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REVIEW

Interleukin-6: Structure–function relationships

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Abstract

Interleukin-6 (IL-6) is a multifunctional cytokine that plays a central role in host defense due to its wide range of immune and hematopoietic activities and its potent ability to induce the acute phase response. Overexpression of IL-6 has been implicated in the pathology of a number of diseases including multiple myeloma, rheumatoid arthritis, Castleman's disease, psoriasis, and post-menopausal osteoporosis. Hence, selective antagonists of IL-6 action may offer therapeutic benefits. IL-6 is a member of the family of cytokines that includes interleukin-11, leukemia inhibitory factor, oncostatin M, cardiotrophin-1, and ciliary neurotrophic factor. Like the other members of this family, IL-6 induces growth or differentiation via a receptor-system that involves a specific receptor and the use of a shared signaling subunit, gp130. Identification of the regions of IL-6 that are involved in the interactions with the IL-6 receptor and gp130 is an important first step in the rational manipulation of the effects of this cytokine for therapeutic benefit. In this review, we focus on the sites on IL-6 which interact with its low-affinity specific receptor, the IL-6 receptor, and the high-affinity converter gp130. A tentative model for the IL-6 hexameric receptor ligand complex is presented and discussed with respect to the mechanism of action of the other members of the IL-6 family of cytokines.

Keywords: cytokine; gp130; interleukin-6; receptor; structure–function; ternary complex

It has been almost a decade since researchers involved in the identification of a number of seemingly unrelated biological growth factors came to the startling conclusion that they had cloned or purified the same protein. Indeed, the molecule that we now call interleukin-6 (IL-6) was originally referred to by such diverse names as interferon-β_2 (Weissenbach et al., 1980; Zilberstein et al., 1986), 26K factor (Content et al., 1982; Haegeman et al., 1986), B-cell stimulatory factor 2 (Hirano et al., 1985), hybridoma growth factor (van Snick et al., 1986; Brakenhoff et al., 1987), plasmacytoma growth factor (Nordan et al., 1987), hepatocyte-stimulatory factor (Gauldie et al., 1987), a hematopoietic factor (Ikebuchi et al., 1987), and cytotoxic T-cell differentiation factor (Takai et al., 1988)—each name reflecting a different biological activity controlled by the same protein. It is now clear that IL-6 plays a central role in diverse host defense mechanisms such as the immune response, hematopoiesis, and acute-phase reactions (for reviews see van Snick, 1990; Akira et al., 1993; Narazaki & Kishimoto, 1994) (Fig. 1). While IL-6 appears to have little to do with the day-to-day "housekeeping" functions of the body, along with other cytokines it represents an important frontline component of the body's armory against infection or tissue damage (Akira et al., 1993; Nicola, 1994). In vivo studies using IL-6 knockout mice demonstrate that, while IL-6-deficient mice develop normally, they have impaired immune and acute-phase responses (Fattori et al., 1994; Kopf et al., 1994; Poli et al., 1994; Ramsay et al., 1994).

In accordance with its functional pleiotropy, IL-6 has been implicated in the pathology of many diseases including multiple myeloma (Kawano et al., 1988; Bataille et al., 1989), rheumatoid arthritis (Hirano et al., 1988), Castleman's disease (Yoshizaki et al., 1989), AIDS (Nakajima et al., 1989; Poli et al., 1990), mesangial proliferative glomerulonephritis (Horii et al., 1989), psoriasis (Grossman et al., 1989), Kaposi's sarcoma (Miles et al., 1990), sepsis (Waage et al., 1989), and osteoporosis (Jilka et al.,

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Abbreviations: CBD, cytokine binding domain; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; Epo, erythropoietin; FN III, fibronectin type III; G-CSF, granulocyte colony-stimulating factor; GH, growth hormone; GM-CSF, granulocyte-macrophage colony stimulating factor; h-, human; IFN-, interferon; IL-, interleukin; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; LIF, leukemia inhibitory factor; m-, mouse; mAb, monoclonal antibody; M-CSF, macrophage colony stimulating factor; OSM, oncostatin M; PRL, prolactin; -R, receptor; s-, soluble.

