Function and Molecular Modeling of the Interaction between Human Interleukin 6 and Its HNK-1 Oligosaccharide Ligands*

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Interleukin 6 (IL-6) is endowed with a lectin activity for oligosaccharide ligands possessing the HNK-1 epitope (3-sulfated glucuronic acid) on some mammalian glycoprotein N-glycans (Cebo, C., Dambrouck, T., Maes, E., Laden, C., Streck, G., Michalski, J. C., and Zanetta, J. P. (2001) J. Biol. Chem. 276, 5685–5691). Using high affinity oligosaccharide ligands, it is demonstrated that this lectin activity is responsible for the early dephosphorylation of tyrosine residues found on specific proteins induced by interleukin 6 in human resting lymphocytes. The gp130 glycoprotein, the signal-transducing molecule of the IL-6 pathway, is itself a molecule possessing the HNK-1 epitope. This indicates that IL-6 is a bi-functional molecule able to extracellularly associate its α-receptor with the gp130 surface complex. Computational modeling indicates that the lower energy conformers of the high affinity ligands of IL-6 have a common structure. Docking experiments of these conformers suggest that the carbohydrate recognition domain of IL-6 is localized in the domain previously identified as site 3 of IL-6 (Somers, W., Stahl, M., and Seehra, J. S. (1997) EMBO J. 16, 989–997), already known to be involved in interactions with gp130.

Interleukin 6 (IL-6) is a pleiotropic cytokine showing essential roles in immunity, hematopoiesis, and inflammation (for reviews, see Refs. 1–4). The detailed mechanism by which the IL-6 binding to its receptor (IL-6Rα) generates a signal remains partially understood, although this signal takes place through a “signal-transducing molecule,” the gp130 glycoprotein in normal human (5, 6). This molecule is considered as a second receptor (IL-2Rβ) by several authors (7–9), the binding of IL-6 to IL-6Rα provoking the association of the later with gp130. Putative domains of direct interactions between these molecules have been described based on X-ray crystallographic data of the gp130 extracellular domain (9) and on site-directed mutagenesis experiments in various subdomains of the molecule (7, 10–16).

These data did not take into account the possibility that IL-6 could be, as other interleukins (17), a bi-functional molecule having, beside a receptor-binding domain, a carbohydrate recognition domain (CRD). As demonstrated for interleukin 2 (IL-2; Ref. 18) in resting human lymphocytes (which do not express the alpha IL-2 receptor (IL-2Rα), when IL-2 is bound to its β-receptor (IL-2Rβ) through its receptor-binding domain, it associates the later to the T cell receptor complex through its CRD. Indeed, in resting human T cells, IL-2 recognizes with a high affinity oligomannosidic N-glycans with 5 and 6 mannose residues found on one N-glycosylated form of CD3. The IL-2 signaling (tyrosine phosphorylation of IL-2Rβ by p56lck) is entirely dependent upon this specific lectin/carbohydrate interaction, and interference with oligomannosides of pathogens could be responsible for severe immunodeficiencies (19, 20). The same lectin activity was shown recently to be involved in the carbohydrate-dependent association between IL-2Rβ and IL-2Rα in a mouse cell line constitutively expressing IL-2Rα (21), a process resembling the second step in the activation process of human lymphocyte occurring after the internalization of the TCR complex (22) subsequent to the initial action of IL-2 on resting cells mentioned above.

Recent studies (17) demonstrated that IL-6 specifically binds to nervous tissue glycoproteins bearing the HNK-1 epitope (glucuronic acid 3-sulfate), the structure having been determined (23, 24). The HNK-1 epitope is an onco-fetal antigen (25–30), especially concentrated at early stages of development on glycoproteins considered as “cell-adhesion molecules”: the N-CAM (31, 32), the J1 glycoprotein (33), the myelin-associated glycoprotein (MAG; 4, 34, 35, 36), the P0 myelin glycoprotein (6, 37, 38), the myelin-oligodendrocyte glycoprotein (MOG; Refs. 39–41, etc.). It is also overexpressed in many cancer cells, but is still expressed in some normal adult cells or tissues (myelin, human NK cells (25, 26)). This epitope is also found on glycolipids (42–44), especially concentrated in human myelin. The binding of IL-6 to the myelin glycoproteins MAG and P0 (17) was reversed specifically using some oligosaccharide aldehydes having the HNK-1 epitope isolated from the mucins of the eggs of Rana temporaria (45). One of these compounds showed a very strong inhibitory property of the binding of IL-6 to the glycoprotein HNK-1 epitope, suggesting that it was a much higher affinity ligand than the glycoprotein HNK-1 epitope. However, other compounds having the glucuronic acid 3-sulfate group were extremely low affinity ligands, suggesting that they did not possess either conformations or additional groups necessary for the binding to IL-6.

