Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 4067–4071, May 1993
Biochemistry

Saturation mutagenesis of the human interleukin 6 receptor-binding site: Implications for its three-dimensional structure

R. SAVINO, A. LAHM, M. GIORGIO, A. CABIBBO, A. TRAMONTANO, AND G. CILIBERTO*

Istituto di Ricerche di Biologia Molecolare, P. Angeletti, Via Pontina Km 30.600, 00040 Pomezia, Rome, Italy

Communicated by Joseph Gall, January 22, 1993

ABSTRACT **Interleukin 6 is a 184-aa polypeptide postulated to belong to the class of helical cytokines. We built a three-dimensional model of human interleukin 6 based on the similarity of its hydrophobicity pattern with that of other cytokines and on the x-ray structure of growth hormone, interleukin 2, interleukin 4, interferon β, and granulocyte–macrophage colony-stimulating factor. The resulting model is a bundle of four α-helices and suggests possible alternative conformations for the 9 C-terminal amino acids; in this region, the importance of Arg-182 and Met-184 for biological activity has been demonstrated [Lutticken, C., Kruttgen, A., Moller, C., Heinrich, P. C. & Rose-John, S. (1991) *FEBS Lett.* 282, 265–267]. Therefore, we generated a large collection of single-amino acid variants in residues 175–181. Analysis of their biological activity in two systems and the receptor binding properties of a subset of the mutants indicates that the entire region is involved in forming the receptor binding surface and supports the hypothesis that this region does not assume an α-helical conformation. Remarkably, we also found a mutant with receptor affinity and biological activity much higher than wild type; the potential therapeutical value of this finding is discussed.**

Interleukin (IL) 6 is a multifunctional cytokine produced by a variety of cell types that acts as a differentiation and growth factor for cells of the immune system, hepatocytes, kidney mesangial cells, hematopoietic stem cells, keratinocytes, and neurons (1, 2). Some aspects of the interaction of IL-6 with target cells have been elucidated through the cloning of two transmembrane molecules that constitute the IL-6 receptor system: the low-affinity 80-kDa IL-6 receptor (3) and the signal transducer protein gp130 (4). However, the features of the interaction between these molecules have remained largely unknown, due to lack of information about their three-dimensional structure and of systematic site-directed mutagenesis. After the x-ray structure of porcine growth hormone (pGH) had been determined (5), Bazan (6) carefully analyzed the hydrophobicity patterns of several cytokines including IL-6 binding to class I receptors. He predicted that these molecules form an antiparallel four-helix bundle, with the same connectivity observed in pGH. The general folding topology of an antiparallel four-helix bundle has been proven to be shared by all helical cytokines for which x-ray or NMR structures have been determined (7–11), although significant local differences can be observed, hinting at the possible existence of structural subclasses.

To assess the compatibility of human (h) IL-6 with this folding pattern, we have built a three-dimensional model of hIL-6 that shows that the predicted topology can be assumed by the hIL-6 sequence with reasonably good internal packing and agreement with the available experimental data. The helix boundaries in our model do not precisely match those previously predicted. More importantly, it is possible that the functionally essential C-terminal region of the molecule, proposed as being included in helix D (6, 12), assumes a nonrepetitive structure similar to that observed in human granulocyte–macrophage colony-stimulating factor (hGM-CSF) (8). To test this possibility, we performed saturation mutagenesis on this region of the molecule and tested the mutants in two biological assays on murine and human cells. Our data show that residues located in the C-terminal region, in addition to those previously identified, are involved in IL-6 receptor binding and are consistent with these residues being in a nonhelical conformation.

MATERIALS AND METHODS

Sequences used are from Releases 21.0 and 31.0 of the Swiss-Prot and EMBL (pIL-6) sequence data bases, respectively. Data bank codes are as follows: hIL-6, IL6_HUMAN; murine (m) IL-6, IL6_MOUSE; rat (r) IL-6, IL6_RAT; pIL-6, SSPRO; human granulocyte (hG) CSF, CSF3_HUMAN; mG-CSF, CSF3_MOUSE; chicken myelomonocytic growth factor, MGF_CHICK. The numbering used for the hIL-6 sequence is shown in Fig. 1. For the multiple sequence alignment, the program PILEUP in the GCG package was used with default parameters (13). The coordinates of hGM-CSF (8) and interferon β (7) are prerelease entries 1GMF and 1IFA of the Brookhaven Protein Data Bank (14). Models were built using the program INSIGHT (15).

