Characterization of an Antagonist Interleukin-6 Dimer by Stable Isotope Labeling, Cross-linking, and Mass Spectrometry*

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The homodimeric form of a recombinant cytokine interleukin-6 (IL-6D) is known to antagonize IL-6 signaling. In this study, spatially proximal residues between IL-6 chains in IL-6D were identified using a method for specific recognition of intermolecular cross-linked peptides. Our strategy involved mixing 1:1 15N-labeled and unlabeled (14N) protein to form a mixture of isotopically labeled and unlabeled homodimers, which was chemically cross-linked. This cross-linked IL-6D was subjected to proteolysis by trypsin and the generated peptides were analyzed by electrospray ionization time-of-flight mass spectrometry (MS). Molecular ions from cross-linked peptides of intermolecular origin are labeled with [12C/12C/12C] + [15N/15N/15N] + [14N/14N/14N] yielding readily identified triplet/quadruplet MS peaks. All other peptide species are labeled with [15N/15N] + [14N] yielding doublet peaks. Intermolecular cross-linked peptides were identified by MS, and cross-linked residues were identified. This intermolecular cross-link detection method, which we have designated “mixed isotope cross-linking” MIX may have more general application to protein-protein interaction studies. The pattern of proximal residues found was consistent with IL-6D having a domain-swapped fold similar to IL-10 and interferon-γ. This fold implies that IL-6D-mediated antagonism of IL-6 signaling is caused by obstruction of cooperative gp130 binding on IL-6D, rather than direct blocking of gp-130-binding sites on IL-6D.

The interleukin-6 (IL-6) cytokine plays a critical role in host defense mechanisms such as T-cell activation, stimulation of B-cell differentiation, acute phase induction in hepatocytes, nerve cell differentiation, and osteoclast turnover (1). Abnormal IL-6 production is associated with a variety of diseases (2) such as rheumatoid arthritis (3), AIDS (4, 5), osteoporosis (6, 7), psoriasis (8), multiple myeloma (9, 10) and Kaposi’s sarcoma (11). Thus the interactions between IL-6 and its associated receptors, the transmembrane glycoproteins IL-6R and gp130 (11), are involved in many diseases such as rheumatoid arthritis (3), AIDS (4, 5), osteoporosis (6, 7), psoriasis (8), multiple myeloma (9, 10) and Kaposi’s sarcoma (11). These interactions involve the formation of a 2:1 complex comprising IL-6, IL-6R, and gp130.

Previously, we have shown that a dimeric form of recombinant IL-6 (IL-6D) is a potent antagonist for IL-6 signaling (16). Recombinant IL-6D binds tightly to soluble IL-6R (sIL-6R) (17). In contrast to the ternary IL-6-sIL-6R complex, IL-6D-sIL-6R complexes bind gp130 weakly and do not show significant biological activity in the signal transducer and activator of transcription 3 (STAT3) phosphorylation assay (16). Natural (glycosylated) human IL-6 is also known to form a dimer that makes up a substantial part of IL-6 in blood or fibroblast secretions (17–19) and has also been shown to interact with membrane-bound IL-6R (15, 20, 59). Recently, glycosylated natural human IL-6, identified by immunoblotting and size exclusion chromatography, was shown to be a survival factor secreted by epithelial cells that inhibited the apoptosis of B-chronic lymphocytic leukemic cells (21). Significantly, recombinant human IL-6D, from Escherichia coli, has been shown to act as a survival factor in a similar way (21). Taken together, these results suggest that natural and recombinant IL-6D may have similar biological activity.

Elucidation of the IL-6D structure will be critical to understanding the basis of its antagonistic properties. Whereas the structure of IL-6D is known to be a 4-helical bundle (22), the structure of IL-6D is unknown. Previous biochemical studies of the sedimentation properties and the unfolding-dissociation relationship of IL-6D (23) have shown it is likely to form a metastable domain-swapped dimer (24, 25) in which adjacent subunits have the IL-6 structure, but contain interchanged α-helical bundle domain elements.