By using this IL-6 high affinity ligand in cultures of resting human lymphocytes, it is demonstrated that the lectin activity of IL-6 is responsible for the IL-6-induced dephosphorylations.
of tyrosine-phosphorylated proteins. One major glycoprotein ligand of IL-6 having the HNK-1 epitope is gp130 itself. Docking experiments of the lower affinity conformers (determined by computational calculations) of the identified oligosaccharide ligands of IL-6 suggest that the CRD of IL-6 overlaps with site 3 of IL-6, previously identified as a site implicated in the association with gp130. These data propose new concepts for the interactions between the IL-6 receptor and its signal-transducing molecule gp130.

MATERIALS AND METHODS

Chemicals—The recombinant human IL-6 (produced in bacteria) and its polyclonal rabbit antibody were from Chemicon International Inc. (Temecula, CA). The monoclonal mouse anti-phosphotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-phosphoserine, anti-phosphothreonine, alkaline phosphatase-labeled rabbit and mouse IgG, normal goat serum, bovine serum albumin (BSA), RPMI 1640 culture medium, Brij97 detergent, phenylmethanesulfonyl fluoride, p-tosyl-arginine methyl ester, nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, α,β-microglobulin, and leupeptin were from Sigma. The serum from the patient with IgM paraproteinemia was kindly provided by Dr. N. Baumann (Laboratoire de Neurobiologie, Hôpital de la Salpetrière, Paris). MAG was purified from adult R. temporaria (i) For testing the effects of IL-6 and of IL-6 and its signal-transducing molecule gp130. Therefore it was necessary to determine whether the HNK-1 glycoprotein ligand found on P0 and MAG and the higher affinity ligand of the eggs of R. temporaria oligosaccharide alditol. For these two molecules, the random search technique was used as implemented in the Sybyl version 6.6 software to sample the conformational space (Tripos, Inc., St. Louis, MO (www.tripos.com)). The OPLS All-Atom Force Field (56) was used except for the sulfated N-linked GlcA for which a charge determination was performed using quantum chemical procedures available in the Jaguar software (Jaguar Version 4.0; Schrödinger, Inc., Portland, OR 97204 (www.schrodinger.com)). The Density Functional Theory was used with the B3LYP hybrid method and the 6-31G basis set. The molecular electrostatic potential was computed on a spherical grid and fit to a set of point charges placed at the atomic centers. The fit was constrained to also reproduce the dipole moment. Fourteen torsional angles of rotatable bonds were changed for the glycoprotein ligand (compound IV in Fig. 1B), 14 and 17 for compounds I and II, respectively, which consist of the glycosidic linkages and the CH2OH, NHCOCH2COOH, and SO3 groups. In both cases, 20,000 configurations were examined using the bump checking (with a Van der Waals scaling factor of 0.7) and energy as criteria. For compound IV, 40311 conformers were found, among which the 10 of lowest energy (in a 6 kcal/mol range) were retained for docking with human IL-6. For compound II only 149 conformers were found due to its low flexibility as compared with compound IV. Again the 10 lowest in energy were kept for docking (in a 10 kcal/mol range). The docking with the SO3− sulfated group found in the IL-6 crystal as a template (9). A random search was performed after replacing the corresponding sulfate in the IL-6 crystal by the oligosaccharide-bound sulfate group. No new conformers were found.