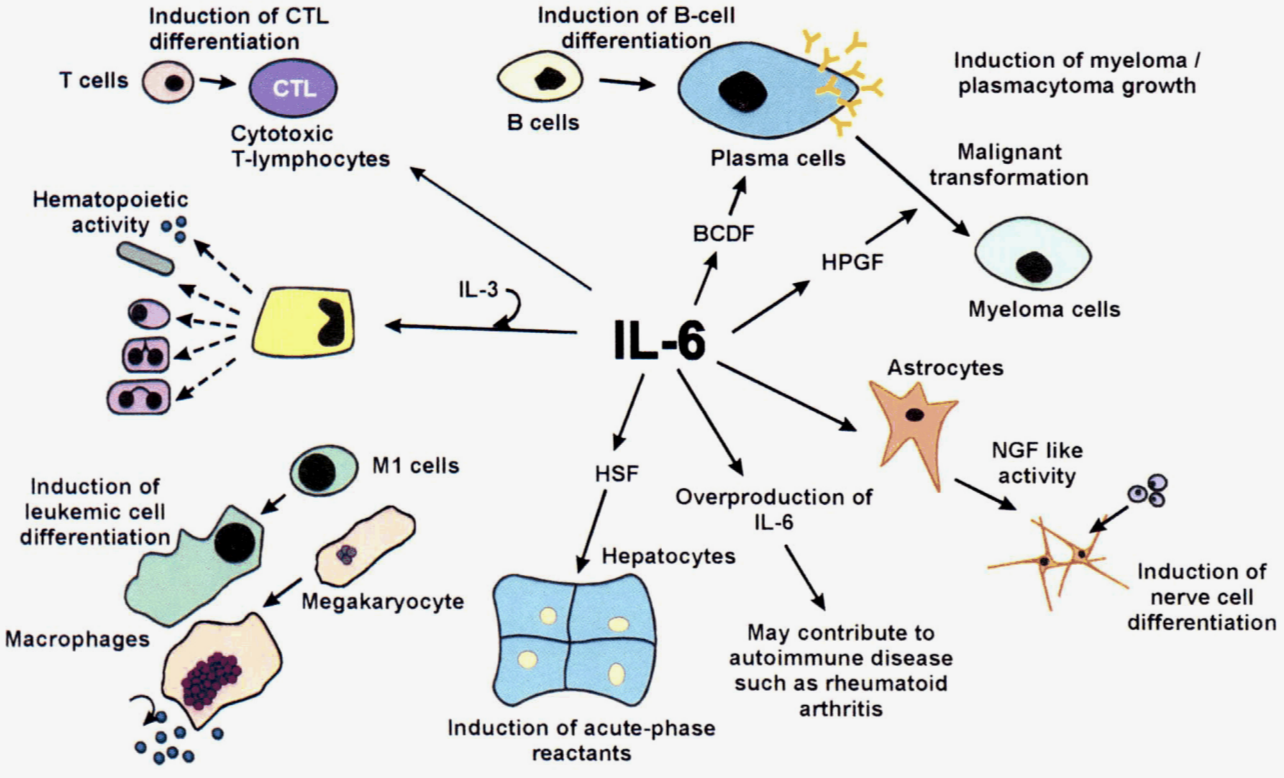

Fig. 1. Biological activities of interleukin-6. The figure shows some of the biological activities of IL-6, as discussed in the introduction.

1992; Poli et al., 1994). Given the association of abnormal IL-6 production and clinical disorders (for reviews see Akira et al., 1993; Hirano, 1994; Klein et al., 1995), there is intense interest in both understanding the biochemical mechanisms controlled by IL-6, and in the development of functional agonists and antagonists as potential therapeutic agents in the treatment of IL-6-associated diseases.

IL-6 exerts diverse proliferative, differentiative, and maturation events depending on the nature of the target cell. Such functional pleiotropy is a common feature of most cytokines and growth factors that have a role in the immunohemopoietic system (Metcalf, 1993; Nicola, 1994). Since a single cell often responds to numerous cytokines and growth factors that act in synergy, overlapping biological activities (functional redundancy) or modulation between such cytokines may occur either at their receptors or along their intracellular signal transduction pathways. It has also been shown that different cytokines can show similar or identical functions on the same cell (for reviews see Nicola, 1989; Kishimoto et al., 1992; Metcalf, 1993). Unlike classical hormones, cytokines are produced locally by a variety of cells that are widespread in the body. They also act locally and are found in the circulation only in low levels. For example, in healthy individuals, serum IL-6 is usually <10 pg/mL (Akira et al., 1993). One widely held view is that the local production and action of cytokines may be an economical way for the body to use the same cytokine to effect pleiotropic biological activities (Nicola, 1994). The relatively recent finding that distinct cytokines that display overlapping biological activities have receptor systems that consist of a unique, low affinity ligand-binding α-chain and a common, signal-transducing high affinity-converting β-chain has been a major step forward in our understanding of cytokine action (for reviews see Kishimoto et al., 1994; Nicola, 1994). For example, IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), IL-11 (for a review see Kishimoto et al., 1994), and cardiotrophin-1 (CT-1) (Pennica et al., 1995) share a β-chain called gp130. Similarly, IL-2, IL-4, IL-7, IL-9, IL-13, and IL-15 share the γ subunit (p64) of the IL-2 receptor, and IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) share a subunit called β_c (for reviews see Miyajima et al., 1992; Voss & Robb, 1994). These shared receptor β-subunits, in part, provide a molecular basis for the functional redundancy of cytokines.