Here, we investigate the arrangement of domain-swapped IL-6D chains within IL-6D using a technique based on cross-linking and mass spectrometry. Although the established techniques of x-ray crystallography and NMR spectroscopy yield high resolution data, often this takes months or years to obtain (26, 27). Techniques in mass spectrometry (MS) combined with cross-linking (28–30) or chemical labeling techniques such as hydrogen/deuterium exchange (31, 32) have been evaluated for rapid low-resolution three-dimensional study of proteins (30) or protein complexes (33, 34). Cross-linking/MS methods involve chemically or photochemically cross-linking a protein complex (35), followed by digestion of the cross-linked complex and MS analysis of the resulting peptide mixture (36). Cross-linked peptides can be identified by parent ion mass and/or the fragmentation pattern produced by tandem mass spectrometry (MS/MS), thereby locating adjacent protein regions and enabling assembly of low-resolution models of proteins or protein complexes. Cross-linking/MS experiments are

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Generally fast and do not require large quantities of protein (30). For these reasons, cross-linking/MS methods have potential as a low-resolution counterpart to x-ray or NMR methods for rapid determination of interacting surfaces between proteins (29) and the topology of proteins within complexes (33).

Despite the advantages of cross-linking methods, namely high molecular weight capability, speed, and the small quantities of protein required, the large number of peptide species that are seen from the digestion of cross-linked proteins makes it difficult to identify relevant intermolecular cross-linked peptides from MS data. This problem has been partially addressed by “tagging” methodologies that allow rapid visual MS location of cross-linked species within complex peptide mixtures (34, 37–39). For example, the use of a 1:1 mixture of undeuterated and deuterated (d5/d4-labeled) cross-linking reagent readily allows mass spectrometric detection of all cross-linked species by the presence of d5/d4-isotope tags (39). However, for studying interactions between proteins, even these tagging methodologies fail short in that they fail to distinguish inter- and intra-cross-linked peptides. This results in cross-linked peptide species being tagged that do not yield useful information on intermolecular interactions, such as intramolecular cross-linked peptides or peptides modified by partially hydrolyzed species being tagged that do not yield useful information on cross-linked peptides. This results in cross-linked peptide intra-

Here we present a method for visualizing intermolecular cross-linked peptides in the IL-6 homodimer that we have designated mixed isotope cross-linking (MIX). Applied to IL-6D, the MIX method requires preparation of uniformly 15N-labeled and unlabeled (14N) IL-6, combined as a 1:1 mixture and re-associated to form a population of 14N-, mixed 14N/15N-, and 15N-labeled IL-6D. Cross-linking and mass spectrometric peptide mapping on this mixture allows intermolecular cross-linked peptides to be identified easily as they form distinctive triplet or quadruplet mass spectrum peaks because of the distribution of 14N- and 15N-labeled peptides within these cross-linked peptides. In contrast, all intramolecular cross-linked and noncross-linked peptides are seen as doublet mass spectrum peaks. This ability to discriminate between inter- and intra-cross-linked species makes the MIX technique a uniquely useful new tool for studying intermolecular interactions. We describe the application of this technique to determine proximal intermolecular residues within the homodimeric form of IL-6 and to deduce the mode of three-dimensional domain swapping, based on the known structure of monomeric human interleukin-6 (22).

EXPERIMENTAL PROCEDURES

Materials—Trifluoroacetic acid (HPLC/Spectro Grade) and bis(sulfosuccinimidyl)suberate (BS3) were from Pierce. Sequencing grade trypsin (EC 3.4.21.3) was from Boehringer Biochemicals. HPLC-grade solvents were from Mallinckrodt, and all other buffers and reagents (Analar grade) were from BDH. All buffers and solutions were prepared with deionized water purified by a tandem Millipore Q and Milli-Q RO system (Milipore).