RESULTS

The IL-6 Lectin Activity Is Responsible for Early Tyrosine Dephosphorylations of Quiescent Human Lymphocytes—To examine the function of the lectin activity of IL-6 for glycoses having the HNK-1 epitope, a population of quiescent human lymphocyte was incubated with IL-6 for 15 min at 37 °C, and the phosphorylation pattern was analyzed using anti-phosphotyrosine, anti-phosphoserine, and anti-phosphothreonine monoclonal antibodies. As shown in Fig. 1A, lane 2, the IL-6-treated material showed a significant decrease of phosphotyrosine residues of tyrosine-phosphorylated proteins of Mv77,000, 73,000, 69,000, 60,000, and 56,000, respectively, whereas no significant variations were observed using the two other antibodies (not shown). When the samples containing IL-6 were co-incubated with IL-6 ligands isolated from the mucins of the eggs of R. temporaria (Fig. 1B), the decrease in tyrosine phosphorylation was suppressed, and the profile of the tyrosine-phosphorylated proteins was identical to the profiles of untreated cells (Fig. 1A, lanes 3 and 4). This indicated that, at low concentrations (1 ng/ml for compound II, i.e. 1 nM and 0.8 μg/ml, i.e. ~1 μM for compound I), the IL-6 oligosaccharide ligands were able to inhibit the earliest dephosphorylations already observed (57) in the IL-6-dependent signaling. In contrast, compound III was ineffective at the concentration of 10 μM.

gp130 (IL-6Rβ) Has a HNK-1-Containing Glycan—The previous data suggested that the lectin activity of IL-6 was of fundamental importance for the IL-6-dependent association between IL-6Rα and its signal-transducing molecule gp130. Therefore it was necessary to determine whether the HNK-1 epitope was present in molecules of the gp130 surface complex, especially on gp130. This point was analyzed in the following way. Resting human lymphocytes (not supplemented with IL-6) were lysed in a mild detergent (55), supplemented with a stronger detergent mixture (known to dissociate surface molecules complex) (55), and incubated in the absence of an antibody serum from a human patient with IgM paraproteinemia (43). The immunoprecipitated material was submitted to SDS-PAGE, blotted on nitrocellulose, and the blots were revealed using a mouse monoclonal anti-gp130 antibody followed by an APK-labeled anti-mouse IgG antibody. As shown in Fig. 2B, the anti-HNK-1 immunoprecipitate actually contained a high Mv glycan.
Fig. 1. A, effect of IL-6 and of IL-6 plus its oligosaccharide ligands (compounds I and II) on the tyrosine phosphorylations of resting human lymphocytes. Human resting lymphocytes were incubated for 15 min in the presence (lane 2) or not (lane 1) of 20 ng/ml IL-6 or in the presence of the same amount of IL-6 plus 1 ng/ml compound II (lane 3) or 1 mg/ml compound I (lane 4). The total cell lysate was submitted to 13% SDS-PAGE followed by transfer on nitrocellulose and immunodetection of phosphotyrosine residues. B, structures of the oligosaccharide ligands (compounds I and II) and nonligands (compound III) of IL-6 isolated from the mucins of the eggs of R. temporaria and of the glycoprotein HNK-1 epitope (compound IV; Ref. 22). The arrow in compound IV indicates the portion of the glycan used for computational modeling.

The representations of the lower energy conformations of the glycoformations, since its energy was 7 kCal/mol lower than the other ligand, compound II, showed that they

Fig. 2. Evidence for the presence of an HNK-1 epitope on gp130. The detergent lysate of resting lymphocytes was immunoprecipitated by addition of the serum of a patient with IgM paraproteinemimia, submitted to 10% SDS-PAGE, and revealed using the monoclonal anti-gp130 antibody followed by an AKP-labeled anti-mouse IgG antibody. A, immunoprecipitation with normal human serum. B, immunoprecipitation with the serum from a patient with IgM paraproteinemimia. Note that a single band of Mr 130,000 was specifically revealed. C and D, silver staining of the immunoprecipitates obtained on HepG2 (C) and human lymphocyte (D) cell lysate. * in D indicates the position of the heavy chain of the IgM. Note the relative abundance of the material immunoprecipitated using the anti-HNK-1 serum as compared with human resting lymphocytes.