In this report, we review the functional characteristics of IL-6, focusing on its interactions with the IL-6 receptor (IL-6R) and gp130.

Structure of IL-6 and the gene

The IL-6 gene is located at chromosome 7p21 (Sehgal et al., 1986) and 5 (Mock et al., 1989) in the human and mouse genomes, respectively. The genes for human (Zilberstein et al., 1986; Yasukawa et al., 1987), mouse (Tanabe et al., 1988), and rat IL-6 (Northemann et al., 1989) have been cloned and sequenced, and all contain four introns and five exons. Within the protein coding region of the gene, the positions of exon/intron boundaries, exon lengths, and location of cysteine residues within exons are conserved across species. However, differences occur at the 5′ boundary of exon 1 and the 3′ boundary of exon 5, which lie outside the coding region. This conservation of genomic structure is also observed in the gene for granulocyte colony-stimulating factor (G-CSF), which is related to IL-6 by sequence homology (Tanabe et al., 1988).

The cDNAs for many species of IL-6 have been cloned and sequenced (Fig. 2). IL-6 from all species studied thus far contains a predicted hydrophobic signal sequence of 24 to 28 residues at the N-terminus of the full length protein (Fig. 2). N-terminal sequencing of the mature human and mouse proteins confirms that the signal peptide is cleaved (Hirano et al., 1985; van Snick et al., 1986; Nordan et al., 1987; van Damme et al., 1987; Simpson et al., 1988a). There is some evidence for differential cleavage of hIL-6 from different sources, in which the mature protein can differ by two residues at the N-terminus (van Damme et al., 1987; May et al., 1991a). In addition, differential cleavage was predicted for feline IL-6 (Bradley et al., 1993). Recently, Kestler et al. (1995) reported the existence in peripheral blood mononuclear cells of an alternatively spliced hIL-6 mRNA transcript lacking exon 2, encoding an N-terminally truncated protein. The complete primary structure of the mature natural mouse protein was established independently using Edman degradation methodologies (Simpson et al., 1988a).

The sequence identity amongst the IL-6 species identified to date ranges from 34% (mouse vs sea otter) to 97% (mangabey vs macaque), or 54% to 99%, respectively, if we consider like, rather than identical amino acids. Amongst species there is considerable amino acid sequence identity in the central portion of the molecule (residues 40–100) and the region close to the C-terminus (residues 165–184). The degrees of similarity between the predicted protein sequences from different species are shown in Table 1.

Post-translational modifications of natural IL-6

Natural human IL-6 is a single-chain glycoprotein with a relative molecular mass (M_r) ranging from 21K to 30K depending on the cellular source and method of preparation (Hirano et al., 1985; Cayphas et al., 1987; van Damme et al., 1987, 1988). This heterogeneity is due to extensive post-translational modification. Under strongly reducing and denaturing conditions, the modified forms of natural IL-6 tend to cluster around two molecular weights. The 23–25K forms are exclusively O-glycosylated, while the 28–30K forms are both N- and O-glycosylated (May et al., 1988a, 1991a). Of the two potential N-glycosylation sequons (Asn-Xaa-Ser/Thr) in human IL-6 (Asn 45 and Asn 144; see Fig. 2), only one (not yet identified) is apparently utilized (Gross et al., 1989). In addition, most forms of natural IL-6 are phosphorylated at multiple serine residues, although the extent of phosphorylation is very tissue specific (May et al., 1988b, 1989a). Sulfation is also common, although it is unclear whether this is by attachment to sugar moieties or tyrosine side-chains (May et al., 1989a, b). Natural murine IL-6 also exhibits heterogeneity with its M_r ranging from 22K to 29K, and with variable charge (pI = 5–7) (van Snick et al., 1986, 1987). This heterogeneity in charge and mass, which diminishes upon treatment with neuraminidase, is attributable to variations in glycosylation (van Snick et al., 1986). Murine IL-6 lacks N-linked glycosylation sequons (Fig. 2), but is O-glycosylated exclusively at Thr 140 (Simpson et al., 1988a). The post-translational modification of IL-6 appears to have little or no effect on its biological activity, as evidenced by the identical activity of IL-6 derived from different recombinant sources with that of natural IL-6 (see below).