Expression and Purification of IL-6M, IL-6D, and Mixed 14N/15N-Labeled IL-6D—Recombinant human IL-6 was expressed in E. coli as a recombinant fusion protein consisting of the first six N-terminal amino acids of bacterial β-galactosidase (Thr-Met-Ile-Thr-Asn-Ser) and residues 48 to 212 of immature human IL-6 (Swiss-Prot P05231) as described elsewhere (42). Purified recombinant [15N]IL-6 was prepared as described by Morton et al. (41). 400 µg of monomeric, dissociated 14N/15N-labeled IL-6D was isolated from this mixture by preparative size exclusion chromatography (SEC) at 25 °C as previously detailed for [15N]IL-6 (14). The column was developed with 20 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, and proteins were detected by absorbance at 215 nm. Both IL-6M and IL-6D were >98% homogenous based on the 2 s/spectrum over the spray voltage of 2.8 kV, and curtain and nebulizer gas flow rates were set at 1.6 and 0.4 liter/min, respectively. Mass spectra were acquired for 2 s/spectrum over the m/z range 300–2000 in profile mode.

Tandem Mass Spectra—MS/MS spectra were acquired on a Q-Tof 2 mass spectrometer (Micromass) by infusion of samples collected off-line from RP-HPLC after diluting 1:1 with 50% aqueous methanol, 0.1% formic acid to enhance sensitivity. Conditions used were: cone voltage, 45 V; collision gas, argon; collision energy, 15 to 35 V. The ESI voltage used was 3.5 kV and the ion source was maintained at 80 °C.

Assignment of Mass Spectra—Where a mass peak was shown by isotopic labeling (as described below) to be an intermolecular cross-linked peptide, potential cross-linked peptide species corresponding to the parent mass were identified using a Java program “X-Link” (available on request, from T.T.). X-Link generates a searchable list of all possible cross-linked peptide molecules from a given set of protein sequences, including modifications by partially hydrolyzed cross-linkers. All possible cross-linked species having a mass within 0.5 Da of the observed molecular mass were considered, and the correct species was identified by comparing the expected fragmentation patterns to observed MS/MS data.

Molecular Modeling—Molecular models of IL-6D were generated using the average NMR structure of monomeric IL-6 (Protein data bank code 1IL6) (25). NMR structural data was used in preference to the parent mass were identified using a Java program “X-Link” (available on request, from T.T.). X-Link generates a searchable list of all possible cross-linked peptide molecules from a given set of protein sequences, including modifications by partially hydrolyzed cross-linkers. All possible cross-linked species having a mass within 0.5 Da of the observed molecular mass were considered, and the correct species was identified by comparing the expected fragmentation patterns to observed MS/MS data.

Molecular Modeling—Molecular models of IL-6D were generated using the average NMR structure of monomeric IL-6 (Protein data bank code 1IL6) (25). NMR structural data was used in preference to the parent mass. Molecular models of IL-6D were generated using the MODELLER program (46) with two IL-6 monomers used as the templates. The five N-terminal residues of the model, which had no template structure available, were positioned in a manner consistent with the observed N terminus cross-linking. In addition, to enable the crossover of helices, residues 127–135 in the C-E loop were released from the IL-6D structure constraints and were allowed to move freely. Apart from the C-E loop, the two IL-6D-like domains of the IL-6D model are structurally identical to IL-6M.
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**Fig. 1.** Schematic of the MIX technique applied to homodimeric protein (IL-6). 14N-Labeled (light) and 15N-labeled (dark) IL-6 were dissociated in 50% acetonitrile, mixed together in ~1:1 molar ratio, lyophilized, reconstituted by dissolving in H2O, and purified using SEC. A mixture of (statistically distributed) [15N/15N,15N/14N,14N/14N]IL-6-D was isolated, which was cross-linked with BS3, digested with trypsin, and then analyzed by RP-HPLC/MS. Isotopically labeled peptides co-elute, giving intermolecular cross-linked peptides a distinctive triplet/quadruplet signature because of the distribution of 14N and 15N in the labeled peptide distribution within these cross-linked peptides. Intramolecular species such as intramolecular cross-linked peptides, chemically modified peptides, and unmodified peptides all appear as doublet peaks.

