Role of Disulfide Bonds in Biologic Activity of Human Interleukin-6*

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We have examined the functional importance of the two disulfide bonds formed by the four conserved cysteines of human interleukin (IL-6). Using a bacterial expression system, we have synthesized a series of recombinant IL-6 mutants in which the constituent cysteines of human interleukin-6 were replaced by other amino acids. Each mutant was partially purified and tested in four representative bioassays. While mutants lacking CysG43 and CysG54 retained activity similar to nonmutated recombinant IL-6, the activity of mutants lacking CysG43 and CysG54 was significantly reduced, especially in assays involving human cell lines. These results indicate that the first disulfide bond of human interleukin-6 is not required for maintenance of normal biologic activity. However, the fact that mutants lacking CysG42 and CysG52 were more active than corresponding cysteine-free mutants indicates that the disulfide bond formed by these residues contributes to biologic activity in the absence of the second disulfide bond. Competition binding studies with representative mutants indicate that their affinity for the human IL-6 receptor parallels their biologic activities on human cells.

Interleukin-6 (IL-6)† is a cytokine which plays a central role in regulation of the body’s response to injury and infection (For recent reviews see Refs. 1–4). Human IL-6 is released by a number of cell types including: B-cells, T-cells, monocytes, keratinocytes, endothelial cells, and astrocytes. Biologic activities of IL-6 include stimulation of differentiation of B-cells and T-cells, stimulation of proliferation of hemopoietic stem cells, and stimulation of release of acute-phase reactants from hepatocytes. Biologically active rIL-6 has been expressed in a number of systems including Escherichia coli, transfected mammalian cells, and cell-free translation systems (5–7).

Several groups have carried out structure-function analysis on human IL-6 by examining the effects of mutations on the biologic activities of rIL-6 (8–12). Using this approach, it has been demonstrated that deletion of 28 amino acids from the amino terminus of mature human IL-6 does not significantly reduce biologic activity (8), while deletion of three amino acids from the carboxyl terminus results in almost total loss of activity (11). We reported previously that the biologic activity of human IL-6 is abolished by internal 20-amino acid deletions anywhere along the length of the protein, with the exception of the extreme amino terminus (12).

The importance of disulfide bonds in maintaining the biologically active conformation of IL-6 is suggested by the fact that the four cysteines of human IL-6 (CysG43, CysG54, CysG42, and CysG52) are completely conserved in other, homologous proteins: murine and rat IL-6, human and murine granulocyte colony-stimulating factor (G-CSF), and chicken myelomonocytic growth factor. Furthermore, it has been demonstrated that the cysteines in human IL-6 form two disulfide bonds (CysG43-CysG54 and CysG42-CysG52) and that analogous disulfide bonds are present in murine IL-6 and human G-CSF (13, 14). It has also been reported that replacement of any one of the cysteine residues in human G-CSF causes loss of activity (15).

In line with these findings we showed that the activity of a cysteine-free IL-6 mutant (rIL-6S) is reduced to less than 0.1% on human cells with respect to a nonmutated control. Although this mutant retains significantly more activity when tested on mouse and rat cell lines (16).

In the present study, we used our previously established bacterial expression system to examine the relative functional importance of the two disulfide bonds of human IL-6 for maintenance of biologic activity. Individual disulfide bonds were removed by replacing pairs of cysteines with either nonpolar (alanine), polar (serine), or charged amino acids (aspartate and arginine), and the resulting mutants were tested in four representative bioassays. We also examined the effects of removing disulfide bonds on specific binding of rIL-6 to human cells. We report here that mutants lacking CysG43 and CysG54 retained activity similar to a nonmutated control in all of the bioassays. In contrast, biologic activity was decreased in mutants lacking CysG43 and CysG52, and this decrease was greater for activity on human than on rodent cells. In general, the effects of removing cysteine pairs on the specific binding of IL-6 mutants to human cells paralleled their biologic activity. Biologic activity of mutants was not correlated with the type of amino acids used for substitution of CysG42 and CysG54. However, in mutants lacking CysG43 and CysG54, activity was consistently highest when cysteines were replaced by alanines and lowest when charged residues were used for substitutions.