Generation of Substitution Mutants. The generation of substitution mutants in positions 177, 178, and 179 has been described (16). Mutants in positions 175, 176, 180, and 181 were generated using the same PCR strategy illustrated in figure 1 of Fontaine *et al.* (16). Some of the mutant cDNAs were also subcloned into *Escherichia coli* expression vector pT7.7 (17).

Expression of Mutant Proteins, Bioassays, and *in Vitro* Binding Assays. Mutant proteins were expressed, as described, either by production in *E. coli* (18, 19) or by *in vitro* transcription and translation (19), and assayed for their ability to stimulate transcriptional activation of a reporter gene on human hepatoma cells (Hep 3B) and growth of mouse B-cell hybridoma 7TD1. Human Hep 3B cells were transfected with the plasmid −219 CRP–CAT, containing the IL-6 responsive element of the C-reactive protein promoter gene, by using the calcium phosphate precipitation technique (20), and induced with increasing amounts of mutant or wild-type (wt) hIL-6. Cell extracts and chloramphenicol acetyltransferase assays were performed as described (21). IL-6 hybridoma assays were performed as described (19, 22). All assays were carried out in duplicate or triplicate, with serial dilutions of two to four preparations of each mutant. The activities of the mutants, expressed as a percentage of wt hIL-6, were calculated as the ratio between the amount of wt hIL-6 and the

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Abbreviations: GH, growth hormone; IL, interleukin; GM-CSF, granulocyte–macrophage colony-stimulating factor; G-CSF, granulocyte CSF; h, human; p, porcine; r, rat; m, murine; wt, wild type.

*To whom reprint requests should be addressed.

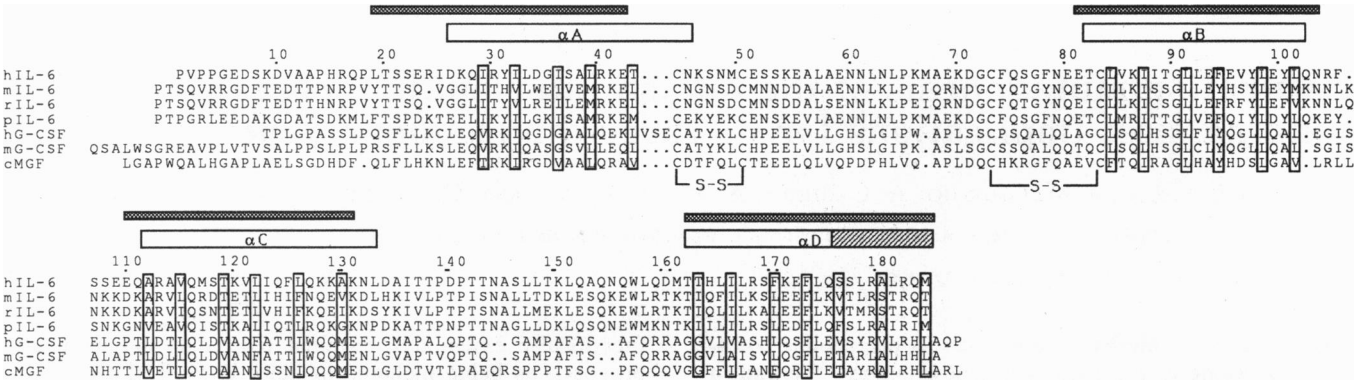

FIG. 1. Multiple alignment of the seven cytokine sequences. Only the mature peptides (as stated in the corresponding data base entry) are shown (for pIL-6, the length of the mature peptide was decided by homology to the other IL-6 sequences). The conserved hydrophobic pattern indicated by boxed residues predicts four helices (A, B, C, and D) represented by open bars. The dashed part of helix D corresponds to the last 9 C-terminal residues for which an alternative conformation is possible. Shaded bars indicate the secondary structure assignment according to Bazan (12). cMGF, chicken myelomonocytic growth factor.

amount of the various mutants necessary to give half-maximal stimulation in each assay. *In vitro* binding assays and competition experiments were performed as described by Fiorillo *et al.* (19).

