL-15 (0.5 μl) was applied to targets along with a sinapinic acid matrix (0.5 μl of 10 mg/ml solution in 50:50 acetonitrile/water), allowed to air dry, and then analyzed with a Finnigan Mat LaserMat time-of-flight laser desorption mass spectrometer (San Jose, CA).

Pharmacokinetics—Experiments were performed to compare the circulation half-lives of IL-15 and PEG-IL-15 (PEG:lysine ratio of 10:1). Groups (3 mice per group) of 10–12-week-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were injected intravenously with 10 μg of protein in a total volume of 150 μl. Following the injections, mice were sacrificed and blood samples were collected at 3, 5, 15, and 30 min and 1, 2, 4, 6, 10, 20, 40, and 48 h via cardiae puncture, and the plasma was analyzed by CTL2-2 competitive inhibition assay, CTL2-2 proliferation assay, and IL-15 ELISA. The ELISA used a murine monoclal antibody capture antigen (M111, Immunex designation) and a murine monoclonal antibody for detection (M112, Immunex designation). These antibodies were found to bind to both the PEGylated and unmodified forms of IL-15, but with different affinities. Therefore, amino acid analysis standards of both IL-15 and PEG-IL-15 were used as controls in the ELISA experiments. Pharmacokinetic parameters of the PEGylated and unmodified forms of IL-15 in the blood were determined and the apparent elimination rate constant (K) and half-life (t1/2) for each form of the molecule were calculated using a pharmacokinetics half-life program on a RS/1 system. The log linear portion of the blood concentration/time curve was used to calculate K with t1/2 determined as t1=K×ln2. In vivo half-life values are calculated as t1/2 = S.E., where S.E. indicates the standard error in fitting the log linear line to the data points in calculating the K value. The distribution (t DS) and elimination (t DS) half-lives were calculated using a biphasic pharmacokinetics program that related the respective log linear concentration/time curves to specific K and t values.

In Vitro CTL2-2 Bioactivity—Bioactivity of IL-15, IL-2, PEG-IL-15, PEG-IL-2, IL-15DS, and IL-15Q108S were assessed using a modified CTL2-2 cell [3H]thymidine incorporation assay (23). CTL2-2 competitive inhibition assays were performed by adding suboptimal amounts of IL-15 (final concentration, 50 pg/ml) or IL-2 (20 pg/ml) to all assay wells after serial dilution of samples and prior to addition of cells.

Sequence Alignment and Molecular Modeling—The amino acid sequences for hIL-15 and hIL-2 have been previously reported (21, 24). In spite of the overwhelming clues to the structural similarities between these cytokines based on their functional similarities and common receptor usage, the amino acid sequences show only weak sequence identities. This is not uncommon among members of the helical cytokine family, of which IL-2 is a member (25, 26). Those amino acid sequences that are conserved between IL-2 and IL-15 were used for alignment. In particular, amino acid residues of each cytokine presumed to define the α helical structure were aligned first. Secondary consideration was given to the conserved cysteine and other conserved residues found in the loop regions that connect each α helix. This alignment strategy required the placement of gaps in either the IL-2 or IL-15 sequence in the loop regions that connect the α helices.

A three-dimensional computer model of IL-15 was generated using J. Eisenman and C. Beers, unpublished results.
FOLDER, a distance geometry based homology modeling package (27, 28). For the model, a “template” sequence was defined that included the structurally conserved amino acid residues within the $\alpha$ helices as defined by other cytokines within the four-helix bundle cytokine family (25). Other amino acids included in the template sequence were short loop regions between or flanking the $\alpha$ helices. The IL-15 model was then generated using the template sequence and the established coordinates for IL-2 (PDB code 3ink) (29, 30). Amino acids in the template sequence of IL-2 were the only residues used to derive structural constraints between atoms of the corresponding amino acids in the IL-15 sequence. For all the other amino acids of IL-15, for which there are no corresponding amino acids in the template sequence, only chemical, steric and loop closure constraints were used to define spatial locations (27).

RESULTS

Physical Characterization of PEG-IL-15 and PEG-IL-2—SDS-polyacrylamide gel electrophoresis analysis and SE-HPLC were used to characterize and separate PEG-IL-15 and PEG-IL-2 species, respectively. Heterogeneous mixtures of PEG-IL-15 and PEG-IL-2 were produced, depending on the PEG:lysine ratio used in the conjugation reaction (Fig. 1). At high PEG:lysine ratios, significant smearing of the bands on the gels was observed. SE-HPLC was used to separate PEGylated species for further analysis (Fig. 2), and MALDI-TOF mass spectroscopy of PEG-IL-15 (PEG:lysine ratio of 100:1 in reaction mixture) confirmed at least seven distinct species ranging in size from 14,000 to approximately 50,000 kDa in intervals of about 5,000 kDa (Fig. 3).

