A monomeric form of human interleukin 10 (IL-10M1) has been engineered for detailed structure-function studies on IL-10 and its receptor complexes. Wild type IL-10 (wtIL-10) is a domain swapped dimer whose structural integrity depends on the intertwining of two peptide chains. wtIL-10 was converted to a monomeric isoform by inserting 6 amino acids into the loop connecting the swapped secondary structural elements. Characterization of IL-10M1 by mass spectroscopy, size exclusion chromatography, cross-linking, and circular dichroism shows that IL-10M1 is a stable α-helical monomer at physiological pH whose three-dimensional structure closely resembles one domain of wtIL-10. As previously reported, incubation of wtIL-10 with a soluble form of the IL-10Ra (sIL-10Ra) generates a complex that consists of 2 wtIL-10 molecules and 4 sIL-10Ras. In contrast, IL-10M1 forms a 1:1 complex with the sIL-10Ra. Characterization of the interaction using isothermal titration calorimetry confirmed the 1:1 stoichiometry and yielded a dissociation constant of 30 nM with an apparent enthalpy of −12.2 kcal/mol. Despite forming a 1:1 complex, IL-10M1 is biologically active in cellular proliferation assays. These results indicate that the 1:1 interaction between IL-10M1 and IL-10Ra is sufficient for recruiting the signal transducing receptor chain (IL-10Rβ) into the signaling complex and eliciting IL-10 cellular responses.

Interleukin-10 (IL-10) is a pleiotropic cytokine that inhibits cell-mediated immune responses while enhancing humoral immunity (1, 2). The immunosuppressive properties of IL-10 are largely derived from its ability to inactivate macrophages by suppressing the synthesis of pro-inflammatory cytokines (including IL-1, IL-6, IFN-γ, and tumor necrosis factor-α) and by inhibiting the expression of cell surface molecules involved in antigen presentation and co-stimulation (3–5). IL-10 is also a powerful growth and differentiation factor for B-lymphocytes, mast cells, and thymocytes (6). These immunostimulatory functions may have negative effects in some instances, as IL-10 has been implicated as an autocrine growth factor in certain B-cell malignancies (7, 8). Thus, antagonists of IL-10 may be useful in treating B-cell lymphomas as well as infections where cell-mediated immune responses are suppressed by IL-10 (9).

Cellular responses to IL-10 require at least two cell surface receptors, IL-10Ra and IL-10Rβ (10–12). Both receptors are members of the class II cytokine receptor family (13, 14). The extracellular domains of the receptors are responsible for binding to IL-10. The intracellular domains of IL-10Ra and IL-10Rβ are associated with Jak1 and Tyk-2 kinases, respectively (15). Kinase activation and subsequent IL-10-induced biological activities are dependent on the formation of a complex between IL-10, IL-10Ra, and IL-10Rβ (11). It is believed that IL-10Ra acts as the primary high affinity receptor for IL-10 and that signaling results from the recognition of this initial complex by IL-10Rβ.

Characterization of the interaction between IL-10 and the IL-10Ra has been facilitated by the production of large quantities of the extracellular domain of IL-10Ra (sIL-10Ra). The apparent dissociation constant between IL-10 and immobilized sIL-10Ra is approximately 500 pM (16). Since IL-10, like IFN-γ, contains 2 identical domains it was expected to bind 2 sIL-10Ra molecules as observed in the crystal structure of the IFN-γ receptor complex (17). However, incubation of the sIL-10Ra with IL-10 generates a stable complex that consists of 2 IL-10 molecules and 4 sIL-10Ras (16). The stoichiometry and affinity of the sIL-10Rb for this complex as well as the significance of the 2:4 IL-10:sIL-10Ra complex in IL-10 signaling is currently unknown.

The crystal structure of IL-10 revealed a symmetric homodimer composed of two α-helical domains oriented at 90° to one another (18, 19). The structural integrity of each domain is dependent on the intertwining of α-helices from each peptide chain such that the first four helices of one chain (A–D) associate with the last two helices (E and F) of the other. The domains of IL-10 share structural similarity with the monomeric type-I (IFN-α, IFN-β, and IFN-γ) and dimeric type-II interferons (IFN-γ) that together form the class II cytokine family (20–23).