lectin activity of IL-6 was essential for its biological function; (ii) this function was the extracellular association between the IL-6Rα complex and the gp130-containing complex, HNK-1-containing glycan being present on gp130 itself. Therefore, it was of interest to make a theoretical model of the interaction between IL-6 and its ligands. The compound II isolated from R. temporaria egg mucins was the higher affinity ligand so far identified. Consequently, it was considered as the reference structure for the determination of the conformation of IL-6 oligosaccharide ligands. Because IL-6 bound to the P0 and MAG glycoproteins possessing the HNK-1 epitope on specific N-glycans of known structures (24), the extended HNK-1 part of this compound (compound IV in Fig. 1B) was taken as the putative endogenous glycoprotein ligand of IL-6. Based on studies (17) of different O-glycans isolated from the eggs of Rana arvalis (49) and of R. temporaria (45), it was clear that the 3-O-SO3H group on GlcA was a fundamental determinant of the interaction between IL-6 and its ligand. The presence of an additional residue (Gal and/or more complex sequences) on the Galβ1,4 of compound II (compound III in Fig. 1B) strongly suppressed the affinity for IL-6. The same was observed when the Galβ1,4 of compound II was replaced by a Galβ1,3. The absence of the Fucα1,2 residue linked to the Galβ1,3 of compound II (compound I) decreased the affinity by a factor of about 104. Therefore, it was suggested that these two compounds possessed in common a special conformation allowing the specific interaction with a domain of IL-6. One possibility was that, beside the common nonreducing carbohydrate sequence SO3H-3-GlcA of compound II, these compounds had a hydrophobic residue (methyl group of Fuc and acetamido group of GlcNAc, respectively) increasing the affinity for IL-6. Consequently, two key structures were chosen for computational conformational analysis: compound II and the compound IV reduced to the Manα1,6 branch (Fig. 1B).

The representations of the lower energy conformations of the R. temporaria ligand, compound II (Fig. 3), showed that they corresponded to very rigid structures. The conformation shown in Fig. 3A was significantly different from all other conformations, since its energy was 7 kCal/mol lower than the other...
lower energy conformation shown in Fig. 3B. It presented a very rigid and compact tripod-like structure, contrasting with the other low energy conformation showing a more extended structure. The glucuronic acid 3-sulfate motif was rotated by about 90° relative to the previous compound, whereas the acetamido group of GalNAc-ol was rotated by 180° relative to the previous compound. The two lowest energy conformations of the glycoprotein HNK-1 epitope (portion of compound IV) were found to be very similar (Fig. 3, C and D) and presented very close energetic levels. These two structures presented a great similarity with the lowest energy conformer of R. temporaria for the motif SO₃H-3-GlcAβ1,3Galβ1. This was evidenced in Fig. 4, where these groups were placed in similar positions. Interestingly, in all these compounds, the methyl groups were in opposition to the sulfate group and at different distances from the sulfate group in the R. temporaria and in the glycoprotein ligand of IL-6. This suggested that the hydrophobic residues were not involved directly in the binding to IL-6. Rather, these experiments suggested that the important domain for the interaction with IL-6 was the conformation of the SO₃H-3-GlcAβ1,3Galβ1 motif. This was perfectly compatible with the fact that the addition of a residue of Galβ on the hydroxyl in position 4 of Gal inhibited the interaction with IL-6. It might be also understood why that the absence of the Fuc residue in compound II (compound I) dramatically decreased the binding of IL-6, modifying the conformation of the SO₃H-3-GlcAβ1,3Galβ1 motif of interaction with IL-6. Indeed, the lower energy conformers of compound I showed different conformations as compared with those of compounds II and IV. Especially, the Gal residue vicinal to the GlcA residue of compound I was turned by 180° compared with that of the previous compounds, explaining its reduced affinity for IL-6 compared with compound II.

Docking of Oligosaccharide Ligands into the IL-6 Carbohydrate Recognition Domain—The biochemical, crystallographic, and computational data obtained on the ligands of IL-6 (Refs. 7, 9, and 15 and this work) allowed us to determine important features for the putative carbohydrate recognition domain of IL-6. Indeed, the three-dimensional structure of IL-6 obtained by x-ray crystallography showed 4 sulfate groups constituting putative binding sites for the sulfate group of the HNK-1 common to all IL-6 ligands. One of these sulfate groups (SO₄²⁻) was strongly hydrogen-bonded to Glu¹⁵⁶, suggesting that this position could be a privileged site for the binding of the sulfate group of the HNK-1 epitope. Furthermore, this sulfate-binding site was close to a water-exposed tryptophane residue (Trp¹⁵⁷), an amino acid frequently encountered in the CRD of lectins (58), and especially in the CRD of calcium-independent lectins (59).