RESULTS

Model Building. Fig. 1 shows a multiple alignment of hIL-6 with the only other cytokine sequences in the sequence data bases showing significant amino acid similarity (17% or more identical residues) over the entire length of the mature polypeptide. From the conserved hydrophobicity pattern indicated in the figure, we derived a secondary structure assignment consisting of four helices, as predicted by Bazan (6, 12), but with some differences in their boundaries and lengths. To select the most likely orientation of the four helices in the bundle, we obtained the best superposition of the helix axes of the two available structures (Protein Data Bank entries, 1GMF and 1IFA). The superposition was used as a guideline throughout the modeling. Intermediate values of interhelix angles between the two structures were taken as a tentative starting point. The sequence was threaded through the helices, selecting the register that gave the best internal hydrophobic packing. Both the interhelix angles and the side-chain conformations (initially set to the most frequent rotamers) were subsequently manually optimized and subjected to a limited energy refinement (15) (Tables 1 and 2). The solvent-accessible surface of the final model did not reveal any sizeable internal cavity.

Due to the lack of both experimental data and significant sequence similarity to the helical cytokines of known structures, we could not reliably model the long loops A–B and C–D connecting the hIL-6 helices (Fig. 2). However, the presence of two disulfide bridges in hIL-6, between Cys-45 and Cys-51 and between Cys-74 and Cys-84 (23), allowed us to tentatively position the first 5 and the last 9 residues of the A–B loop. The predicted length of the hIL-6 A–B loop is ≈10 aa longer than the equivalent regions in hGM-CSF, hIL-4, or hIL-2, where the A–B and C–D loops pack compactly, forming a small β-sheet, whereas in pGH, hGH, and interferon β, the A–B loop has about the same length as hIL-6 (30–35 residues) and is not involved in tight interactions with the C–D loop. Therefore, we would expect a conformation more similar to the latter for the hIL-6 A–B loop, since it can more easily accommodate deletions or insertions.

Bazan (12) proposed that helix D extends up to the C terminus of IL-6 and that a major receptor-binding determinant (called the D2 motif) is situated in the middle of helix D. The structure of the recently determined complex between hGH and its receptor (extracellular domain of the hGH receptor) shows that in this molecule the receptor-binding site extends from the D2 motif toward the N-terminal part of helix D (9). In contrast, in hIL-6, residues C-terminal to the D2 motif are known to be important determinants in receptor binding (24). Therefore, a different conformation for the C-terminal region of IL-6 cannot be excluded. Our model does not allow us to discriminate between the two alternative conformations schematically shown in Fig. 2. This question was, therefore, addressed through site-directed mutagenesis.

Saturation Mutagenesis of the hIL-6 C Terminus. To gain insight about both the structure and function of the hIL-6 C-terminal region, we generated a series of single-amino acid substitutions in this region. We focused our attention on residues 175–181, Gln-Ser-Ser-Leu-Arg-Ala-Leu. The last 3 aa Arg-182, Gln-183, and Met-184 have been mutated by others (24, 25).

Mutants were generated at the cDNA level by PCR and sequenced over the entire PCR-amplified region. For analysis of biological activity, we selected 45 variants, of which 3 unexpectedly had 2-aa substitutions instead of 1: 169Asn/177Val, 105Ile/180Thr, and 108Asn/181Cys (see Fig. 3). For each position, 5–8 mutants were examined for their biological activity to identify the features necessary for proper functioning.