The IL-10 dimer is thought to be the result of an evolutionary mechanism of protein oligomerization often referred to as 3D domain swapping (24). This suggests that IL-10 evolved from a monomeric protein by exchanging structural domains (α-heli-
ces E and F for IL-10) with another monomer to create the dimer. Stability studies with IL-10 have established that significant amounts of a monomeric form of IL-10 can be generated at low protein concentrations, increased temperature (including 37 °C), or under extreme conditions such as low pH or high concentrations of guanidine HCl (25). However, under physiological conditions the monomeric form of IL-10 is not stable and does not have the same domain structure as the IL-10 dimer (25). Furthermore, biological assays of the denatured samples show a linear correlation between the percent dimer present and IL-10 biological response suggesting that the dimer is the active species in signaling.

Since IL-10 is likely to have evolved from a monomeric species, its quaternary structure must be very important for generating IL-10-induced cellular activities. To test this hypothesis, we engineered an IL-10 monomer (IL-10M1) by inserting six amino acids (GGGSGG) into the inter-domain linker region of IL-10 between residues Asn116 and Lys117. Our studies show that IL-10M1 is stable under native conditions (pH 7.0, 25 °C) and structurally similar to one domain of IL-10. IL-10M1 binds to sIL-10Ra with a 1:1 stoichiometry, representing one-fourth of the 2 IL-10/sIL-10R complex observed for the IL-10 dimer. Surprisingly, IL-10M1 was shown to be biologically active and was unable to antagonize IL-10 in cellular proliferation assays. Our studies show that IL-10M1 is suitable for x-ray crystallographic studies of 1:1 IL-10 receptor complexes, protein-folding studies, and for defining the energetic contribution of each residue in the IL-10/sIL-10R binding interface.

**EXPERIMENTAL PROCEDURES**

**Cloning of wtIL-10 and IL-10M1**—All restriction enzymes were purchased from New England Biolabs. The wtIL-10 cDNA sequence (ATCC 6891) was PCR-amplified using Pfu polymerase (Stratagene) with the 5′ primer 5′-TGTTAAGCATATGACCCCAGGCCAGGACC-3′ and the 3′ primer 5′-CCGCTCGAGTACGTTTCGTATCTTCATTGTT-3′. The PCR product was digested with NdeI and XhoI and subsequently ligated into the pET-32 expression vector (Novagen) to generate pET-32-wtIL-10.

IL-10M1 was engineered using the previously determined crystal structure of wtIL-10 (26). Possible internal peptide sequences were modeled using the computer graphics program CHAIN (26). The expression plasmid for IL-10M1 was generated from the ligation of two DNA fragments into pET-32 to generate pET32-IL-10M1. DNA fragment 1 was PCR-amplified from the 5′ primer 5′-AAGAGTTCGCTACGTAATGC-3′ and the 3′ primer 5′-AATGATCCACACCGTTCTACAGGAAGAATTG-3′ and digested with NdeI and XhoI. DNA fragment 2 was PCR-amplified from the 5′ primer 5′-ATGGATCCGGGTGAAGAACGAGCAAGGCCTGGA-3′ and the 3′ primer 5′-CCGCTCGAGTACTCTCTGATCTTTCTTCTGCT-3′ and digested with BamHI and XhoI. It codes for IP residues 1–116 and IL-10M1 residues 117–160. PCR product was digested with NdeI and XhoI and subsequently ligated into the pET-32 vector expression vector (Novagen) to generate pET-32-wtIL-10M1.

**Expression and Purification of wtIL-10 and IL-10M1**—Cells containing the pET-32-wtIL-10 or pET-32-IL-10M1 plasmids were grown in Luria Broth media to an optical density of 0.6 and then induced with 1 mM isopropyl-β-D-thiogalactoside for 3 h. The cells were collected by centrifugation, and the inclusion bodies were purified by sonication in 100 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 8.0. IL-10M1 and wtIL-10 were refolded using conditions previously reported for wtIL-10 (19). Briefly, inclusion bodies were solubilized in 6 M guanidine HCl containing 5 mM DTT. Refolding was initiated by a 10-fold dilution of the solubilized inclusion bodies into a solution containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA, 2 mM reduced glutathion, and 2 mM oxidized glutathion. Purification of the refolded material was achieved by sequential ion exchange chromatography on HS-20 and HQ-10 columns (PE Biosystems) followed by an additional gel filtration procedure. Each purification step was analyzed by SDS-PAGE to assist in pooling fractions.