Therefore, the glycoprotein HNK-1 (portion of compound IV) conformer was computationally docked into the IL-6 molecule, placing the sulfate group of HNK-1 at the position of SO₄²⁻. Computational analysis of the lower energy structure of the complex allowed placing the HNK-1 glycan as shown in Fig. 5. In this position, the sulfated glycan appeared to be specifically hydrogen-bonded to the protein. Indeed, besides three hydrogen bonds involving SO₄²⁻ with Glu¹⁵⁶, the calculations of the interatomic distances indicated the possibility of two strong hydrogen bonds between the nitrogen atom of the side chain of Asn¹⁵⁵ and the hydroxyl groups of the C-4 and C-6 carbon atoms of the galactose residue part of the SO₃H-3-GlcAβ1,3Galβ1 motif (3.08 and 2.86 Å, respectively). A third weak hydrogen bond (3.43 Å) could be formed between the oxygen atom of the amido group of Asn¹⁵⁵ and the oxygen atom of the C-2 carbon atom of GlcA. Furthermore, the two other pyranic rings of the HNK-1 ligand (GlcNAcβ1,4 and Manα1,6) were found in the vicinity (mean distance 5 and 4.5 Å for GlcNAc and Man, respectively) from the indolic cycle of Trp¹⁵⁷, a situation allowing strong interactions between the pyranic rings and Trp. Additional hydrogen bonds could be formed between the oxygen atoms of the C-4 of Gal and C-2 of GlcA and a water molecule (H₂O⁵⁷ (9)), itself hydrogen-bonded to the nitrogen atom of the acetamido group of Asn¹⁵⁵.

**DISCUSSION**

Importance of the Lectin Activity of IL-6 for Its Biological Function—The signaling pathway of IL-6 involves the association of the molecular complex containing its receptor (IL-6Rα) to the surface molecular complex including the signal-transducing gp130 glycoprotein (IL6-Rβ). Several studies (60–63) demonstrated that this IL-6-induced association between the two types of molecular complexes initiated a reduction of specific phosphotyrosine residues of several proteins. This was due to a specific phosphatase, SHP-2, endowed with two binding sites for phosphotyrosine residues (62, 64–67) associated with the gp130 complex. In a previous paper, we demonstrated (17) that IL-6 has, as several other cytokines, a calcium-independent lectin activity. Indeed, IL-6 recognizes glycans having the HNK-1 epitope as a major determinant. Since this property was never detected before, the question was asked to find out...
the biological role of the lectin activity of IL-6. The present study demonstrates that the lectin activity of IL-6 is essential for the first steps of its signaling pathways. Indeed, the addition of minute amounts (~1 nM) of the high affinity oligosaccharide ligand (compound II) completely inhibited the early dephosphorylations of phosphotyrosine residues induced by IL-6 on resting human lymphocytes. The inhibition of tyrosine dephosphorylations was dose-dependent, and the effect of the IL-6 on resting human lymphocytes. The inhibition of tyrosine dephosphorylations was identical to their ability to inhibit the biological role of the lectin activity of IL-6.

Although the consequences on the early tyrosine phosphorylations mechanisms on resting human lymphocytes are different from that observed for IL-2 (dephosphorylation for IL-6 instead of tyrosine phosphorylations; Ref. 18), the lectin activity of these two cytokines is necessary for the specific associations between the interleukin receptor and the signal-transducing complex. Based on the discovery of different lectin activities of several cytokines, these observations could lead to the definition of a general mechanism of action of cytokines on resting and/or normal human lymphocytes in the specific association of their receptors to signal-transducing molecules.

The data observed for the immunoprecipitates of the cancer cell line HepG2, in which more than 20 different immunoprecipitated subunits were detected at similar levels as gp130, might explain the extreme responsiveness of these cells to IL-6. However (68, 69), these cells did use other signaling systems not involving gp130. The overexpression of the HNK-1 epitope in HepG2 cells might result in a polysemous signaling.

Putative Localization of the CRD of IL-6—Because of the importance of the biological function of the lectin activity of IL-6, we decided to go further into the definition of the conformation of its oligosaccharide ligands and of its carbohydrate recognition domain. IL-6 belongs to a family of cytokines, including interleukin-11, the ciliary neurotrophic factor, the oncstatin M, and the cardiotrophin-1, sharing similar features in their structural organization (9). Interleukin-6 is a protein of 184 amino acid residues long containing four α-helices organized in a classical four-helix bundle and a supplementary mini-helix E located in the CD loop (66). Studies based on site-directed mutagenesis (7, 15) and/or crystal analysis (9) were performed on IL-6 and led to the design of three sites of crucial importance for the binding of the cytokine to IL-6Rα, on the one hand, and to gp130, on the other hand.