Pharmacokinetics—A comparison of the circulating serum concentrations of IL-15 and PEG-IL-15 as measured by ELISA is shown in Fig. 4. Based on these data, pharmacokinetic half-lives were measured for IL-15 ($t_{1/2a} = 0.9 \pm 0.3$ min; $t_{1/2b} = 16.0 \pm 1.7$ min) and PEG-IL-15 ($t_{1/2a} = 87.6 \pm 8.2$ min; $t_{1/2b} = 2445 \pm 167$ min). Also, the circulation half-life of IL-15 was estimated by CTLL-2 proliferation assay ($t_{1/2a} = 1.0 \pm 0.7$ min; $t_{1/2b} = 16.5 \pm 4.7$ min), and the circulation half-life of PEG-IL-15 was estimated by CTLL-2 inhibition assay ($t_{1/2a} = 107 \pm 41$ min; $t_{1/2b} = 859 \pm 133$ min). PEG-IL-15 clearly was eliminated more slowly than un-PEGylated IL-15; $t_{1/2b}$ was slower by a factor of 52 (by CTLL-2 analysis) or 153 (by ELISA).

In Vitro Bioassay Characterization—CTLL-2 bioassays were used to measure the bioactivity of each of the molecules investigated in this study (Fig. 5). Biological activity was measurable for IL-2, IL-15, and PEG-IL-2 (PEG:lysine ratio of 100:1). Similar specific activities were measured for IL-15 and IL-2, whereas the bioactivity of PEG-IL-2 was lower than these by 2 orders of magnitude. No bioactivity could be measured for PEG-IL-15 (PEG:lysine ratio of 100:1), IL-15D8S, or IL-15Q108S. Nonconjugated PEG did not inhibit CTLL-2 proliferation at concentrations used in the PEGylated cytokine assays (data not shown). Intermediate bioactivities were measured for PEG-IL-15 and PEG-IL-2 with PEG:lysine ratios of 1:1, 3:1, and 10:1 (data not shown). The ability of PEGylated forms of IL-15 and IL-2 and mutated forms of IL-15 to specifically in-
hibit the bioactivity of unmodified IL-15 or IL-2 was measured. The specific inhibitory effects of PEG-IL-15, IL-15D8S, and IL-15Q108S on the biological activity of a standard solution of IL-15 (50 pg/ml) are clearly demonstrated (Fig. 6). No such inhibitory effect was found for PEG-IL-2 in this assay (Fig. 7).

It should be noted that the mutated forms of IL-15 were able to inhibit IL-15 binding at concentrations of approximately three orders of magnitude lower than those required to inhibit IL-15 binding with PEG-IL-15. A 2000–5000-fold molar excess of PEG-IL-15 (based on protein weight) was required to obtain 100% inhibition of IL-15 induced bioactivity, whereas only a 10–20-fold molar excess of IL-15D8S or IL-15Q108S was required to obtain 100% inhibition of IL-15 induced bioactivity. None of the cytokines investigated in this study were able to inhibit the biological activity of a standard solution of IL-2 (20 pg/ml) (Fig. 7).

Sequence Alignment of IL-15 and IL-2—The sequence alignment for huIL-15 and huIL-2 is shown in Fig. 8. A disulfide bridge is conserved between IL-15 (Cys42-Cys86) and IL-2 (Cys58-Cys105), and one disulfide bridge unique to IL-15 (Cys35-Cys85) has been proposed (1). The structural significance of the sequence alignment can be evaluated by the number and site of identities within the four blocks defining the four α helices. The conserved EFLXXXXXXQXXI (Xs are nonidentical amino acids) in the D-helix, the conserved CXXEL in B-helix, the conserved cysteine within the loop that connects the C-helix and D-helix, and conserved DLX in the A-helix further support the expected structural similarities between the two cytokines. Conservation of XDX in the A-helix and XQX in the D-helix also suggests similar receptor binding modes between these two cytokines because XDX and XQX of IL-2 are known to be involved in β and γ receptor binding, respectively. Furthermore, the level of identities found within the four helices are no lower than those between other helical cytokines that are known to belong to helical cytokine family using crystal structure analysis (25).