**Protein Concentrations**—Protein concentrations for all experiments were determined from the UV absorbance at 280 nm to obtain mg/ml using the appropriate ε1% data for wtIL-10 and sIL-10R. An ε1% for wtIL-10 and IL-10M1 of 0.47 was used as previously reported (27). The calculated ε1% value for sIL-10R is 1.6.

**MALDI-TOF Mass Spectroscopy**—Mass analysis was carried out on a Voyager Elite mass spectrometer with delayed extraction technology (PerSeptive Biosystems, Framingham, MA) operating in a reflector mode. The acceleration voltage was set at 25 kV, and 50–100 laser shots were summed. Samples were mixed 1:10 with sinapinic acid dissolved in acetonitrile, 0.1% trichloroacetic acid (1:1). Apomyoglobin was used as an internal standard.

**Size Exclusion Chromatography**—Receptor complexes were formed by incubating IL-10M1 or wtIL-10 with sIL-10Ra at different molar ratios for 1 h at 4 °C. The complexes were purified by gel filtration using two Superdex 200 (10 × 300 mm) columns (Amersham Pharmacia Biotech) linked in tandem that were equilibrated in 20 mM Tris-HCl, 150 mM NaCl, pH 8.0, at a flow rate of 0.35 ml/min. 100-μl volumes were injected onto the column. Apparent molecular weights were calculated using standard curves of the gel filtration standards (Bio-Rad). The extracellular fragment of the IL-10Rα (sIL-10Rα) for gel filtration chromatography and ITC experiments and neutralization studies was produced in Drosophila S2 cells (Invitrogen and SmithKline Beecham).

**Circular Dichroism**—Circular dichroism spectra were measured using an Aviv model 62DS spectropolarimeter. Reported spectra were averaged from the three scans from the resulting spectrum taken in an identical manner. Data collection was made every 0.5 nm using a 1-s averaging time and a 1.5-nm bandwidth. Sample concentrations were 2 mg/ml in 20 mM sodium phosphate buffer, pH 7.0, in an 80-μl sample cell with a 0.1-mm path length. Melt experiments were done at 0.04 mg/ml in 20 mM sodium phosphate, pH 7.0, in a 3.5-ml stirred sample cell, with a 1-cm path length. The temperature was changed by 0.5 ° increments to 95 °C and monitored by a sample cell temperature probe. At each set temperature the signal at 222 nm was averaged for 10 s using and 1.5-nm bandwidth.

**Isothermal Titration Calorimetry**—Titration calorimetry was carried out using a VP-ITC calorimeter (Microcal, Inc., Northampton, MA) at 25 °C. Prior to ITC analysis protein solutions were extensively dialyzed against 150 mM NaCl and 20 mM sodium phosphate/pipes (sodium diphosphate) (pH 7.1) with 100 units/ml Pfu Taq DNA polymerase and 1.5 mM IPTG (isopropyl-β-D-thiogalactoside). The IL-10M1 or wtIL-10 solution was titrated into a 1.39-ml cell containing a 6.5 μM solution of Drosophila S2-derived sIL-10Rα. After an initial injection of 0.5 μl, which was not used in data fitting, 12 injections of 20.5 μl each were made at 240-s intervals. A blank titration was also performed in which ligand was injected into buffer. The data were fit to a single binding site model using Origin (version 5.0; Microcal, Inc.) after subtracting a linear regression of the integrated peak areas from the blank run (28).

**Precipitation Assays**—IL-10M1 or wtIL-10 was diluted into assay medium consisting of RPMI 1640, 1% fetal bovine serum, 2 mM Glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM 2-mercaptoethanol. Samples were dispensed into 96-well microtiter plates (Becton Dickinson) in duplicate wells and serially diluted 3-fold across the plates. Ba8.1c1 or BaMr 29-1 cells were then added to the assay plates at a final density of 1.5 × 104 cells/well. After incubation for 48 h at 37 °C, 5% CO2, medium and can be measured spectrophotometrically at a wavelength of 490 nm with a reference wavelength of 570 nm. For determining specific activities, the concentration of cytokine that induced half-maximal biological response was determined as the cytokine concentration that caused 50% inhibition of a fixed amount of cell proliferation. Specific activities were determined both in the presence and absence of 1 μM PDTC for IL-10M1 or IL-10M1 was performed by addition of various concentrations of sIL-10Rα.