Post-translational modifications of recombinant IL-6

Like most cytokines, IL-6 is found in the circulation in low abundance (Akira et al., 1993), necessitating its large-scale production for structure–function studies by recombinant means. IL-6 has been expressed in rabbit reticulocyte lysate (Geiger et al., 1988), *Escherichia coli* (Brakenhoff et al., 1987; Tonouchi et al., 1988; Krüttgen et al., 1990a; Yasueda et al., 1990; Arcone et al., 1991a; Grennet et al., 1991; Zhang et al., 1992; Hammacher et al., 1994), *Saccharomyces cerevisiae* (Guisez et al., 1991), *Pichia pastoris* (this laboratory, unpublished data), *Aspergillus nidulans* (Contreras et al., 1991), *Xenopus laevis* (Fontaine et al., 1991), baculovirus insect cells (Matsuura et al., 1989) and Chinese hamster ovary (CHO) cells (Eisenthal et al., 1993; Orita et al., 1994).

IL-6 expressed in yeast is not post-translationally modified. However, glycosylation occurs in some of the expression systems based on higher species. Human IL-6 expressed in baculovirus shows heterogeneity and apparent glycosylation (Matsuura et al., 1989). CHO cell-expressed hIL-6 is N-glycosylated at Asn 45 and O-glycosylated at Thr 138 (Orita et al., 1994), whereas hIL-6 expressed by *X. laevis* oocytes is only N-glycosylated at Asn 144 (Fontaine et al., 1991). Regardless of the glycosylation profile or N-terminal heterogeneity, natural IL-6 and that produced by recombinant means from these different sources have essentially the same biological activity (Brakenhoff et al., 1987; van Damme et al., 1987; Geiger et al., 1988; Simpson et al., 1988b).

Oligomeric forms of IL-6

Higher M_r forms of fibroblast-derived natural IL-6 have been observed with a range of 45K to 85K (May et al., 1991b). These forms have been shown to be oligomeric complexes of post-translationally modified IL-6, the individual components of which can be identically or differently modified (May et al., 1991b). The subunits of these oligomers are not linked by intermolecular disulfide bonds. Depending on the assay used, the activities of these oligomeric forms are equivalent to, or less than, that of monomeric hIL-6 (May et al., 1991b). Higher molecular weight forms of IL-6 have also been observed for other species including feline IL-6 (Ohashi et al., 1989; Goitsuka et al., 1990). Recombinant forms of IL-6, expressed in different systems, can also form multimers. For instance, *E. coli*–expressed hIL-6 can form relatively stable non-covalent dimers as purification artefacts (Ward et al., 1996). Although dimeric recombinant hIL-6 has a stronger binding affinity for the IL-6R than monomeric recombinant hIL-6, or natural monomeric hIL-6 (Wijdenes et al., 1991), it is impeded in its IL-6R-dependent interaction with gp130, and, therefore, has a lower biological activity than monomeric hIL-6 (Ward et al., 1996). Human IL-6 expressed by baculovirus in insect cells has also been shown to form dimers (Matsuura et al., 1989).

Physicochemical characterization of IL-6

The physicochemical characterization of IL-6 to date has been fairly limited. Circular dichroism spectroscopy was used to show that recombinant IL-6 has a high α-helix content. The secondary structural compositions of human and mouse IL-6 expressed in *E. coli* are predicted to be 67% α-helix, 15% β-sheet, 18% β-turn and random coil (Krüttgen et al., 1990a), and 52% α-helix, 10% β-sheet, 19% β-turn and 19% random coil (Zhang et al., 1992), respectively. Estimations of the thermostability of IL-6 at physiological pH are hampered by the non-two-state equilibrium unfolding under these conditions. In particular, recombinant mIL-6 produces unfolding intermediates that tend to associate (Ward et al., 1995b). These intermediates have the same spectral properties as the ag-