Molecular Modeling—Ribbon models of IL-2 and IL-15 are illustrated in Fig. 9, A–D. The side chains of amino acid residues of IL-2 that are known to influence binding to the α, β, and γ receptor subunits are highlighted in Fig. 9A. As a result
sequence alignment and spatial comparison between IL-2 and IL-15, residues on IL-15 hypothesized to interact with $\beta$ and $\gamma$ receptor subunits were identified (Asp$^8$ and Gln$^{108}$, respectively). The positions of single point mutations made for IL-15D8S and IL-15Q108S are illustrated in Fig. 9B. Potential PEGylation sites (lysine residues and the N terminus) are highlighted for IL-2 and IL-15 in Fig. 9C and D, respectively. In comparing these figures, it is evident that potential PEGylation sites are absent in the region of IL-15 that, by comparison with IL-2, may bind IL-15R$\alpha$. Also, potential PEGylation sites in the region of IL-15 which may bind IL-15R$\beta$ were noted. On the basis of these observations, we hypothesized that PEG-IL-15 (as well as IL-15D8S and IL-15Q108S) could still bind to IL-15R$\alpha$ but would not signal through the $\beta\gamma$ receptor complex.
DISCUSSION

Our initial goal for PEGylation of IL-15 was to extend its circulation half-life in a manner similar to that reported for IL-2 (31, 32). Toward this end, both IL-15 and IL-2 were PEGylated with lysine-specific, succinimidyl carbonate-activated PEG. The physical characteristics of PEG-IL-15 were found to be very similar to those of PEG-IL-2. Specifically, SDS-polyacrylamide gel electrophoresis and SE-HPLC demonstrated similar distinct banding patterns that were attributable to the number of PEG conjugates on each protein molecule (Figs. 1 and 2). Also, pharmacokinetic studies carried out in a murine system demonstrated a significant enhancement of the circulation half-life of PEG-IL-15 in comparison with unmodified IL-15 (Fig. 4), an observation similar to that reported in the literature for PEG-IL-2 (31).

However, despite the physical similarities of PEG-IL-15 and PEG-IL-2, the biological activity of IL-15 was significantly altered by PEGylation, whereas the activity of IL-2 was largely unaffected by PEGylation. Specifically, PEG-IL-15 was inactive in the CTLL-2 bioassay (Fig. 5) but was able to competitively inhibit the biological activity of unmodified IL-15 (Fig. 6). By comparison, the biological activity of PEG-IL-2 was only slightly reduced relative to unmodified IL-2 (Fig. 5).

In order to account for the discrepancies in the biological activity of PEG-IL-15 and PEG-IL-2, a critical analysis of the spatial orientation of the binding domains of each cytokine was carried out. The sequences of IL-15 and IL-2 were aligned (Fig. 8), and a molecular model of IL-15 was constructed (Fig. 9). On the basis of this model, we hypothesized that IL-15 and IL-2 could bind to their respective α subunits and to their and common β and γ receptor subunits through a similar spatial orientation of each cytokine. To test this hypothesis, mutagens were generated at positions Asp8 or Gln108, which by analogy with mutagenesis studies carried out on IL-2, would interfere with β or γ receptor subunit binding, respectively.

The resulting muteins, IL-15DS8 and IL-15Q108S, were tested for their biological activity in vitro. As with PEG-IL-15, each of the muteins was inactive in the CTLL-2 bioassay (Fig. 5) but was able to competitively inhibit the biological activity of unmodified IL-15 (Fig. 6). These results supported the model of IL-15 and confirmed our rationale for mutating IL-15 at positions Asp8 and Gln108. The antagonistic activity of IL-15DS8 and IL-15Q108S, as well as that of PEG-IL-15, could best be explained by the model. Disruption of binding to either the β or the γ receptor subunit would prevent signal transduction and cell proliferation, while still allowing competitive binding to the αβγ receptor complex through IL-15α. In further support of this conclusion, neither IL-15DS8 nor IL-15Q108S was able to inhibit the bioactivity of IL-2, which binds to a unique IL-2α subunit but signals through the common βγ receptor subunit complex (Fig. 7).

That PEGylation of IL-15 could disrupt β or γ receptor subunit binding is seen by the presence of two lysine residues at positions Lys10 and Lys11 (Fig. 9D). PEG conjugation to either of these residues could sterically interfere with β receptor binding to the region of IL-15 containing Asp8. Equally important in the IL-15 model is the observation that no lysine residues are present in the region hypothesized to bind to the α receptor subunit (approximately between residues 21 and 33). The closest lysine residues to the hypothesized α receptor subunit binding domain are Lys13 in helix A and Lys15 near the carboxyl end of the strand separating helix A and B.