EXPERIMENTAL PROCEDURES

Construction of Genes Encoding rIL-6 Mutants—The construction of the gene encoding rIL-6S, in which the four naturally occurring cysteines of human IL-6 are replaced by serines, has been described previously (16). Briefly, this gene was assembled from 22 synthetic oligonucleotides and initially cloned into a modified pBS M13- cloning vector (Stratagene). The natural stop codon of this gene was converted to a serine codon by cassette mutagenesis to allow expression as a fusion protein. The gene encoding an IL-6 analogue with the natural pattern of cysteines (rIL-6S) was constructed by cassette mutagenesis of the rIL-6S gene as described previously (12).

Genes in which individual pairs of cysteine codons were replaced by pairs of serine codons were constructed by a modification of the
recombinant circle polymerase chain reaction method (17). The plasmids p365 and p469, which contain the genes encoding rIL-6s and rIL-6, respectively, were each amplified with two separate primer sets. One primer set, designated a, was used to amplify the entire length of each plasmid with the exception of the region encoding Cys² and Cys¹⁰ in Cys² as described previously. The other primer set, designated b, encoded the region encoding Cys² and Cys¹⁰ in the case of p365. The products of this type of reaction are designated as p365a or p469a, respectively, and are used as templates in the reaction used in the isolation of the hybridoma growth factor activity of Interleukin-6 (12). Briefly, cells were washed and resuspended in binding medium at 10⁷ cells/ml. The appropriate amounts of labeled and unlabeled rIL-6 in 40 μl of binding buffer were added to 100 μl of cells in a microcentrifuge tube. ice-cold binding buffer was added to each tube to a final volume of 200 μl. The reaction was stopped by transferring the mixture to a 200-fold excess of unlabeled IL-6. After gel purification, products p365a and p469b were combined, denatured, and annealed to produce recombinant circles with two single-stranded gaps. The single-stranded DNA across the first gap encoded Cys² and Cys¹⁰ from p469, while the single-stranded DNA across the second gap encoded Ser⁷¹ and Ser⁷⁴ from p365. After gel purification, products p365a and p469b were combined, denatured, and annealed to produce recombinant circles with two single-stranded gaps. The single-stranded DNA across the first gap encoded Cys² and Cys¹⁰ as described previously (12). The rIL-6 variants were cleaved from β-galactosidase in the presence of a constant concentration of 125I-labeled IL-6 and were incubated with U937 cells (21). U937 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU of penicillin G/ml, 100 μg of streptomycin/ml. Cells were passed by dilution in fresh maintenance medium when cell densities reached 5 × 10⁵ cells/ml.

Binding assays-Binding studies were performed using a human monocytoid cell line (U937) (21). U937 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU of penicillin G/ml, 100 μg of streptomycin/ml. Cells were passed by dilution in fresh maintenance medium when cell densities reached 5 × 10⁵ cells/ml.

For use in binding studies, rIL-6 was purified to apparent homogeneity by filtration on a Sephadex G-10 (Aldrich) column and the specific activity of labeled rIL-6 was determined by measuring IL-6-stimulated secretion of fibrinogen from human (HEP 3B2) and rat (FAZA 967) hepatoma cells. Fibrinogen was quantitated using a sandwich ELISA specific for human or rat fibrinogen.

Quantitation of biologic activities was carried out as described previously. Briefly, activity in the hybridoma growth assay was defined as the concentration of IL-6 needed to cause half-maximal proliferation, while activity in the hepatocyte stimulation and B-cell differentiation assays was defined as the concentration of IL-6 needed to cause doubling or quadrupling of secretion of fibrinogen or IgM, respectively. For calculation of activities, dose-response curves were plotted on a semilogarithmic scale, and a computer program was used to fit the approximately linear portion of each curve with a second order polynomial. Activities for each assay are expressed as a percentage of the activity of nonmutated rIL-6 in the same assay.