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                 -20       -10         1        10        20
Human      MNSFSTSAFGPVAFSLGLLLVLPAAFPAPVPPGEDSKDVAAPHRQPLTSSERIDKQ
Mangabey   MNSFSTSAFGPVAFSLGLLLVLPAAFPAPVLPGEDSKDVAAPHSQPLTSSERIDKH
Macaque    MNSFSTSAFGPVAFSLGLLLVLPAAFPAPVLPGEDSKNVAAPHSQPLTSSERIDKH
Sea Otter  ---RSTSAFSPVAFSLGLLLVMATAFPTPGPLGGDSKDDATSNRPPLTSADKMEDF
Mink       --------------------------------------------------------
Dog        MNSLSTS-----AFSLGLLLVMATAFPTPGPLAGDSKDDATSNSLPLTSANKVEEL
Seal       ---RFTSAFSPVAFSLGLLLVMATAFPTPGPVGGESQADATSNRPPLTSPDKMEEF
Cat        MTFLSTSAFSPLAFSLGLLLVVATAFPTPGPLGG----DATSNRLPLTSADKMEEL
Pig        MNSLSTSAFSPVAFSLGLLLVMATAFPTPGRLEEDAKGDATSDKMLFTSPDKTEEL
Orca       ---RFTS-----AFSLGLLLVTATAFPTPGPLGEDFKDDTTSDRLYLTSPDKTEAL
Cow        MNSRFTSAFTPFAVSLGLLLVMTSAFPTPGPLGEDFKÑDTTPGRLLLTTPEKTEAL
Sheep      MNSLFTSAFSPLAVSLGLLLVMTSAFPTPGPLGEDFKÑDTTPSRLLLTTPEKTEAL
Mouse      MKFLSARDFHPVAF-LGLMLVTTTAFPTSQVRRGDFTEDTTPNR-PVYTTSQVGGL
Rat        MKFLSARDFQPVAF-LGLMLLTATAFPTSQVRRGDFTEDTTHNR-PVYTTSQVGGL
consensus  mnsxstsafxpvAfsLGLlLvxxtAFPtpgplgedxkxdatxxrxpltsxxkxexl

           30        40        50        60        70        80
Human      IRYILDGISALRKETCÑKSNMCESSKEALAENNLNLPKMAEKDGCFQSGFNEETCL
Mangabey   IRYILDGISALRKETCÑRSNMCDSTKEALAENNLNLPKMAEKDGCFQSGFNEDTCL
Macaque    IRYILDGISALRKETCÑRSNMCESSKEALAENNLNLPKMAEKDGCFQSGFNEDTCL
Sea Otter  IKFILGKISALRNEMCDKYNKCEDSKEVLAENNLNLPKLAEKDRCFQSRFNQETCL
Mink       ---------------------------AENNLKLPKLAEKDKCFQSQFNQETCM
Dog        IKYILGKISALRKEMCDKFNKCEDSKEALAENNLHLPKLEGKDGCFQSGFNQETCL
Seal       IKYILGKISALRKEMCDKYNKCEDSKEALAENNLRLPKLAEKDGCFQSGFNQETCL
Cat        IKYILGKISALKKEMCDNYNKCEDSKEALAENNLNLPKLAEKDGCFQSGFNQETCL
Pig        IKYILGKISAMRKEMCEKYEKCENSKEVLAENNLNLPKMAEKDGCFQSGFNQETCL
Orca       IKYILGKISAMRKEMCEKYDKCENSKEALAENNLNLPKMAEKDGCFQSGFNQETCL
Cow        IKRMVDKISAMRKEICEKNDECESSKETLAENKLNLPKMEEKDGCFQSGFNQAICL
Sheep      IKHIVDKISAIRKEICEKNDECENSKETLAENKLKLPKMEEKDGCFQSGFNQAICL
Mouse      ITHVLWEIVEMRKELCNGNSDCMNNDDALAENNLKLPEIQRNDGCYQTGYNQEICL
Rat        ITYVLREILEMRKELCNGNSDCMNSDDALSENNLKLPEIQRNDGCFQTGYNQEICL
consensus  IkyilxkIsalrkExCxkxnxCexskeaLaENnLnLPkmaekDgCfQsgfNqetCl

              90        100       110       120       130
Human      VKIITGLLEFEVYLEYLQNRFESS-EEQARAVQMSTKVLIQFLQKKAKNLDAITTP
Mangabey   VKIITGLLEFEVYLEYLQNRFESS-EEQARAVQMSTKVLIQLLQKKAKNLDAITTP
Macaque    VKIITGLLEFEVYLEYLQNRFESS-EEQARAVQMSTKVLIQFLQKKAKNLDAITTP
Sea Otter  TRITTGLQEFQIHLKYLESNYEGN-KDNAHSVYISTKHLLQTLRPM--NQIEVTTP
Mink       TRITTGLQEFQIHLKYLEANYEGN-KNNAHSVYISTKHLLQKLRPM--NQVEVTTP
Dog        TRITTGLVEFQLHLNILQNNYEGD-KENVKSVHMSTKILVQMLKSKVKNQDEVTTP
Seal       TRITTGLLEFQIHLKYIQANYEGN-KEDANSVYISTKLLVQMLMKKVKSQDEVTTP
Cat        TRITTGLQEFQIYLKFLQDKYEGD-EENAKSVYTSTNVLLQMLKRKGKNQDEVTIP
Pig        MRITTGLVEFQIYLDYLQKEYESN-KGNVEAVQISTKALIQTLRQKGKNPDKATTP
Orca       MRITTGLLEYQIYLDYLQNEYEGD-KEAIEAVQISSKALAQILRQKVKNPDEVTTP
Cow        IRTTAGLLEYQIYLDYLQNEYEGN-QENVRDLRKNIRTLIQILKQKI--ADLITT-
Sheep      IKTTAGLLEYQIYLDFLQNEFEGN-QETVMELQSSIRTLIQILKEKI--AGLITT-
Mouse      LKISSGLLEYHSYLEYMKNNLKDNKKDKARVLQRDTETLIHIFNQEVKDLHKIVLP
Rat        LKICSGLLEFRFYLEFVKNNLQDNKKDKARVIQSNTETLVHIFKQEIKDSYKIVLP
consensus  xritt GLlEfqiyLxylqnxyegn-kexaxxvqxstkxLiqxlxxkxknxdxittp