**RESULTS**

**Experimental Strategy—**The experimental strategy used in this study is shown in Fig. 1. Purified [15N]- and [14N]IL-6 in dissociating solvent (50% acetonitrile) were mixed, then lyophilized and re-dissolved to form a mixture of isotopically labeled and unlabeled IL-6 monomers and dimers. Homogenous [15N/15N,15N/14N,14N/14N]IL-6-D was isolated, which was cross-linked with BS3, digested with trypsin, and then analyzed by RP-HPLC/MS. Isotopically labeled peptides co-elute, giving intermolecular cross-linked peptides a distinctive triplet/quadruplet signature because of the distribution of 14N and 15N in the labeled peptide distribution within these cross-linked peptides. Intramolecular species such as intramolecular cross-linked peptides, chemically modified peptides, and unmodified peptides all appear as doublet peaks.

The cross-linking reaction was confirmed by SDS-PAGE to be specific for dimeric IL-6, as a predominant band of relative molecular mass (Mr) 40,000 was seen on cross-linking IL-6p, whereas for cross-linked IL-6p only a Mr, 20,000 band was observed (Fig. 2, inset). The refolding conditions used for [15N/15N,15N/14N,14N/14N]IL-6-D were identical to those previously used to isolate, assay, and characterize IL-6p, through biological and biophysical assays (16). Previous cross-linking studies on the related cytokine erythropoietin (47) showed that the level of BS3 cross-linking here is unlikely to affect protein structure. The cross-linking reaction was confirmed by SDS-PAGE to be specific for dimeric IL-6, as a predominant band of relative molecular mass (Mr) 40,000 was seen on cross-linking IL-6p, whereas for cross-linked IL-6p only a Mr, 20,000 band was observed (Fig. 2, inset).

**Peptide Mapping of Cross-linked** [15N/15N,15N/14N,14N/14N]IL-6p—Following cross-linking, proteolysis and on-line LC/MS of the [15N/15N,15N/14N,14N/14N]IL-6p, three easily recognizable triplet or quadruplet MS peaks were observed. The molecular ions corresponding to these peaks were designated as the intermolecular cross-linked species X1, X2, and X3, and otherwise chemically identical peptides within cross-links. The MS peak shape of intermolecular cross-linked peptides is determined by the difference between the number of nitrogen atoms in the cross-linked peptides. If this difference is small, the two possible mixed [15N/12N]-labeled cross-linked peptide species will have similar molecular masses, resulting in a triplet MS peak comprised of 14N-/14N-, 14N/15N-, and 15N/15N-labeled species. If this difference is larger, then the two mixed 14N/15N-labeled cross-linked peptide species will have distinguishable masses by MS, resulting in a quadruplet MS peak comprised of 14N/14N-, 14N/15N-, 15N/14N-, and 15N/15N-labeled species. All other species, namely intramolecular cross-linked and noncross-linked peptides, were seen as 14N- and 15N-labeled forms, resulting in doublet MS peaks. This allowed quick visual identification of molecular ions from intermolecular cross-linked peptides. The identities of the cross-linked tryptic peptides in these intermolecular species were then assigned based on parent mass using the in-house program X-Link.