Binding assays were carried out as described in (19). Briefly, cells were washed and resuspended in binding medium at 10⁷ cells/ml. The appropriate amounts of labeled and unlabeled rIL-6 in 40 μl of binding buffer were added to 100 μl of cells in a microcentrifuge tube, and the mixtures were rocked at 4 °C. After 3 h, 500 μl of ice-cold binding buffer were added to each tube, and the resulting mixture was transferred to another tube containing 500 μl of a mixture of 40% dioxylaldehyde and 60% dibutylphthalate. After centrifugation at 10,000 × g for 2 min, the supernatant was removed by aspiration, and the tips of the tubes, containing the pellets, were cut off and radioactivity measured in a γ counter. Specific binding was measured by subtracting the radioactivity bound in the presence of a 200-fold excess of unlabeled IL-6.

The maximal binding capacity of 125I-IL-6 was determined as described previously (22) by measuring the binding of a constant amount of IC50 of unlabeled IL-6 on increased concentrations of labeled IL-6. The specific radioactivity of labeled IL-6 was determined by self-displacement analysis as described previously (22). Briefly, 10⁵ U937 cells were incubated with 205,000 cpm of 125I-IL-6 and various amounts of either unlabeled IL-6 or unlabeled IL-6. Bound and free radioactivity was measured. A log-log plot of the ratio of bound/free activity versus the amount of labeled or unlabeled IL-6. The specific radioactivity was calculated as the ratio of the amount of radioactivity to the amount of unlabeled IL-6 required to obtain the same bound/free ratio. For calculation of bound/free ratios, the concentration of free ligand was determined by measuring the high radioactivity remaining on top of the phthalate mixture after the centrifugation. Data for Scatchard analysis and competition studies were generated by measuring binding in the presence of a constant concentration of 125I-IL-6.
varying concentrations of partially purified, unlabeled rIL-6 proteins. Scatchard and competition data were analyzed with the LIGAND program (23).

RESULTS

Construction of Genes Encoding rIL-6 Mutants—In order to study the functional role of individual disulfide bonds in human IL-6, we examined four classes of mutants of human IL-6: (a) a mutant containing the four cysteines of natural human IL-6 (Fig. 1A); (b) mutants in which all four of the natural cysteines were replaced by other amino acids (Fig. 1B); (c) mutants in which only Cys4 and Cys52 were replaced (Fig. 1C); and (d) mutants in which only Cys41 and Cys46 were replaced (Fig. 1D). Our primary goal was to selectively preclude the formation of individual disulfide bonds without disrupting other structural features of the protein. It was therefore desirable to replace the natural cysteines of human IL-6 with an amino acid which resembles cysteine as closely as possible in terms of size and chemical properties. In previous structure-function studies, disulfide bonds have most commonly been disrupted by replacement of cysteines with alanine or serine, which resemble cysteine in having a single-carbon side chain. However, the polarity of the cysteine side chain is intermediate between that of alanine, which is nonpolar and serine, in which the sulfur of the cysteine side chain is replaced with a more electronegative oxygen. While serine has been found to replace cysteine more often in corresponding proteins of homologous organisms (24), paired alanines seem a rational choice for replacement of disulfide bonds, which are nonpolar. Therefore, for each of the classes of mutants described above, we constructed one variant in which the appropriate cysteines were replaced with serines and another variant in which they were replaced with alanines. We also constructed a third variant for each class of mutants, in which cysteines were replaced with pairs of oppositely charged amino acids (aspartate and arginine). This latter group of mutants provided a means of assessing the functional consequences of nonconservative cysteine substitutions.

The genes encoding two of the variants described in this report, rIL-6 and rIL-6S, were assembled using synthetic oligonucleotides as described previously (12, 16). The genes encoding the other eight rIL-6 variants were constructed by mutagenesis of the genes encoding rIL-6 and rIL-6S. The rIL-6 protein, which contains the four natural cysteines of human IL-6, was used as a standard of comparison in all bioassays and binding studies.

As described above, one class of rIL-6 mutants consisted of three variants which contained no cysteines. In rIL-6A, rIL-6S, and rIL-6DR both pairs of cysteines were replaced by alanines, serines, or aspartate-arginine pairs, respectively. In the second class of mutants, formation of the first disulfide bond was prevented by replacing Cys41 and Cys46 with serines in rIL-6acc, with alanines in rIL-6acc, and with aspartate and arginine, respectively, in rIL-6acc. In the third class of mutants, formation of the second disulfide bond was prevented by replacing Cys41 and Cys46 with serines in rIL-6css, with alanines in rIL-6css, and with aspartate and arginine respectively, in rIL-6cdr.