           140       150       160       170       180
Human      DPTTÑASLLTKLQAQ-NQWLQDMTTHLILRSFKEFLQSSLRALRQM     Y00081
Mangabey   EPTTÑASLLTKLQAQ-NQWLQDMTTHLILRSFKEFLQSSLRALRQM     L26032
Macaque    EPTTÑASLLTKLQAQ-NQWLQDMTTHLILRSFKEFLQSNLRALRQM     L26028
Sea Otter  DPTTDASLQALFKSQ-DKWLKHTTIHLILRRLEDFLQFSLRAIRIM     L46804
Mink       NPTTDSSLQALFKSQ-DKWLKHVTIHLILRSLEDFLQFSLRAIRIM     L34165
Dog        DPTTDASLQAILQSQ-DECVKHTTIHLILRSLEDFLQFSLRAVRIM     U12234
Seal       DPTTDTSLQAILKAQ-DKWLKHTTIHLILRSLEDFLQFSLRAVRIM     L46802
Cat        VPTVEVGLQAKLQSQ-EEWLRHTTIHLTLRRLEDFLQFSLRAVRIM     D13227
Pig        NPTTNAGLLDKLQSQ-NEWMKNTKIILILRSLEDFLQFSLRAIRIM     M80258
Orca       DPTTÑASLMNNLQSQNDDWMKNTKIILILRSLENFLQFSLRAIRIK     L46803
Cow        -PATNTDLLEKMQSS-NEWVKNAKIILILRNLENFLQFSLRAIRMK     X57317
Sheep      -PATHTDMLEKMQSS-NEWVKNAKVIIILRSLENFLQFSLRAIRMK     X68723
Mouse      TPISNALLTDKLESQ-KEWLRTKTIQFILKSLEEFLKVTLRSTRQT     M24221
Rat        TPTSNALLMEKLESQ-KEWLRTKTIQLILKALEEFLKVTMRSTRQT     M26744
consensus  xPttnaslxxklqsq-xewlkxxtihliLrslexFLqfslRaxRim
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Table 1. *Percentage sequence identities (upper triangle) and similarities (lower triangle), for various IL-6 species*[a]

	Human	Mangabey	Macaque	Sea Otter	Mink	Dog	Seal	Cat	Pig	Orca	Cow	Sheep	Mouse	Rat
Human		95	96	55	54	58	59	57	59	60	52	51	39	39
Mangabey	98		97	52	53	55	55	54	57	56	49	48	38	37
Macaque	98	99		52	52	55	56	55	57	56	50	49	38	38
Sea Otter	70	69	69		90	76	82	76	68	69	52	48	34	35
Mink	71	71	70	94		72	80	70	66	65	49	46	37	37
Dog	75	75	74	88	86		80	78	68	71	56	53	36	39
Seal	73	72	72	89	90	90		78	66	70	53	51	36	38
Cat	74	73	72	86	80	87	86		68	68	53	51	36	39
Pig	76	75	74	79	75	82	81	81		81	65	61	39	39
Orca	77	76	75	80	77	85	83	81	92		69	66	40	40
Cow	71	70	69	67	66	73	69	69	77	82		88	39	39
Sheep	69	68	68	66	66	73	70	68	77	83	94		38	38
Mouse	57	57	57	54	58	60	56	58	60	63	62	62		85
Rat	58	58	57	57	60	62	57	61	62	65	61	61	92	

[a]Values (expressed as percentages) are calculated from the sequence alignments shown in Figure 2 based on the sequence of mature hIL-6. Conservative substitutions are included and, where sequences differ in length, percentage values are based on the shorter of the two sequences being compared.

gregates that form at high protein concentrations and elevated temperatures (Morton et al., 1995), suggesting that this aggregation also results from the association of partially unfolded forms of mIL-6. At pH 4.0, where unfolding induced by urea follows a two-state process, recombinant wild-type mIL-6 has a thermostability of 9.0 kcal/mol (Ward et al., 1995b) compared with 14.5 kcal/mol for the related recombinant human growth hormone (hGH) (Brems et al., 1990). A variant of human IL-6, lacking the 22 N-terminal amino acids and the disulfide bond between Cys 44 and Cys 50 (these residues being substituted for serine), was shown to retain the properties of the full-length protein (Breton et al., 1995) and have a stable, protease resistant, molten globule-like conformation at pH 2.0 (de Filippis et al., 1996). The unfolding of this A-state follows a non two-state process with highly stable secondary structure (de Filippis et al., 1996).