**Cross-linking—**The purified [15N/15N,15N/14N,14N/14N]IL-6-Dp was judged homogeneous based on co-elution with unlabeled purified IL-6p on analytical SEC (Fig. 2). Furthermore, the [15N/15N,15N/14N,14N/14N]IL-6-Dp could be cross-linked in an identical way to homogeneous [15N]IL-6p, as seen on SDS-PAGE (Fig. 2, inset). The refolding conditions used for [15N/15N,15N/14N,14N/14N]IL-6-Dp were identical to those previously used to isolate, assay, and characterize IL-6p, through biological and biophysical assays (16). Previous cross-linking studies on the related cytokine erythropoietin (47) showed that the level of BS3 cross-linking here is unlikely to affect protein structure. The cross-linking reaction was confirmed by SDS-PAGE to be specific for dimeric IL-6, as a predominant band of relative molecular mass (Mr) 40,000 was seen on cross-linking IL-6p, whereas for cross-linked IL-6p only a Mr, 20,000 band was observed (Fig. 2, inset).
were found (see below) to cross-link interchain residues, N terminus Lys128, Lys171 and Lys27, and Lys171 and Lys66, respectively (Table I). Importantly, the intermolecular cross-linked peptide species X1 was abundant and was easily observed on a chromatogram, whereas a control digest of cross-linked monomeric IL-6 showed no corresponding intramolecular species resulting from a cross-link between the N terminus and Lys128. The species X2 and X3 were both observed at much lower concentrations. Additionally, 13 tryptic peptides and three cross-linked intramolecular peptides were observed as doublet peaks, which did not yield additional structural information on intermolecular interactions within IL-6D (data not shown). The ratios of integrated peak areas within multiplets, and the separation between multiplet peaks, were consistent with a 14N:15N ratio of 1:0.7. Under ESI-time of flight MS/MS conditions (Q-Tof2), informative fragmentation reactions resulting in sequence coverage of both cross-linked peptides were observed on the 2+ and 3+ ions of the cross-linked peptides. Furthermore, MS/MS spectra of peaks within multiplets displayed isotopic shifts between MS/MS fragment ion peaks consistent with assignments. For example, the triply charged ion of the cross-link species X3 (see Table I) at m/z 765.78 presented as a quadruplet peak under MS (Fig. 3A). Tandem mass spectrometry (MS/MS) on the lowest m/z peak of this quadruplet showed a series of b and y fragment ions from both tryptic peptides joined in the cross-linked species X3 (Fig. 3B). This MS/MS spectrum unambiguously identified the sequences of the cross-linked peptides and the location of the cross-link between them. For the cross-linked species X3, its constituent peptides were Leu-Pro-Lys66-Met-Ala-Glu-Lys, denoted T1, and Ser-Phe-Lys171-Glu-Phe-Leu-Gln-Ser-Leu-Arg, denoted T2, cross-linked via Lys66-Lys171 (Fig. 3C). In addition, MS/MS on the second lightest quadruplet peak of X3 (Fig. 3D) showed the expected sequence ions from the cross-linked peptide 14N[T1]-15N[T2]. These results show that the MIX-tagged mass peaks correspond to cross-linked peptides of intermolecular origin.

**DISCUSSION**

**Structure of IL-6D**—Previously, it has been shown that IL-6D has a frictional coefficient consistent with two IL-6M subunits in an end-to-end arrangement (23). Furthermore, unfolding/dissociation relationships (23) indicate that IL-6D is a metastable dimer and probably exhibits three-dimensional domain swapping, in which the two adjacent domains are IL-6M-like in structure but have domain elements (a-helices and/or loops) interchanged symmetrically via the top or bottom “face” on the IL-6M structure (23, 25). In particular, the structure of the a-helical bundles making up the IL-6M-like domains is expected to be essentially conserved between IL-6D and IL-6M, the only structural difference of note being in the loops that cross between the two domains (23, 25).

Given a three-dimensional domain-swapped structure for IL-6D, the mode of domain element interchange can be identified from the intermolecular cross-links identified through the MIX experiment. The cross-linked intermolecular peptide X1, which links the N terminus to Lys128, was not detected from the control digest of cross-linked (monomeric) IL-6M. Rather, the species X1 was observed only from the digest of the cross-linked dimer, IL-6D. Hence in the IL-6D, the N terminus of one IL-6 chain must be in close proximity to Lys128 of the other chain. This indicates that X1 must originate from a cross-link at the interface of the IL-6 dimer between the two IL-6M-like domains. Otherwise, if the cross-linked peptide X1 were intradomain, then it would also be expected from the control digest of IL-6M because (i) the IL-6M structure is contained within the domain-swapped IL-6D structure (23–25) and (ii) the cross-linked residues in X1 will not be directly involved in the crossover loop region, because Lys128 is part of the C-helix. Consequently, the two IL-6M-like domains in IL-6D must be oriented “head to head,” having the N terminus/C terminus containing faces of the IL-6M subunits adjacent as shown in cartoon models II, III, and IV in Fig. 4A. These cartoon representations show a nonswapped dimer (model I) and all possible modes of symmetrical three-dimensional domain swapping for

| Cross-linked intermolecular peptide designation | Cross-linked residues | Observed mass [M + H]⁺ | Δ (mass accuracy) c parts per million | Fragment ions identified/expected d |
|-----------------------------------------------|-----------------------|------------------------|--------------------------------------|-----------------------------------|
| X1 | N terminus Lys128 | (111–127) (121–129) | 2480.43 | 35.4 | 16/36 |
| X2 | Lys171-Lys66 | (169–179, 25–30) | 2251.25 | 1.4 | 14/30 |
| X3 | Lys171-Lys66 | (169–179, 64–70) | 2295.32 | 33.7 | 13/32 |

a) Cross-links are via ε-amino groups for lysines or the N terminus.