Site 1 is located on the C-terminal part of the α-helix of IL-6 and is implicated in the binding of IL-6 to its α-receptor. The point mutations in site 1 dramatically decrease the biological activity of the cytokine on several cell lines (10–14, 67), decreasing the binding of IL-6 to IL-6Rα. Sites 2 and 3 are implicated in the oligomerization of the gp130/IL-6/IL-6Rα complex with the gp130 complex. gp130 is a glycoprotein possessing the HNK-1 epitope. This association allows the action of a tyrosine phosphatase (Pase).

**Fig. 5. Representation of the results of docking experiments of the Ranaligand of IL-6 into the IL-6 structure (A) and enlargement of the site of interaction of the glycoprotein HNK-1 epitope with IL-6 (B).** Note that the sulfate group of the ligand should interact with highly hydrogen-bonded sulfate group identified by x-ray crystallography. Note that the possibility of two strong hydrogen bonds (blue dashed lines) between the nitrogen atom of the side chain of Asn184 with the hydroxyl groups borne by the C-4 and C-6 carbon atoms of the Gal residue. Note that the pyranose rings of Man and GlcNAc present the possibility of strong interaction with Trp157. This area of IL-6 (site 3) has been shown to be essential for the binding of IL-6 to gp130 and for the biological function of IL-6.
molecules. Site 2 consists of amino acid residues in the A and C helices, whereas site 3 is located in the terminal part of the C-terminal domain and the N-terminal part of the D-helix. Two types of mutants bearing point mutations at sites 2 and 3 were generated (15); the first group possessed fourth amino acid substitutions at site 2 (Y31D/G35F/S118R/V121D). The following point mutations, W157R/D160R and T162D, designed the site 3 variants. Interestingly, combined site 2 and site 3 mutants normally bound to the IL-6Rα but have no biological activity, failing to bind gp130. Site 2 or 3 mutants, although they were able to bind one single molecule of gp130 in the presence of the IL-6Rα, also lack to transduce the intracellular signal generated by the cytokine, similarly unable to form a gp130 dimer. These observations led the authors to identify two independent binding sites for gp130 on the IL-6 molecule (9, 16, 70).

Our data propose site 3 of IL-6 as its carbohydrate recognition domain. Indeed, it possesses a sulfate-binding site, the sulfate group being strongly attached to Gln\(^{156}\) through three hydrogen bonds (the third hydrogen bond involving a protonated form of sulfate is likely at neutral pH because of the partial ionization of this relatively weak acidic group). It possesses a water-exposed Trp\(^{157}\) residue included in a cavity, the function remained unknown, although its replacement by other amino acids eliminated the IL-6-dependent signaling. Such a role of Trp residues in the CRD of lectins is a common feature (58, 59), the indolic ring allowing strong interactions with the pyramidal rings of monosaccharides. Furthermore, two strong hydrogen bonds can be formed between the hydroxyl groups of C-6 and C-4 carbon atoms of Gal substituted in position 3 by SO\(_3\)-H-3-GlcA. These interactions could be stabilized by a third hydrogen bond involving the hydroxyl group of the C-2 carbon atom of GlcA. Based on the use of different glycans isolated from *R. temporaria* and *R. arvalis*, the binding to IL-6 requires a strict conformation of the SO\(_3\)-H-3-Glc\(_{\alpha}\)1,3Gal\(_{\alpha}\)1 motif, corresponding to the lowest energy conformers calculated both for the *R. temporaria* and the glycoprotein HNK-1 epitope. This conformation is not dependent upon hydrophobic interactions involving methyl groups of the ligands, these methyl groups being likely important for directing the conformation of the ligand. Such a specificity of hydrogen bonds in determining the binding or not of a mono- or oligosaccharide and in changing the carbohydrate binding properties of lectins has been demonstrated previously by molecular engineering (58, 71). In this way, the present data reinforce the concepts based on the previous knowledge on the mechanisms and on the determination of the carbohydrate specificities of protein-sugar interactions. Based on the results of docking experiments (Fig. 5), the lower energy conformation of the glycoprotein HNK-1 group showed that the semi-acetalic group of the C-1 carbon atom of Man is turned outside the IL-6 structure, indicating that the interaction observed with a portion of the HNK-1 containing N-glycan will still occur with a complete glycan because of the absence of steric hindrance of the of the more internal parts of the glycan and of the protein to which it is attached (gp130). Furthermore, the data of the docking experiments explained why compound III is not a ligand, and compound I is a poor ligand, of IL-6. Therefore, it may be hypothesized that site 3 of IL-6, defined as a site of interaction with gp130, corresponds to the carbohydrate recognition domain of IL-6.