Chemical modification studies

In the absence of three-dimensional structural information, it is possible to obtain residue-specific information about solvent accessibility and importance for activity or stability through chemical modification and mutational studies.

Disulfide bonds

While IL-6 contains four cysteine residues that are highly conserved in all species (rat, murine, human, feline, canine, porcine, ovine, monkey, mink, seal, orca, sea otter, and bovine), rat IL-6 and dog IL-6 each contain an additional cysteine residue at positions 88 and 157, respectively (Fig. 2). Murine and human IL-6 show the same disulfide bridges (Cys 44–Cys 50 and Cys 73–Cys 83) (Simpson et al. 1988b; Clogston et al. 1989), which are presumably conserved across species. These cysteine connectivities are also observed in the related human granulocyte-colony stimulating factor (G-CSF) (Clogston et al., 1989; Lu et al., 1989), and chicken myelomonocytic growth factor (Leutz et al., 1989). Even when solubilized from inclusion bodies in *E. coli*, IL-6 requires no additional refolding step to ensure correct disulfide bond formation (Zhang et al., 1992).

Both disulfide bonds can be reduced and alkylated under nondenaturing conditions, suggesting that they are solvent exposed. The first disulfide bond (Cys 44–Cys 50) can be selectively reduced (Rock et al., 1994) and plays only a minor role in hIL-6 activity (Snouwaert et al., 1991b; Rock et al., 1994). The second disulfide bond, however, plays a much larger role in hIL-6 activity (Snouwaert et al., 1991b; Rock et al., 1994). The disulfide bonds are apparently responsible for maintaining structural integrity of receptor binding sites rather than conformational stability (Rock et al., 1994). Substitution of the four cysteine residues in IL-6 by other amino acids by recombinant means results in biological activities of less than 0.1% when tested on human cell lines (Snouwaert et al, 1991b; Rock et al., 1994). However, when tested on rat and

Fig. 2. (*Figure is on facing page.*) Alignment of the known IL-6 sequences. The numbering is based on the mature human sequence (Hirano et al., 1986) and the consensus sequence is shown below. Gaps (–) are inserted to maximize alignment. If fewer than 50% of the residues at any position are identical, the consensus residue is shown as x and completely conserved residues are shown in upper case. Amino acids with similar physico-chemical properties are shown in the same color in the alignment. GenBank accession codes for the translated sequences are given at the end of the alignment. The primary structures predicted from the corresponding cDNAs of human (Hirano et al., 1986), mouse (van Snick et al., 1988), rat (Northemann et al., 1989), bovine (Droogmans et al., 1992), pig (Richards & Saklatvala, 1991), sheep (Andrews et al., 1993; Ebrahimi et al., 1995), feline (Bradley et al., 1993; Ohashi et al., 1993), seal, orca, and sea otter (King et al., 1996), monkey (Villinger et al., 1995), mink (Bloom et al., 1994), and dog (Kukielka et al., 1994) IL-6 are shown. The N-terminal sequence of native mIL-6 is Phe-Pro-Thr-Ser-Gln- (Simpson et al., 1988a); of native hIL-6 is Pro-Val-Pro-Pro- (Hirano et al., 1985) and Ala-Pro-Val-Pro-Pro- (van Damme et al., 1988). The discrepancy in N-terminii for hIL-6 may reflect variable cleavage of the mature proteins by contaminating proteases during isolation. Positions of potential N-glycosylation sequons (Asn-Xaa-Ser/Thr) are indicated above the asparagine residue by (~). Note that there is a discrepancy between the reported primary structures for dog IL-6: A cysteine residue at position 157 in the GenBank database corresponds to a tryptophan residue in Kukielka et al. (1994).

mouse cell lines some biological activity is retained (Jambou et al., 1988), possibly due to less strict requirements of conformational integrity for IL-6 ligand binding in these species. In contrast, chemical reduction and alkylation of the disulfide bonds of recombinant mIL-6 reduces the stability of the protein and radically decreases its activity to an extent which is dependent on the type of modifying agent used (Zhang et al., 1997).