Biological Activity of hIL-6 Mutants. Wild-type and mutant hIL-6 molecules were obtained by *in vitro* transcription–translation and the biological activities of the mutants were determined in two assays on cells of human and murine origin. hIL-6 activity on human cells was measured by its ability to enhance transcription from acute-phase gene pro-

Table 1. Interhelix angles and distances in the final model

	Helix B		Helix C		Helix D	
Helix	ϑ, deg.	d, Å	ϑ, deg.	d, Å	ϑ, deg.	d, Å
A	36.8	14.6	−152.2	9.6	−160.8	9.2
B	—	—	−166.0	9.0	−149.4	10.5
C	—	—	—	—	30.2	12.5

ϑ, Interhelix angle; d, distance; deg., degrees.

Table 2. Composition of consecutive layers in bundle core

Layer	Helix A	Helix B	Helix C	Helix D
1	Ile-32	Leu-91	Leu-126	Leu-174
2	Ile-36	—	Leu-122, Ile-123	Phe-170
3	Ile-39	Leu-98	Thr-119	Leu-167
4	Thr-43	Leu-101	Val-115	Thr-163

Layers are approximately one helical turn apart.

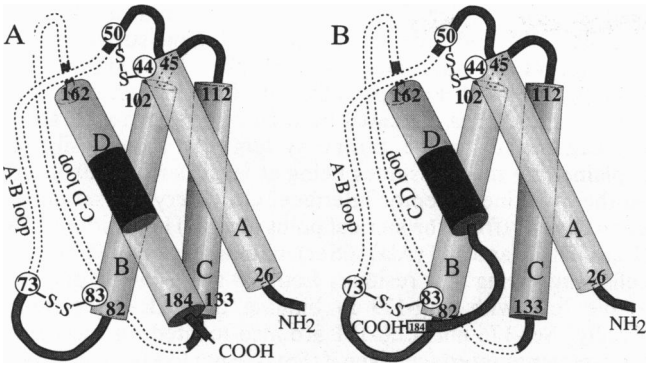

FIG. 2. Schematic representations of the two hIL-6 models with helices represented by cylinders. The shaded area on helix D corresponds to the D2 motif proposed by Bazan (12). Residue numbers correspond to the boundaries of the four helices as given in Fig. 1. The conformation of the A–B and C–D loop has not been assigned, as indicated by the broken lines.

moters in cells of hepatic origin (26); therefore, mutants were tested for their ability to induce chloramphenicol acetyltransferase (CAT) activity from a human C-reactive protein promoter–CAT fusion transfected into the human hepatoma cell line Hep 3B (19). The results obtained in this system are particularly relevant because they depend on the efficient interaction of the cytokine with the homologous receptor system. Mutants were also tested in the classical hybridoma growth factor assay for their ability to stimulate growth of the hIL-6-dependent murine hybridoma cell line 7TD1 (22). The results are shown in Fig. 3. For mutants of Ser-177, Leu-178, and Arg-179, the activity on mouse cells has been described (16) and is reported here to facilitate the comparison of their activity in the two systems.

Gln-175 can be replaced with several other residues (with the exception of Asp) without drastic effects (residual activity, >30%) on mouse cells. On the contrary, small side chains (Gly, Ala, and Asn) are not well-tolerated in human cells (Fig. 3). All mutations of Ser-176 have no detrimental effect. The substitution Ser176Arg is the only one found to be significantly more active than wt hIL-6 (370 ± 90%) on human cells. The most active protein on mouse cells is Ser176Val (480 ± 10% compared to wt hIL-6). Interestingly, Val is the amino acid found in the corresponding position in mIL-6 (Fig. 1) and the mutant behaves exactly like mIL-6, which shows a specific activity roughly 5-fold higher than hIL-6 on murine cells (19). Ser176Pro shows almost full activity when tested on human cells (73 ± 17%) and even higher levels of activity than wt hIL-6 when tested on mouse cells (150 ± 48%). The activity of mutants with substitutions in position 177 and 178 on mouse cells has been described (22) and is included in Fig. 3. Surprisingly, the behavior of these mutants is quite different when they are tested on human cells. At position 177, only Ser177Ala and Ser177Thr preserve wt activity; mutations Ser177Val, Ser177Met, and Ser177Cys reduce biological activity; and mutation Ser177Ile totally abolished activity. At position 178, mutants show an inverse correlation between activity on human cells and bulkiness of the substituent side chain: mutation Leu178Thr is fully active, Leu178His retains only 16% activity, and Leu178Tyr is totally inactive. We have recently shown that the positive charge of Arg-179 is an important determinant in the receptor binding site of hIL-6 (16). Our data now indicate that Ala-180 is also very important, and whereas the conservative Ala180Ser substitution is well-tolerated in both systems, the remaining mutations are all detrimental. Finally, for Leu-181, all amino acid substitutions but Pro are active to various extents.