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2 K. Josephson and M. R. Walter, unpublished data.
RESULTS

Engineering of IL-10M1—The crystal structure of IL-10 revealed a homodimer comprised of two intertwined polypeptide chains that contain six α-helices labeled A–F (18, 19). The two chains tightly associate with one another such that each domain of the dimer consists of helices A–D from one chain and helices E and F from the other. Helices D and E are connected by a 12-residue linker of which the final four residues (Glu115–Asn116–Lys117–Ser118) extend across the dimer interface. The crystal structure of IL-10M1 showing the insertion of helices E and F into the cleft formed from helices A–D (Fig. 1).

Computer modeling was used to identify the location and length of an insertion peptide (IP) that would allow the formation of a stable IL-10 monomer. These studies led to the design of IL-10M1 that contained a 6-residue insertion peptide (IP) Gly-Gly-Gly-Ser-Gly-Gly between Asn116 and Lys117. The predicted structure of IL-10M1 showing the insertion of helices E and F into the cleft formed from helices A–D.

Expression and Purification of IL-10M1 and wtIL-10—IL-10M1 and wtIL-10 are expressed at equal levels in E. coli where they form insoluble inclusion bodies. Both polypeptides are refolded using the conditions previously described for wtIL-10 (19). Purified IL-10M1 and wtIL-10 proteins were estimated to be greater than 98% pure by SDS-PAGE. IL-10M1 and wtIL-10 peptide chains were analyzed by MALDI-TOF mass spectrometry. The observed masses of 19,151.6 (calculated 19,150.8) for IL-10M1 and 18,778.4 (calculated 18,778.5) for wtIL-10 (18). Purified IL-10M1 and wtIL-10 proteins were expressed using the conditions previously described for wtIL-10 (19). Purified IL-10M1 and wtIL-10 proteins were estimated to be greater than 98% pure by SDS-PAGE. IL-10M1 and wtIL-10 peptide chains were analyzed by MALDI-TOF mass spectrometry. The observed masses of 19,151.6 (calculated 19,150.8) for IL-10M1 and 18,778.4 (calculated 18,778.5) for wtIL-10 are consistent with the addition of an initiating N-terminal methionine to each sequence.

IL-10M1 Forms a 1:1 Complex with sIL-10R—IL-10M1 and wtIL-10 receptor complexes were analyzed by size exclusion chromatography to determine the stoichiometry of the IL-10M1:sIL-10R complex. Free IL-10M1 eluted at a volume of 29.7 ml with a calculated molecular weight of 18,600. Free

![Fig. 1. Design of an IL-10 monomer. A, schematic diagram of the IL-10 polypeptide. Each α-helix in the chain is represented as a rectangle and labeled A–F from the N to C terminus. The amino acid sequence for the hinge region between helices D and E is shown for wtIL-10 and for IL-10M1. B, ribbon diagram of the intertwined wtIL-10 dimer (34). C, ribbon diagram of a single wtIL-10 chain or open monomer as described in Ref. 24. D, predicted structure of IL-10M1 showing the insertion of helices E and F into the cleft formed from helices A–D.](image)

![Fig. 2. Analysis of the oligomerization state of IL-10M1. Gel filtration chromatograms for IL-10 (A) and IL-10M1 (B). Samples containing 50 μg of IL-10 or IL-10M1 in a volume of 100 μl were injected onto two Superdex-200 (10 × 300 mm) columns linked in tandem. The column was equilibrated in 20 mM Tris-HCl buffer, 150 mM NaCl, pH 8.0, at a flow rate of 0.35 ml/min. The columns were calibrated by a standard curve produced using gel filtration standards (Bio-Rad). Inset, analysis of cross-linked IL-10 and IL-10M1 by SDS-PAGE. Molecular weight markers are shown in lane 1. Control samples without cross-linker are shown in lanes 2 for IL-10 and lane 4 for IL-10M1. IL-10 and IL-10M1 samples obtained from the gel filtration experiment and subsequently incubated with 1% glutaraldehyde are shown in lanes 3 and 5, respectively.](image)
is believed to be due to experimental errors in determining the isotherm (28). The small deviation of the stoichiometry from 1:1 kcal/mol was derived from least squares fitting of the binding 10M1 and sIL-10R ratio plus an additional peak corresponding to excess sIL-10R. Results in the same size complex as observed for the 1:1 molar ratio of 1:1 (13:13 μM) (B), and IL-10M1 and sIL-10Ra at a molar ratio of 1:4 (13:52 μM) (C). The columns were calibrated by a standard curve produced using gel filtration standards (Bio-Rad). The arrow denotes the position of the 2 wtIL-10:4 sIL-10Ra complex peak.