Expression and Characterization of IL-6 Variants—All mutants were expressed as β-galactosidase fusion proteins in E. coli. This system allowed microgram quantities of rIL-6 to be expressed and partially purified from small bacterial cultures. The partially purified fusion proteins were cleaved with collagenase to release rIL-6, which could be tested directly in bioassays or competitive binding studies. The simplicity of the purification protocol allowed testing several independent protein preparations for each variant.

None of the physical properties on which purification depended were noticeably altered by replacement of cysteines. Although we had found previously that deletion of some 20-amino acid segments of rIL-6 increased the solubility of the IL-6/β-galactosidase fusion protein,2 this property was not affected by removal of cysteines. We also found that rIL-6 and rIL-6S display identical retention times during purification to homogeneity by reversed-phase high performance liquid chromatography, indicating that replacement of cysteines with serines did not alter hydrophobicity.

In order to determine whether appropriate disulfide bonds were present in our rIL-6 variants, representative proteins (rIL-6, rIL-6acc, rIL-6css, rIL-6S) were subjected to polyacrylamide gel electrophoresis under reducing and nonreducing conditions (gel not shown). Whereas the mobilities of these proteins were identical under reducing conditions, the mobilities of the cysteine-containing variants were increased relative to that of rIL-6S under nonreducing conditions. The apparent relative molecular masses of the nonreduced variants, based on their mobilities relative to standards, were 22.00 for rIL-6, 22.30 for rIL-6acc, 22.25 for rIL-6css, and 22.44 for rIL-6S. The decrease in apparent relative molecular mass of the cysteine-containing variants relative to rIL-6S indicates that intramolecular disulfide bonds give these mutants a more compact structure. As expected, this effect is most pronounced for rIL-6, which has the potential to form both of the disulfide bonds present in natural IL-6. The absence of improper intermolecular disulfide bridges is indicated by the fact that the intensity of Coomassie-stained protein bands was similar for reduced and nonreduced samples and by the absence of IL-6 multimer bands in nonreduced samples.

Effects of Disrupting Disulfide Bonds on Biologic Activity—The biologic activities of our rIL-6 disulfide mutants are summarized in Table I. Surprisingly, considering the evolutionary conservation of cysteines in IL-6, mutants in which

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the Cys45-Cys51 disulfide bond was disrupted (rIL-6ACC, rIL-6CSS, and rIL-6DCSSC) had biologic activities similar to those of the nonmutated control in all cases. There was no consistent correlation between the activities of these mutants and the type of amino acid used to replace Cys45 and Cys51.

In contrast, the biologic activities of mutants in which the Cys52-Cys41 bond was disrupted (rIL-6CCA, rIL-6CCS, rIL-6CTDC) were significantly reduced compared to those of the nonmutated control. This reduction in activity was most apparent in the assays using human cell lines. Whereas the activities of these mutants ranged from 3.5 to 66% in the assays using rodent cells, their activities ranged from 0.06 to 2.4% in the assays using human cells. This species specificity is similar to that reported previously for rIL-6S (12). In all assays, rIL-6CCA was more active than rIL-6CSSA, which in turn was more active than rIL-6CTDC. Despite the decreased activity caused by removal of Cys52 and Cys41, these mutants were consistently more active than the analogous cysteine-free variants (rIL-6A, rIL-6S, rIL-6DR). This indicates that, in the absence of the Cys41-Cys52 bond, the Cys45-Cys51 bond does play some role in maintaining biologic activity. The pattern of relative biologic activities for the cysteine-free mutants was similar to that described for mutants lacking only Cys51 and Cys41; that is, for any given assay, rIL-6A was more active than rIL-6S, which in turn was more active than rIL-6DR. As expected, these mutants also retained significantly more activity in rodent cells (0.8–52%) than in human cells (<0.01–0.8%).