From these results the following general conclusions can be drawn. (*i*) Introduction of a negative charge anywhere in

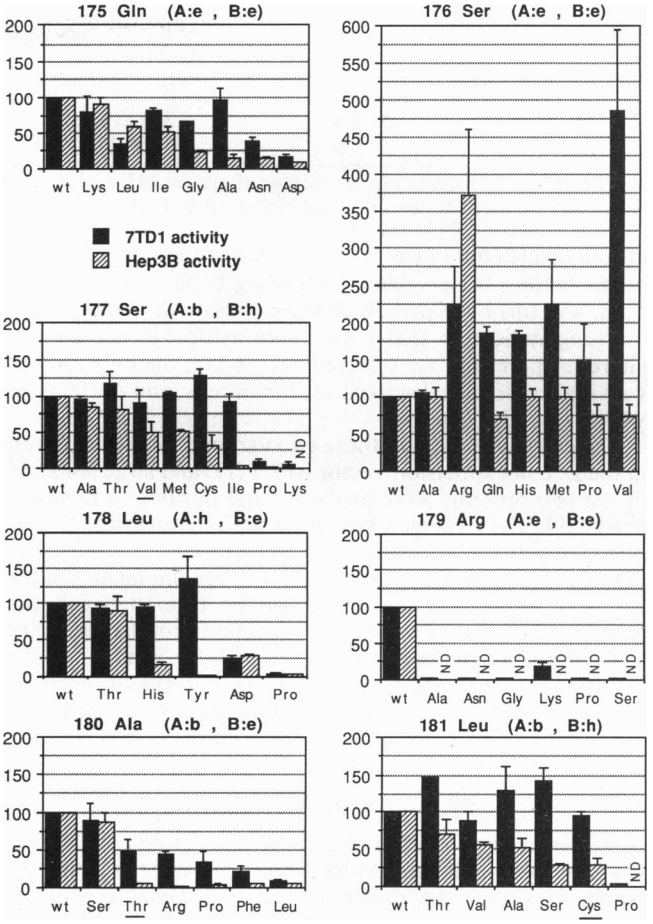

FIG. 3. Biological activity of hIL-6 single-amino acid mutants on mouse hybridoma cells (solid bars) and human hepatoma cells (hatched bars) expressed in percentage of hIL-6 of wt activity. The wt residue in the hIL-6 sequence is shown above each histogram with individual amino acid substitutions indicated below the corresponding bar. The three unexpected double substitutions (see text) Ser177Val/Ser169Asn, Ala180Thr/Phe105Ile, and Leu181Cys/Ser108Asn are underlined. Symbols in parentheses after the residue type refer to the predicted degree of accessibility according to models A and B, respectively. b, Buried; h, half-buried; e, exposed.

the analyzed region always causes a significant drop of biological activity in both cell lines (Fig. 3). (*ii*) With only two exceptions (Gln175Leu and Ser176Arg), the effects of the mutations are more detrimental when the mutants are tested on human cells than on mouse cells. This effect has been observed before (19, 23) and may be attributable to a different degree of tolerance of the murine vs. the human receptor complex. It is well-established that although the mouse 80-kDa receptor efficiently binds hIL-6 and mIL-6, the human receptor recognizes only the human cytokine (19). (*iii*) Single Pro substitutions at any of six of the seven positions are sometimes compatible with residual biological activity (as shown by the behavior of Ser176Pro and Ala180Pro), not to be expected if the region is folded as an α-helix (see *Discussion*).