sIL-10Ra eluted at a volume of 26.62 ml with a calculated molecular weight of 42,600. Fig. 5 shows the chromatograms of IL-10M1 and sIL-10Ra mixed at 1:1 or 1:4 molar ratios. IL-10M1 and sIL-10Ra mixed at a 1:1 ratio elutes from the column at 24.92 ml and has an apparent molecular weight of ~69,000. When the proteins are mixed in this ratio, essentially all of the individual reactants are used to form the receptor complex. Incubation of a 1:4 molar ratio of IL-10M1 and sIL-10Ra results in the same size complex as observed for the 1:1 molar ratio plus an additional peak corresponding to excess sIL-10Ra.

Characterization of the IL-10M1-sIL-10Ra Interaction—Isothermal titration calorimetry (ITC) was used to define further the stoichiometry and dissociation constant between IL-10M1 and sIL-10Ra. Aliquots of 20.5 μl of a 65 μM solution of IL-10M1 were titrated into a 6.5 μM solution of sIL-10Ra at 25 °C. The titration data revealed a stoichiometry of 1.15 mol of IL-10M1 per mol of sIL-10Ra. A dissociation constant \( K_d \) for the complex of 30 nM with an apparent \( \Delta H \) of binding of ~12.2 kcal/mol was derived from least squares fitting of the binding isotherm (28). The small deviation of the stoichiometry from 1:1 is believed to be due to experimental errors in determining the concentrations of the reactants.

IL-10M1 Stimulates Cellular Proliferation—IL-10M1 was tested for its ability to induce proliferation of murine Ba/F3 cells transfected with either the human (Ba8 cells) or murine (BaMr cells) IL-10Ra (Fig. 6). The concentrations of wtIL-10 required for half-maximal responses (EC_{50}) on BaMr and Ba8 cells were ~0.05 and ~0.8 ng/ml, respectively. These concentrations translate to specific activities for wtIL-10 of ~2 × 10^7 units/mg on BaMr cells and ~1.25 × 10^6 units/mg on Ba8 cells as previously reported (30). Surprisingly, IL-10M1 also displayed considerable biological activity with EC_{50} values of ~0.9 ng/ml on BaMr cells and ~7 ng/ml on Ba8 cells. Based on the EC_{50} values reported above, wtIL-10 is approximately 18-fold more active than IL-10M1 on BaMr cells and about 9-fold more active than IL-10M1 on Ba8 cells. The specific activity of wtIL-10 was approximately 16-fold lower on Ba8 cells than BaMr cells. The same trend was also observed for IL-10M1 although the activity difference between the cell lines was only 8-fold. Despite the lower activity of IL-10M1, higher concentrations of IL-10M1 resulted in maximal activity equivalent to wtIL-10 on both cell lines. IL-10M1 did not antagonize wtIL-10 on either cell line (data not shown).

IL-10M1 Activity Is Antagonized by sIL-10Ra—IL-10M1 or wtIL-10 induced cellular proliferation was neutralized by addition of sIL-10Ra to the culture media (Fig. 7). Neutralization of IL-10M1 activity required an ~3.8-fold molar excess of sIL-10Ra on Ba8 cells and a 35-fold molar excess of sIL-10Ra on BaMr cells. A 38- and 343-fold molar excess of sIL-10Ra was required to inhibit wtIL-10 induced proliferation on Ba8 cells and BaMr cells, respectively. These studies show that IL-10M1 biological activity requires the specific interaction with the IL-10Ra. Furthermore, IL-10M1 activity on either cell line is neutralized with approximately 10-fold less sIL-10Ra than required to neutralize wtIL-10. For both IL-10M1 and wtIL-10, 10-fold higher concentrations of sIL-10Ra are required to neutralize their activities on BaMr cells compared with Ba8 cells. This result is consistent with the higher affinity of the murine IL-10Ra for IL-10 than the human IL-10Ra.