Role of Disulfide Bonds in Binding of rIL-6 to Human Cells—The U937 cell line was chosen for use in binding studies for several reasons: (a) results obtained with a human cell line are more likely to provide information about the binding of human IL-6 to its receptor in vivo; (b) U937 cells have been reported to express a relatively high density (2800/cell) of IL-6 receptors (25); (c) U937 cells grow rapidly in suspension, making it relatively easy to obtain the large numbers of cells needed for binding assays; and (d) U937 cells show relatively low nonspecific binding of 125I-IL-6. The general applicability of results obtained with U937 cells to other types of human cells is supported by the fact that the affinity of recombinant human IL-6 for U937 cells is similar to that measured for other human cells (25). The biologic activity of IL-6 on U937 cells, as measured by inhibition of proliferation (26), is also comparable, in terms of the minimum effective concentration and the concentration range over which the effect is observed, to the activities we measure in HEP 3B2 and SKW6.4 cells.

Before using 125I-IL-6 for Scatchard analysis and competition studies, the amount of bindable protein present in the 125I-IL-6 preparation was determined, and its specific activity was calculated. The maximal binding capacity was determined by measuring the binding of a constant amount of labeled IL-6 to an increasing number of U937 cells (22). The extrapolated value of binding to an infinite number of cells indicates that approximately 50% of the labeled IL-6 retained its ability to bind specifically to U937 cells. A specific radioactivity of 106,000 cpm/ng was measured by self-displacement analysis (22), and a similar value was obtained by testing the labeled IL-6 in a hybridoma growth assay using 7TD1 cells.

The data for Scatchard analysis of rIL-6 binding were generated by incubating U937 cells in the presence of a constant concentration of 125I-IL-6 and increasing concentrations of unlabeled rIL-6. Scatchard analysis of the specific binding of 125I-IL-6 to U937 cells indicated the presence of 3790 binding sites/cell with a Kd value of 1.2 nM.

Because the effects of mutations on activity in human cells were relatively independent of the amino acids used to replace cysteines, competition studies were carried out with three representative mutants: rIL-6S, rIL-6SCS, rIL-6CSSS. The relative affinities of these mutants for the human IL-6 receptor were determined by displacement of labeled rIL-6 with unlabeled mutant proteins (Fig. 2). Kd values of each mutant were determined using a curve-fitting program (23). The apparent Kd values determined for mutants were 2.9 nM for rIL-6SCC, 380 nM for rIL-6CSS, and 760 nM for rIL-S. It should be noted that, because mutants rIL-6CSS and rIL-6D did not completely inhibit binding even at high concentrations, the standard error associated with the Kd values determined for these mutants is relatively large (>10% of the determined Kd). The minimum Kd values calculated for these mutants are 340 nM for rIL-6CSS and 680 nM for rIL-6S. The calculated affinities of these mutants generally paralleled their biologic activities on human cells. However, it is interesting to note that the Kd of rIL-6CSS was more than double that of the nonmutated control, despite the fact that the activity of this mutant was not reduced relative to the control.

**DISCUSSION**

Native human IL-6 forms two disulfide bridges which have been assigned as Cys45-Cys51 and Cys41-Cys52. The conservation of these cysteines in proteins homologous to human IL-6 (murine and rat IL-6, murine and human G-CSF, chicken myelomonocytic growth factor) suggests that they may be important in stabilizing the biologically active conformation of this cytokine. In order to gain a better understanding of the role which disulfide bonds play in the function of human IL-6 receptors (25), rIL-6CSSC, and rIL-6CSSS. The conservation of these cysteines in proteins homologous to human IL-6 (murine and rat IL-6, murine and human G-CSF, chicken myelomonocytic growth factor) suggests that they may be important in stabilizing the biologically active conformation of this cytokine. In order to gain a better understanding of the role which disulfide bonds play in the function of human IL-6.
IL-6, we examined the biologic activity and binding characteristics of rIL-6 mutants in which one or both pairs of constituent cysteines were replaced with other amino acids. Surprisingly, removal of the first disulfide bond by replacement of Cys"5 and Cys"54 did not significantly affect biologic activity. In contrast, replacing Cys"54 and Cys"54 caused a significant decrease in activity, especially in human cells. In general, however, mutants containing only Cys"5 and Cys"54 were more active than analogous cysteine-free mutants.