b) Cross-linked peptides are denoted by the start and end residue positions in parentheses. Residue numbering used is for mature human IL-6 (23).

c) Defined as Δ(mass accuracy) = [(measured) − (theoretical)]/(measured) for mass of [M + H]⁺.

d) Number of fragment ions assigned/number of possible b and y fragment ions from the peptide sequence.

**Fig. 3.** MS analysis of cross-linked peak X3 from the digestion of cross-linked 14N/15N, 14N/14N, 14N/15N, 15N/15N IL-6D. Panel A, mass spectrum of triply charged, MIX-tagged quadruplet molecular ion of cross-linked X3 at m/z 765.78 (see Table I) with bars showing selection windows for CID-MS acquisition leading to spectra in panels B and D. Panels B and D, deconvoluted (all peaks from multiply charged species converted to the singly charged equivalents) collision-induced fragmentation spectra on m/z windows of 3 Da on the quadruplet peak formed by X3 (see panel A). Panel B (selection window 764.8–767.3 Da) shows fragmentation of the 14N[T1]-14N[T2] form of the cross-linked peptide, whereas panel D (selection window 768.0–770.2 Da) shows fragmentation of the 14N[T1]-15N[T2] form. T1 and T2 denote tryptic peptides (169–179) and (64–70), respectively (see Table I). The peak “T1-XL” has mass consistent with an unknown fragmentation within the crosslinked moiety derived from BS3. Panel C, possible MS/MS fragmentation schemes from X3, with nomenclature indicated for the peptide fragmentation products. A standard nomenclature is used (54) for fragment ions from cross-linked peptides (55, 56).
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FIG. 4. A, models I-IV are cartoon representations of possible modes of domain swapping between subunits in IL-6A dimer, based on a head to head alignment of IL-6A subunits consistent with the intermolecular crosslink X1 between the N terminus and Lys128 (see Table I). The two IL-6A chains are black and white. The cross-linked peptides X1, X2, and X3 (Table I) are positioned on the cartoon of IL-6A, and shown as intermolecular (red-blue) or intramolecular (blue-blue or red-red). B, ribbon representation of the IL-6A structural model. The D- and E-helices are swapped between IL-6A-like domains as shown in model IV (Fig. 4A). The IL-6A chains are colored dark red and dark blue. C, ribbon representation of the IL-6M-like domains within IL-6D. However, the IL-6D model was constructed with an orientation between IL-6M-like domains similar to that between the “monomer-like” domains in IL-10 (25). IL-10 was selected from the three possible dimeric cytokines mentioned above, on the basis of its elongated shape (45) (the IL-10 inter-domain angle is −135°), for consistency with the known high axial ratio of IL-6A (23). The cross-linking data does not provide information on the relative orientation of IL-6M-like domains within IL-6D. However, the IL-6B chain is swapped relative to the A-helix and A-B loop in each IL-6M-like domain. Only model IV (Fig. 4A) contains this structural feature and is consistent with the intermolecular nature of cross-linked peptides X1, X2, and X3, as shown by the MIX experiment. Furthermore, the domain-swapped models II and III are inconsistent with the conserved intramolecular disulfide bond Cys117-Cys119, which tethers the A-B loop to the B-helix. Model IV (Fig. 4A) is characterized by symmetrical swapping of the two helices nearest to the C terminus, the D- and E-helices, between IL-6M-like subunits in IL-6D. This mode of domain element interchange corresponds closely to that seen in the known structures of the naturally dimeric short chain cytokines IL-10, interferon-γ, and IL-5, which have the “interferon-γ” fold (25) and are distant homologs of IL-6. Moreover, a recent survey of 40 domain-swapped proteins shows that almost all are swapped via regions adjacent to the N or C terminus, which is a property of this model (24).