DISCUSSION

PCR mutagenesis has been used to insert the sequence Gly-Gly-Ser-Gly-Gly-Gly between Asn^{116} and Lys^{117} of the wtIL-10 polypeptide chain. Refolding this mutant under conditions...
transfected with the human IL-10R α, the curves converged to an IC$_{50}$ concentration for sIL-10R IL-10R data points transfected with the murine cell proliferation on BaMr cells (black data points) and wtIL-10 (circles) to induce proliferation. Cell proliferation was monitored after 48 h by optical density measurement following MTS staining.

These experiments taken together suggest that despite eliminating the wtIL-10 dimer interface, IL-10M1 has a structure similar to one domain of the wtIL-10 dimer.

The thermal unfolding data show that IL-10M1 is more stable than wtIL-10. This suggests that the wtIL-10 dimer has evolved to carry out a specific biological function at physiological temperature rather than to achieve optimal thermal stability. Currently it is not clear why wtIL-10 has not evolved a more extensive dimer interface such as observed for IFN-γ or interleukin-5 (29, 31, 32). This is especially curious since wtIL-10 begins to dissociate in vitro at a concentration of ~1 μM but is biologically active at concentrations ~10$^6$-fold (~1 pm) below where dimer dissociation is observed (25). This suggests that the stability characteristics of the wtIL-10 dimer may play an important and yet undefined role in regulating wtIL-10 biological activity.

The interaction between IL-10M1 and sIL-10Rα expressed in Drosophila S2 cells was characterized by gel filtration chromatography and ITC studies. The data reveal the binding stoichiometry for the IL-10M1-sIL-10Rα interaction is 1:1 at all concentrations studied (6–25 μM). As a control, we repeated the efforts of Tan et al. (16) who showed that incubation of wtIL-10 and sIL-10Rα generates a complex of 2 wtIL-10 molecules and 4 sIL-10Rα. Since IL-10M1 is essentially identical to one domain of wtIL-10, we expected that incubation of IL-10M1 with sIL-10Rα might form complexes that contained 2 or 4 IL-10M1 molecules and 2 or 4 sIL-10Rα. However, these larger complexes were not observed over the concentrations studied. Thus, the interaction between IL-10M1 and the sIL-10Rα is not sufficient to form the 2 IL-10-4 sIL-10Rα complex observed with the IL-10 dimer.

Since IL-10M1 forms a 1:1 complex with the sIL-10Rα, we hypothesized that IL-10M1 would not be biologically active. However, the ability of IL-10M1 to induce short term proliferative responses was only about 9-fold lower than wtIL-10 on murine BaF3 cell line expressing the human IL-10Rα (BaF3 cells) and 18-fold less active than wtIL-10 on BaF3 cells expressing the murine IL-10Rα (BaMr cells). Differences in activity between these cell lines is at least partly the result of different receptor components (human IL-10Rα/murine IL-10Rβ on BaF3 or murine IL-10Rα/murine IL-10Rβ on BaMr) displayed on their cell surfaces (11, 30). However, on both cell lines maximal proliferative responses equivalent to wtIL-10 could be achieved with higher concentrations of IL-10M1 (Fig. 6). Our experiments do not rule out the possibility that in the biological assay IL-10M1 might dimerize on the cell surface.
However, this seems unlikely since IL-10M1 is observed as a monomer at concentrations much higher (~13 μM) than required for IL-10M1 activity (~0.5 nM).

This leads to the question of the importance of the wtIL-10 dimer for generating wtIL-10 biological responses. One advantage for the wtIL-10 dimer is its ability to simultaneously bind two IL-10Rαs. Based on the crystal structure of wtIL-10 and the IFN-γ receptor complex, the orientation of the wtIL-10 dimer domains (90°) is optimized for this 2-fold interaction that is lost in IL-10M1. As a result, wtIL-10 binds to the IL-10Rα ~60-fold more avidly than IL-10M1 (0.5 versus 30 nM). Although this is an important difference between wtIL-10 and IL-10M1, it is interesting that viral IL-10 with an apparent dissociation constant of ~500 nM (16-fold higher than IL-10M1) has the same specific activity as wtIL-10 (30). The decreased biological activity of IL-10M1 versus wtIL-10 may be the result of the different receptor complexes they form with the IL-10Rα. The 2IL-10-2IL-10Rα complex generated by the dimer provides a way to cluster four IL-10Rαs on the cell surface. The ability of the dimer to cluster four IL-10Rαs may enhance IL-10Rβ binding and subsequently wtIL-10 biological activity. Since IL-10M1 only forms a 1:1 interaction with IL-10Rα, the ability to recruit IL-10Rβ should be significantly diminished.