The apparently nonessential role of the Cys"54-Cys"51 bond of human IL-6 raises the question of why the constituent cysteine residues have been conserved in homologous cytokines through 200 million years of evolution. One possible explanation is that this bond has a function not detected by our bioassays. For example, the presence of the Cys"54-Cys"51 bond might increase the half-life of IL-6 in vivo by providing protection from degradative enzymes, either directly or by enhancing binding to α2-macroglobulin (27). The absence of such degradative enzymes in our bioassay system would prevent the detection of such an effect. On the other hand, the Cys"54-Cys"51 bond might play a role in folding or secretion of IL-6 from mammalian cells and would, therefore, be functionally unimportant in our bacterial expression system.

Another interesting possibility is that the first disulfide bond of IL-6 has been conserved because it is resistant to loss by random mutation. Presumably this bond arose because it was important for activity in an ancestral cytokine which gave rise to IL-6, G-CSF, and myelomonocytic growth factor. Support for this idea comes from a published report that the analogous disulfide bond is required for biologic activity in human G-CSF (15), although the data on which this report is based have not yet been published. If the only function of this bond was to stabilize the biologically active conformation of the cytokine, it is difficult to explain why this bond should be conserved in mouse, rat, and human IL-6 for more than 60 million years after losing this function. Generally, the loss of such a disulfide during evolution would involve substitution of both constituent cysteines with other residues, as exemplified in the family of serine proteases. The low statistical probability of mutating both cysteines simultaneously suggests that this substitution process is sequential. However, if the presence of an unpaired cysteine in the Cys"54 or Cys"51 position of IL-6 is detrimental to expression or activity, as has been reported for several other engineered proteins, than natural selection would prevent substitutions at either of these positions. Thus it is possible, in theory at least, that a nonfunctional disulfide bond may have become “locked” into the structure of IL-6.

There are several possible explanations for the greater functional importance of the second disulfide bond of human IL-6. One possibility is that the second disulfide bond makes a relatively greater contribution to the thermodynamic stability of the active conformation of IL-6. In theory, disulfide bonds stabilize the properly folded conformation of a protein by lowering the entropy of the unfolded state. Using a previously described method (28), it can be calculated that the two disulfide bonds of human IL-6 should theoretically decrease the entropy of the unfolded state by 8.1 kcal/mol at 37 °C. Because this stabilizing effect increases with the length of the loop created by a disulfide bond, the theoretical decrease in entropy due to the Cys"54-Cys"54 bond (4.3 kcal/mol) is less than that due to the Cys"54-Cys"51 bond (3.8 kcal/mol). However, the relatively small difference in the values calculated for these bonds suggests that the importance of the second disulfide arises from its position in the protein, rather than from a general stabilization of the folded state.

In the absence of crystallographic data, only indirect evidence can be used to support the idea that the functional importance of the second disulfide bond of IL-6 arises from its particular location in the protein. Despite the lack of an x-ray structure for IL-6, a general structural model has been proposed for a group of cytokines including IL-6, based on similarities in predicted secondary structure, matching locations of known exon boundaries, and receptor homology (29, 30). The members of this group, referred to as helical cytokines, are proposed to adopt a conformation similar to that of growth hormone and prolactin. This proposed structure consists of a bundle of four antiparallel α helices, designated A to D, and associated connections: long A-B and C-D loops and a short B-C loop. A putative receptor-binding surface, which includes parts of helix A, helix D, and the A-B connecting loop, has been proposed for these helical cytokines based on mutagenic studies of growth hormone (31). If an analogous receptor binding region exists for IL-6, it would include the second disulfide bond, which would anchor the receptor-binding portion of the A-B loop to helix B. In contrast, the first disulfide bond of IL-6 would be located in a region of the A-B loop outside of the proposed binding region. Interestingly, the region of the growth hormone A-B loop which is proposed to be analogous to the first disulfide loop of IL-6 is alternatively spliced out at the DNA level with no deleterious effect on structure. One argument against the general applicability of this model, however, is that the fact that the first disulfide bond of G-CSF is required for activity (15). Hopefully, future studies will help to better define the underlying principals governing the structure-function relationships of IL-6 and other cytokines.

Acknowledgments—We thank Michelle Emfinger for valuable technical assistance and Kang Zhang for oligonucleotide synthesis.

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