In contrast to IL-15, no lysine residues exist on IL-2 in regions that would interfere with either β or γ receptor subunit binding, and two lysine residues (Lys35 and Lys43) are known to interact with the IL-15α subunit (Fig. 9C) (9–11). These observations are also consistent with the in vitro biology results in which PEG-IL-2 retains biological activity (Fig. 5) but is unable to act as a specific antagonist in vitro in the presence of unmodified IL-2 (Fig. 7).

For the bioassay and pharmacokinetic studies, we chose to use forms of PEGylated molecules that were heavily PEG-modified in order to avoid heterogeneous mixtures of non-PEG and PEG-modified proteins. However, even in mixtures that were highly PEGylated, characterization by MALDI-TOF mass spectroscopy identified a heterogeneous population of PEGylated species (Fig. 3). Because multiple species of PEG-IL-15 conjugates were present in these samples, the specific activity could be expected to be reduced (i.e. some species of IL-15 may exist that have not been PEG conjugated at either position Lys10 or position Lys11). It should also be considered that a single chain of PEG may be extended in solution such that a chain attached at one site may interfere with the protein-receptor interactions at another distant site. Each of these possibilities is consistent with our CTLL-2 bioassay data, in which the concentration of PEG-IL-15 required to inhibit the activity of unmodified IL-15 was approximately 100-fold higher than that required for similar activities of IL-15DS8 and IL-15Q108S (Fig. 6).

The technique of protein PEGylation is usually considered in the context of extending the half-life of circulating proteins, reducing protein antigenicity, or improving the resistance of a given protein to proteolysis. In this study, we have demonstrated the potential use of PEG as a probe for protein structure and function. The observation that PEG can selectively interfere with a specific biological function has been reported previously in the literature. For example, in the process of PEGylating IgG, the complement-fixing activity of this molecule can be reduced, whereas its ability to bind antigen remains unaltered (19). Also, in the process of PEGylating granulocyte-macrophage colony-stimulating factor, neutrophil priming can be maintained or even enhanced, whereas the colony-stimulating activity of this molecule is reduced (20). In fact, the knowledge that PEGylation of proteins can interfere with protein-ligand interactions in general has led investigators to introduce cysteine residues in recombinant proteins to enable site-specific PEGylation and reduce the possibility of steric interference from PEG (33, 34). Site-directed mutagenesis has also been used to eliminate lysine residues in locations where lysine-specific PEGylation could interfere with specific protein function (35).

We suggest that protein PEGylation may also be used as a complementary technique to protein engineering in order to sterically control protein-ligand interactions. Protein-ligand interactions typically involve many amino acids over the surfaces of proteins: e.g. lysozyme/antibody (36), trypsin/trypsin inhibitor (37), and growth hormone/receptor (38). By traditional protein engineering techniques, amino acid residues must be systematically mutated over a wide target region in order to uncover key residues or combinations of residues that are critical to protein function. In the case of protein PEGylation, the extended polymer chain may serve to sterically interfere with protein-protein interactions over a fairly large region on the surface of a protein molecule. In the particular example of IL-15 described in this paper, the likely PEGylation of residues Lys10 and/or Lys11 were sufficient to block the action of the nonadjacent critical residue, Asp8.

The potential exists (by introducing specific amino acid residues in critical regions) to PEG modify other ligands that bind multisubunit receptors in order to make specific antagonists (e.g. growth hormone, TNF, IL-2, IL-4, etc.). These molecules should have the added attributes conferred by PEG conjugation.
tion, including extended serum half-life. This approach could be particularly useful when single point mutations are insufficient to block the binding of specific receptor subunits.