Our studies strongly argue that the interaction of one domain of wtIL-10 with one IL-10Rα and one IL-10Rβ is sufficient for cellular proliferative responses. Thus, this ternary complex is all that is required for activation of the intracellular kinases, Jak1 and Tyk2. From our present data, we predict that each domain of wtIL-10 in the 2:4 wtIL-10 receptor complex independently activates the Jak kinases responsible for signal transduction. Said another way, phosphorylation events occur only between receptors associated with the same domain of wtIL-10 instead of across the 2-fold axis of the dimer. Currently, our predictions are only for wtIL-10 proliferative responses. This is because Riley et al. (33) recently reported that different portions of the IL-10Rα intracellular domain are responsible for wtIL-10-induced proliferative and cytokine inhibitory activities. Experiments to determine the ability of IL-10M1 to suppress cytokine synthesis are currently under way.

IL-10M1 provides an important tool for studying the structural, stoichiometric, and energetic requirements necessary for IL-10-induced receptor activation. Since IL-10M1 forms a 1:1 interaction with sIL-10Rα it provides a simplified complex, compared with the 2:4 wtIL-10-2IL-10Rα complex, for detailed biophysical studies. The 1:1 complex will be most helpful to our efforts to obtain crystals of the IL-10 receptor complex for high resolution x-ray diffraction studies and to define the energetic contributions of residues in the IL-10/sIL-10Rα binding interface without the complications of the larger 2:4 complex. Additional protein folding and NMR studies on IL-10M1 are also under way. The design and characterization of IL-10M1 represents our first efforts to antagonize wtIL-10 activity by changing its quaternary structure. Our current and future studies outlined above should provide new insights for modulating the IL-10 signal transduction pathway.