Changes in Activity on Human Cells Correlate with Variations in the Affinity for the hIL-6 Receptor. Our mutagenesis of the cluster of hIL-6 residues 175–181 generates variants with unaltered, strongly impaired, or improved biological activity on human cells. To establish whether these changes are related to modifications in hIL-6 receptor recognition, we selected some of these mutants for a detailed analysis of their binding to purified recombinant hIL-6 receptor (19). We wanted to compare mutants that (*i*) had the same amino acid

Table 3. Comparison of receptor binding and biological activity

Mutation	Biological activity, % of wt hIL-6	hIL-6R binding, % of wt hIL-6
Ser176Arg	370 ± 90	320 ± 87
Ala180Arg	<0.1	<0.1

Affinity for the soluble hIL-6 receptor of mutant hIL-6 produced in *E. coli* compared to the observed biological activity.

introduced (to minimize variations due to the type of substitution made), (*ii*) produce contrasting behaviors in human cells, and (*iii*) have preserved their activity on mouse cells, a strong indication that their conformation is not grossly altered. Therefore, we selected Ser176Arg, the only mutant with higher biological activity in human cells, and Ala180Arg, which is totally inactive.

The cDNAs coding for these two variants were transferred in the *E. coli* expression vector pT7.7 (17) and large amounts of the two proteins were produced and purified to homogeneity (19). They showed biological activity identical to that obtained with the *in vitro*-synthesized proteins (Table 3). The binding of the mutant proteins to the recombinant soluble hIL-6 receptor was calculated as the ratio between the amount of unlabeled wt hIL-6 and mutant protein necessary to displace 50% of receptor-bound wt ^{125}I-labeled hIL-6. The results (Table 3) show that there is agreement between biological activity of the mutant on human Hep 3B cells and their affinity for the soluble hIL-6 receptor.

DISCUSSION

Our present knowledge of structure–function relationships in the large group of molecules known as helical cytokines emphasizes the importance of the C-terminal region for receptor binding and biological activity (27–30). This has also been demonstrated for hIL-6, by immunological evidence [binding of neutralizing monoclonal antibodies (31)] and by limited site-directed mutagenesis (24). Our three-dimensional model of hIL-6 shows that this important region of the molecule can have one of two alternative conformations: either the terminal helix D extends to (or close to) the very end of the chain, as seen in pGH, hGH, hIL-4, or hIL-2 (5, 7, 9–11), or helix D is one or two turns shorter as in hGM-CSF (8) and the last part of the chain adopts a nonhelical conformation. In the absence of x-ray crystallographic or NMR studies, our saturation mutagenesis approach was directed to select one of these two possibilities.

Our data, besides showing that the C-terminal region carries several determinants of receptor binding, either through direct contacts or by stabilizing the local structure of the cytokine, strongly favor the alternative that the last 9 residues (aa 176–184) are not folded as a continuous α-helix. This conclusion is based on the following evidence:

(*i*) Ser176Pro has normal or even higher than wt activity in human and murine cells, respectively. Moreover, the mutant protein can be immunoprecipitated as well as wt hIL-6 by MabB1 (data not shown), which recognizes a discontinuous epitope formed by amino acids Pro-18 to Asp-26 in the N terminus and Leu-181 to Met-184 at the C terminus (31). Therefore, from all the data available so far, this mutation does not seem to alter or change the relative positioning of the N and C termini, an observation that is hardly compatible with residue 176 being in a canonical helical environment. α-Helices containing prolines, in general, show kinks (32). For hIL-6, even a small kink would significantly alter the local structure around Pro-176 and affect receptor binding and possibly recognition by the conformational MabB1 (31).