The question remains as to how these data can fit with previous studies (biochemical and structural) and, especially, with those obtained with recombinant soluble forms of IL-6 receptors (72). Indeed, in the presence of a soluble form of gp130, the complex IL-6-IL-6Rα, which is present on the form of a heterodimer, is transformed into a hexamer. This is likely due to the fact that the soluble gp130 associates into dimers spontaneously and can bind two IL-6-IL-6Rα complexes. The question remained to explain how the lectin activity could participate to the formation of these hexamers. The recombinant soluble form of gp130 used in the previous studies for detecting the formation of hexamers (73–76) was produced in Chinese hamster ovary cells. This point seems of importance, since these cells are (as HepG2 cancer cell types) competent for the synthesis of the HNK-1 epitope. Therefore, it may be suggested that the formation of the hexamer is directed by the presence of one N-glycan possessing the HNK-1 epitope on each gp130 molecule, i.e. two glycans endowed with the HNK-1 epitope per dimer of soluble gp130. This allows the fixation of two IL-6 molecules bound to IL-6Rα and, consequently, the formation of the hexamer comprising two gp130, two IL-6 and two IL-6Rα molecules. The localization of the CRD of IL-6 in site 3 of the molecule fits with the theoretical model of the formation of the hexamer proposed by Somers et al. (9). The recombinant soluble form of gp130 used in the previous studies possessed three potential N-glycosylation sites (Asn\(^{13}\), Asn\(^{39}\), and Asn\(^{109}\)) susceptible to having the HNK-1 epitope. Based on the stoichiometry of the molecules in the hexamers (74), it is suggested that only one of these N-glycosylation sites has the HNK-1 epitope (otherwise the stoichiometry would have been changed to higher order of associations). However, it is not certain that one of these three N-glycosylation sites found on the soluble recombinant form of gp130 is actually the same as that bearing the HNK-1 epitope on the complete gp130 produced by quiescent human lymphocytes. Indeed, there is a general consensus that membrane anchoring plays important roles in the pattern of N-glycosylation of glycoproteins. Therefore, it may be that, in human lymphocytes, the HNK-1 epitope could be built on N-glycans present on more internal parts of the complete gp130. Although the precise signals in the polypeptide chain necessary for the synthesis of the HNK-1 epitope are not known, it may be that, if such signal sequence exist, it would be the same in the soluble gp130 and in the total membrane-bound gp130. Therefore, it is possible that the HNK-1 bearing N-glycan would be the same in the two types of molecules, a point that could be easily solved by site-directed mutagenesis of the individual three potential N-glycosylation sites of the soluble gp130. A question remains to know how other cytokines, which use the same gp130 signal-transducing molecule, can associate their receptors to gp130. Based on the three-dimensional structures, it is unlikely that they recognize also the HNK-1 epitope, although they could be able to associate their receptor with gp130 through other specific lectin activities. The high level of N-glycosylation of gp130 may be a key for understanding different carbohydrate-dependent association with the cytokine receptors.

Therefore, this study proposes new concepts in the mechanism of the IL-6 function and structure. The involvement of the HNK-1 epitope in the function of IL-6 may be of importance for pathology, since HNK-1 is especially expressed in the nervous tissue and more specifically expressed in myelinizing cells in adult brain (28, 29, 39). This overexpression of the HNK-1 epitope could be a clue for understanding demyelinating diseases. For example, the knockout of the IL-6 gene in mice suppresses the experimental allergic encephalomyelitis and its associated demyelination observed in specific mouse strains sensitive to experimental allergic encephalomyelitis (77–79).

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