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REFERENCES
1. de Waal Malefyt, R., Yssel, H., Roncalio, M.-G., Spits, H., and de Vries, J. E. (1992) Curr. Opin. Immunol. 4, 314–320
2. Moore, K. W., O’Garra, A., de Waal Malefyt, R., Vieira, P., and Mosmann, T. R. (1993) Annu. Rev. Immunol. 11, 165–190
3. Ho, A. S., and Moore, K. W. (1994) Ther. Immunol. 3, 173–185
4. de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G., and de Vries, J. E. (1993) J. Exp. Med. 177, 1249–1244
5. Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M., and O’Garra, A. (1991) J. Immunol. 147, 3815–3822
6. Rousett, P., Garcia, E., Defrance, T., Peronne, C., Vezzio, N., Hsu, D. H., Kastelijn, R., Moore, K. W., and Banchereau, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1890–1893
7. Emilie, D., Touboul, R., Raphael, M., Peuchmair, M., Devergne, O., Rea, D., Coumbaras, J., Crevy, F., Lebecq, M., Joubi, I., and Galanaud, P. (1992) Eur. J. Immunol. 22, 2897–2942
8. Khatri, V. P., and Caligiuri, M. A. (1998) Cancer Immunol. Immunother. 46, 239–244
9. Howard, M., O’Garra, A., Ishida, H., De Waal Malefyt, R., and De Vries, J. (1992) J. Clin. Immunol. 12, 239–247
10. Liu, Y., Wei, S. H., Ho, A. S., de Waal Malefyt, R., Moore, K. W. (1994) J. Immunol. 152, 1821–1819
11. Kotenko, S. V., Krause, C. D., Izotova, L. S., Pollack, B. P., Wu, W., and Pestka, S. (1997) EMBO J. 16, 5894–5903
12. Spencer, S. D., Di Marco, F., Hooey, J., Pitts-Meek, S., Bauer, M., Ryan, A. M., Sordat, B., Gibbs, V. C., and Auge, M. (1998) J. Exp. Med. 187, 571–578
13. Ho, A. S. Y., Liu, Y., Khan, T. A., Dze, H.-H., Bazan, J. F., and Moore, K. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11267–11271
14. Bazan, J. F. (1990) Cell 61, 753–754
15. Finhloom, D. S., and Winstock, K. D. (1995) J. Immunol. 155, 1079–1090
16. Tan, J. C., Broom, S., Kong, H., DiGiacomo, R., Dolphin, E., Baldwin, S., Narula, S. R., Zavodny, P. J., and Chou, C.-C. (1997) J. Biol. Chem. 272, 12096–12101
17. Walter, M. R., Windsor, W. T., Nagabhushan, T. L., Lundell, D. J., Lunn, C. A., Sordat, B., Gibbs, V. C., and Auge, M. (1998) J. Mol. Biol. 289, 229–240
18. Zdanov, A., Schalk-Hihi, C., Gustchina, A., Tsang, M., Weatherbee, J., and Woldawer, A. (1995) Structure 3, 591–601
19. Sprang, S. R., and Bazan, J. F. (1993) Curr. Opin. Struct. Biol. 3, 815–821
20. Radhakrishnan, R., Walter, L. J., Hruza, A., Reichert, P., Trotta, P. P., Nagabhushan, T. L., and Walter, M. R. (1996) Structure 4, 1453–1463
21. Ramaswamy, R., Walter, L. J., Subaranian, P., Johnson, H., and Walter, M. R. (1999) J. Mol. Biol. 1, 151–162
22. Bennett, M. J., Schluhmeier, M. P., and Eisenberg, D. (1995) Protein Sci. 4, 2455–2468
23. Syto, R., Murgolo, N. J., Braswell, E. H., Mui, P., Huang, E., and Windsor, W. T. (1998) Biochemistry 37, 16943–16951
24. Sack, J. S. (1988) J. Mol. Biol. 6, 224–225
25. Windsor, W. T., Syto, R., Tsrabopoulos, A., Zhang, R., Durkin, J., Baldwin, S., Paliwal, S., Mui, P., P., P., Pramanik, B., Trotta, P. P., and Tindall, S. H. (1993) Biochemistry 32, 8807–8815
26. Wiseman, T., Williston, S., Brands, J. F., and Lin, L. N. (1989) Anal. Biochem. 170, 131–137
27. Li, J., Cook, R., Doyle, M. L., Hensley, P., McNulty, D. K., and Chakravarti, I. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6694–6699
28. Li, J., de Waal, R., Bierie, F., Parham, C., Bridon, J.-M., Banchereau, J., Moore, K. W., and Xu, J. (1997) J. Immunol. 158, 604–613
29. Dickason, R. P., and Huston, D. P. (1996) Nature 379, 652–655
30. Milburn, M. V., Hassell, A. M., Lambert, M. H., Jordan, S. R., Proudfoot, A. E., Graber, P., and Wells T. N. (1993) Nature 363, 172–176
31. Riley, J. R., Takeda, R., Akira, S., and Schreiber, R. D. (1999) J. Biol. Chem. 274, 16513–16521
32. Carson, M. (1991) J. Appl. Cryst. 24, 958–961

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Additions and Corrections

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Evidence for triacylglycerol synthesis in the lumen of microsomes via a lipolysis-esterification pathway involving carnitine acyltransferases.

Khaled A. H. Abo-Hashema, Max H. Cake, Glen W. Power, and Doug Clarke

Page 35581: The text relating to Fig. 6 unintentionally omitted mentioning that Fig. 6 was based on a similar diagram in Owen et al. (Owen, M. R., Corstorphine, C. C., and Zammit, V. A. (1997) Biochem. J. 323, 17–21). The reason for including this figure was to provide a rationale for the radiolabeling pattern that was observed, not to claim intellectual precedence. Nevertheless, to our knowledge, our study provides the first direct demonstration of triacylglycerol synthesis within the microsomal lumen.

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Design and analysis of an engineered human interleukin-10 monomer.

Kristopher Josephson, Ruth DiGiacomo, Stephen R. Indelicato, Abiye H. Iyo, T. L. Nagabhushan, Matthew H. Parker, and Mark R. Walter

Dr. Iyo’s name was misspelled. The correct spelling is indicated above.