A molecular model of IL-6D was constructed (Fig. 4B) based on the cross-linking data and the NMR structure of IL-6M (22) (see “Experimental Procedures”), in which IL-6M-like domains were connected to one another via the C-E loops. One IL-6M-like domain was composed of the A-, B-, and C-helices from one chain and the D- and E-helices of the other chain. The cross-linking data does not provide information on the relative orientation of IL-6M-like domains within IL-6D. The five N-terminal residues, which do not appear in the NMR structure of monomeric IL-6, are displayed in a conformation consistent with the cross-linking data (Fig. 4B). This model satisfies the distance constraints, namely <24 Å between Ca-Ca atoms of lysines and the N terminus, imposed on IL-6D by the cross-linking of ε-amino groups by BS3 (30, 48) as seen in cross-linked peptides X1, X2, and X3.

Implications for the Inhibitory Role of IL-6D—IL-6D in vivo has diverse roles in mediating proliferative signals to B- and T-cells in the immune system. Signaling occurs via sequential binding to a two-receptor system made up of the membrane-bound proteins IL-6R and gp130. IL-6 first binds to IL-6R for the IL-6-IL-6R complex to recruit gp130 in the second step, which then forms a hexameric complex active in signaling and consisting of two molecules each of IL-6, IL-6R, and gp130 (14).

The topology of this hexameric IL-6 receptor complex is not yet known. Mutagenesis and structural studies on IL-6 have shown that it contains one site for IL-6R binding, site I, and two sites for gp130 binding, sites II and III (22, 44). Site I consists of the C-terminal end of the D-helix, the C-terminal part of the long AB-loop, and the N-terminal part of the B-helix (49). Site II is a region halfway along the A- and C-helices, whereas site III is located on the DE-loop. This topic has also been reviewed by Simpson et al. (12).

Previously, recombinant IL-6D has been shown to inhibit IL-6 signaling (16, 23), which potentially has therapeutic applications. This inhibition is likely to be biologically important, as IL-6 in vivo is partly homodimeric (17–19) and recent studies suggest that naturally occurring and recombinant IL-6D may have similar biological activity (21). Thus, IL-6D in vivo seems likely be a natural inhibitor for IL-6 signaling. Recombinant IL-6D has been shown to bind strongly to the soluble extracellular domain of IL-6R (sIL-6R) to form a stable IL-
The proposed structure of IL-6$_{c}$ can also account for its inhibitory nature with respect to IL-6 signaling (16). In structural terms, the lack of strong binding of soluble gp130 to the stable IL-6$_{c}$ (sIL-6R) complex is likely to be because of restriction of the relative orientations of gp130-binding sites II and III on IL-6$_{c}$ (50) within IL-6$_{c}$ (sIL-6R) complex, which precludes cooperative soluble gp130 binding. This is consistent with the model of IL-6$_{c}$ obtained from the cross-linking data (Fig. 4C), because no gp130-binding sites on IL-6 (51, 52) appear to be directly blocked by IL-6 dimerization, their relative orientations are constrained. Furthermore, the orientation of IL-6$_{c}$-like domains within the IL-6$_{c}$ molecule is very different from that seen for the viral IL-6 homolog, vIL-6, within the crystal structure of the 2:2 vIL-6:gp130 complex, which is capable of signaling (53). In this complex, the vIL-6 chains are widely separated, the minimum distance between them being ~35 Å (for comparison, IL-6$_{M}$ is ~30 Å × 30 Å × 50 Å). Thus, constrained orientation of gp130-binding sites within IL-6$_{c}$ rather than dimerization induced blocking of IL-6 receptor-binding sites as previously proposed (23), seems likely to account for the IL-6$_{c}$-mediated inhibition of IL-6 signaling. A more detailed structural understanding of the inhibitory role of IL-6$_{c}$ must await the three-dimensional structures of the signaling (IL-6$_{c}$) and inhibitory (IL-6$_{c}$) complexes with their associated receptors. Although the full description of the IL-6$_{c}$ structure awaits the project, and A. W. Burgess and G. E. Reid for critical comments on the manuscript.

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