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REFERENCES

1. Grabstein, K. H., Eisenman, J., Shanebeck, K., Rauch, C., Srinivasan, S., Pung, V., Beers, C., Richardson, J., Schoenborn, M. A., Abdieh, M., Johnson, L., Alderson, M. R., Watson, J. D., Anderson, D. M., and Giri, J. G. (1984) Science 264, 965–968
2. Carson, W. E., Giri, J. G., Lindemann, M. J., Linett, M. L., Ahdieh, M., Paxton, R., Anderson, D., Eisenmann, J., Grabstein, K., and Caligiuri, M. A. (1994) J. Exp. Med. 180, 1395–1403
3. Giri, J. G., Abdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L. S., Cosman, D., and Anderson, D. M. (1994) EMBO J. 13, 2922–2930
4. Armitage, R. J., Macduff, B. M., Eisenman, J., Paxton, R., and Grabstein, K. H. (1995) J. Immunol. 154, 483–490
5. Kennedy, M. K., and Park, L. S. (1996) EMBO J. 15, 3654–3663
6. Anderson, D. M., Kumaki, S., Abdieh, M., Friend, D. J., Loomis, A., Shanebeck, K., DaRose, R., Cosman, D., Park, L. S., and Anderson, D. M. (1995) EMBO J. 14, 3654–3663
7. Anderson, D. M., Kumaki, S., Abdieh, M., Bertels, J., Tonetsku, M., Loomis, A., Giri, J., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Valentine, V., Shapiro, D. N., Morris, S. W., Park, L. S., and Cosman, D. (1995) J. Biol. Chem. 270, 28862–28869
8. Weir, M. P., Chaplin, M. A., Wallace, D. M., Dykes, C. W., and Hobden, A. N. (1988) Biochemistry 27, 6883–6882
9. Weigel, U., Meyer, M., and Sebald, W. (1989) Eur. J. Biochem. 180, 295–300
10. Sauer, K., Nachman, M., Spence, C., Bailon, P., Campbell, E., Tsien, W.-H., Kondas, J. A., Hakimi, J., and Ju, G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4636–4640
11. Ju, G., Collins, L., Kaffka, K. L., Tsien, W.-H., Chizzonite, R., Crowl, R., Bhatt, R., and Kilian, P. L. (1987) J. Biol. Chem. 262, 5723–5731
12. Collins, L., Tsien, W.-H., Seals, C., Hakimi, J., Weber, D., Bailon, P., Hockings, J., Greene, W. C., Toome, V., and Ju, G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7709–7713
13. Liang, S.-M., Thatcher, D. R., Liang, C.-M., and Allet, B. (1986) J. Biol. Chem. 261, 334–337
14. Zarrawi, S. M., Vega, F., Jr., Doyle, E. L., Huyhhe, B., Flaherty, K., McKay, D. B., and Zarrawi, G. (1993) EMBO J. 12, 5113–5119
15. Katre, N. V., Knauf, M. J., and Laird, W. J. (1987) Proc. Natl. Acad. Sci. USA 84, 1487–1491
16. Katre, N. V. (1990) J. Immunol. 144, 209–215
17. Delgado, C., Francis, G. E., and Fisher, D. (1992) Crit. Rev. Ther. Drug Carrier Sys. 9, 249–304
18. Suzuki, T., Kanbara, N., Tomono, T., Hayashi, N., and Shinohara, I. (1984) Biochim. Biophys. Acta 788, 245–255
19. Knu¨sli, C., Delgado, C., Malik, F., Démine, M., Tejedor, M. C., Irvine, A. E., Fisher, D., and Francis, G. E. (1992) Br. J. Haematol. 82, 654–661
20. Brandhuber, B. J., Boone, T., Kenney, W. C., and McKay, D. B. (1987) Science 238, 1707–1709
21. Giri, J. G., and Paxton, R. J. (1996) in Human Cytokines, Handbook for Basic and Clinical Research (Aggarwal, B. B., and Gutterman, J. U., eds) Vol. II, pp. 135–145, Blackwell Science, Cambridge, MA
22. Price, V. Mochirski, D., March, C. J., Cosman, D., Delee, M. C., Klinek, R., Cleveenger, W., Gillis, S., Baker, P., and Urdal, D. (1987) Gene 55, 287–293
23. Gillis, S., Ferm, M. M., Ou, W., and Smith, K. A. (1978) J. Immunol. 120, 2927–2932
24. Taniguchi, T., Matsuí, H., Fujita, T., Takaoka, C., Kashima, N., Yoshimoto, R., and Hamuro, J. (1983) Nature 302, 305–310
25. Rozwarski, D. A., Gronenborn, A. M., Clare, G. M., Bazan, J. F., Bohm, A., Wlodawer, A., Hatada, M., Karplus, P. A. (1994) Structure 2, 159–173
26. Wlodawer, A., Pavlovsky, A., and Gusterichina, A. (1993) Protein Sci. 2, 1373–1382
27. Srinivasan, S., March, C. J., and Sudarsananam, S. (1993) Protein Sci. 2, 277–289
28. Sudarsananam, S., March, C. J., and Srinivasan, S. (1994) J. Mol. Biol. 241, 143–149
29. Amit, A. G., Mariuzza, R. A., Phillips, S. E. V., and Poljak, R. J. (1986) Science 233, 747–753
30. Janin, J., and Chothia, C. (1976) J. Mol. Biol. 100, 197–211
31. de Vos, A. M., Uitsch, M., and Kossiakoff, A. A. (1992) Science 255, 306–312