(*ii*) Our previous mutagenesis studies showed that Arg-179 is on the hIL-6 surface and is involved in receptor binding (16). The results presented here indicate that the adjacent residues Ser-176, Ser-177, Leu-178, Ala-180, and Leu-181 are also exposed and face the receptor binding surface. The behavior of mutants such as Ser177Ile, Leu178Tyr, and Ala180Arg, which are not active at all in human cells but correctly folded as indicated by their normal or moderately reduced activity in the murine system, can most easily be explained by these residues being at least partially exposed on the cytokine–receptor interface. Only very conservative substitutions (from the sterical point of view) in positions 178 (Leu178Thr) and 180 (Ala180Ser) maintain activity in human cells, suggesting that residues Leu-178 and Ala-180 play an active role with Arg-179 in binding the hIL-6 receptor. Finally, Ser-176 and Leu-181 are also located on the cytokine–receptor interface, even if they are not likely to be direct contact points in the human system because of the high number of substitutions that maintain activity in human cells. This is suggested by the behavior of mutants Ser176Val and, to a lesser extent, Leu181Thr: in both cases restoring the murine sequence in the respective positions causes a specific increase in activity in mouse cells. All these data favor our model B (the nonhelical conformation), which also better fits the mutation data with respect to the predicted accessibility of the individual residues (Fig. 3).

While this report was in preparation, Leebeek *et al.* (33) described a set of mutants on the hIL-6 15 terminal amino acids, in which one, two, or at most three substitutions per each position were generated and analyzed using similar assay conditions. The data on the biological activity of their mutants are compatible with our model. In particular, they also find that the Ser176Pro mutant (Ser177Pro in their numbering) is fully active, contrary to previous reports by others (24). In addition, their data confine the receptor binding site to residues C-terminal to Gln-175.

In our collection of 45 hIL-6 mutants, we also find a strong up mutant (Ser176Arg, 3- to 4-fold higher activity on human cells). Receptor binding studies with this mutant confirm that increased activity is due to increased receptor affinity. This result, while strengthening the hypothesis that Ser-176 is on the cytokine–receptor interface, is of practical interest, given the potential of using hIL-6 in the therapy of neoplastic diseases and thrombocytopenia (34). The clinical use of receptor super binders, such as our Ser176Arg, therefore, could allow a decrease in the dosage of cytokine administered to the patient.

Our model has been helpful in designing the mutants described in this report that allowed us to choose between two possible conformations for the C-terminal part of the molecule. We intend to follow a similar strategy to solve the ambiguities that still remain about the A–B and C–D loops and to extend the mapping of the binding site to other parts of the hIL-6 molecule to obtain a more complete picture of the interactions with the hIL-6 receptor. The results from homolog scanning mutagenesis (19) indicate that there are also receptor binding determinants in the region of residues 28–92, which, in our model, is located in the end part of the A–B loop. In hGH, hIL-2, and hGM-CSF, the end part of the A–B loop is indeed part of the receptor binding site (9, 27, 35). Interestingly, in hIL-6, Cys-73 and Cys-83, which form the only disulfide bridge essential for biological activity, also map in this region (23). Engineering additional disulfide bridges at appropriate positions into the IL-6 molecule could be one way to probe the closeness of parts of the structure to test our model.

Once the coordinates of the hGH–receptor complex or of a similar complex are available, it might also be possible to extend the prediction to the way in which the cytokine binds to its receptor, an even more interesting question. The extensive hydrophobic interactions between receptor loops and cytokine in hGH–receptor involve unusual partners such as Trp and Asp or Lys and Arg (14). This suggests that surface

complementarity, rather than charge complementarity, is the predominant mode of recognition and demonstrates that, even with the knowledge of the detailed three-dimensional structure of one cytokine–receptor complex, to predict those contacts for other cytokine–receptor systems will not be a trivial problem.

We thank Janet Clench and Maurizio Sollazzo for critically reading the manuscript, Yves Cully for art work, Veronique Fontaine for experimental support, and Franco Di Padova for the gift of MabB1. This project was supported in part by EEC Grant Bridge B1OT-CT91-0260 and by a "Consiglio Nazionale delle Ricerche (CNR)" grant Biotechnology and Bioinstrumentation to G.C.

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