Methionine and tyrosine

The five methionine residues of hIL-6 are differentially susceptible to oxidation. Met 49 is the residue that is the most readily oxidised to methionine sulfoxide by chloramine T, followed by Met 117, Met 184, and Met 161 (Nishimura et al., 1991a). Only Met 67 is resistant to oxidation, while Met 161 appears to be important for receptor binding (Nishimura et al., 1991a). Of the three tyrosine residues in hIL-6, only Tyr 31 is readily iodinated, which has no discernible effect on receptor binding (Nishimura et al., 1990).

Tryptophan

Murine IL-6 contains two tryptophan residues, Trp 34 and Trp 157. In recombinant mIL-6, Trp 34 and Trp 157 have a relatively low fluorescence emission maximum, suggesting that their side-chains are solvent-exposed (Ward et al., 1993b). Both residues can be readily modified under non-denaturing conditions by 2-nitrophenylsulfenylchloride to produce a molecule that is only slightly less stable than unmodified mIL-6 and maintains a high percentage of its biological activity (Zhang et al., 1993). The fluorescence of Trp 34 is quenched at low pH (Ward et al., 1993b). Studies on mIL-6 mutants and corresponding synthetic peptide analogues established that this is due to the quenching of Trp 34 fluorescence by the neighboring protonated His 31 residue (Ward et al., 1993b, 1995b; Matthews et al., 1997). The associated equilibrium unfolding intermediates of mIL-6 (see section on physicochemical properties) have unusual fluorescence characteristics, attributable to burial of tryptophan side chains (Ward et al., 1995b). In line with this, mIL-6 mutants with Trp 34 substituted for alanine have increased two-state unfolding behavior, implying that the region near His 31 and Trp 34 is involved in the protein–protein interactions that lead to the aggregation of mIL-6. These studies therefore suggest that it may be possible to engineer more stable analogs of mIL-6 that do not undergo the time-dependent aggregation. The single tryptophan residue of hIL-6 (Trp 157) is conserved in most species (see Fig. 2) and the fluorescence of recombinant hIL-6 closely resembles that of Trp 157 in recombinant mIL-6 (Matthews et al., 1997). Trp 157 in hIL-6 is readily modified and lies close to a receptor binding region (site III; cf. Determination of the active site, below) (Nishimura et al., 1991a; Brakenhoff et al., 1994).

IL-6 and helical cytokine structure

Early predictive studies of cytokine sequences (Parry et al., 1988, 1991; Bazan, 1990a, 1991) and the relationships among their receptors (Bazan, 1989, 1990b, 1990c), when combined with the early crystallographic studies of GH (Abdel-Meguid et al., 1987) and IL-2 (Brandhuber et al., 1987), led to the prediction that many cytokines would have a four-α-helix bundle structure. Subsequently, the three-dimensional structures of 14 different helical cytokines (some receptor-bound) have been solved by X-ray diffraction and NMR (Table 2) confirming this prediction. These structures have been reviewed by several authors (Boulay & Paul, 1993; Sprang & Bazan, 1993; Wlodawer et al., 1993; Rozwarski et al., 1994; Mott & Campbell, 1995), and structural comparisons are made in many of the papers cited in Table 2. Recently, the NMR assignments and secondary structures of hIL-6 (Nishimura et al., 1996; Xu et al., 1996) and OSM (Hoffman et al., 1996) were also shown to be consistent with the four-α-helix bundle structure. Although the three-dimensional structure of IL-6 has not yet been published, it is clear from these studies that it shares the helical cytokine fold. (See Note added in proof.)

The helical cytokine structures have a left-handed (Presnell & Cohen, 1989) up-up-down-down four-helix bundle topology. In terms of the "right hand rule" of physics, "left-handed" means that helix C is to the left of helices A and B, when the thumb of one hand is oriented parallel to the N- to C-terminal direction of helix A. With respect to the direction of the A helix, the B helix is parallel, while the C and D helices are antiparallel. If helix A is considered to go "up" then, in sequential order, the directions of the other helices are up-up-down-down, so that the A and B helices and the C and D helices are diagonally opposite in the bundle. Further details of these structures led them to be classified into the short- and long-chain class (class 1) cytokines and the interferon-like (class 2) cytokines (Boulay & Paul, 1993; Sprang & Bazan, 1993). The cytokine family encompassing IL-6, IL-11, LIF, OSM, and CNTF, as well as IL-12, erythropoietin (Epo), prolactin (PRL), G-CSF, and GH, belong to the long-chain class of four-α-helical bundle proteins.

Several differences distinguish short- and long-chain cytokines. The sequence length is generally <150 residues for short-chain cytokines and >160 residues for the long-chain class. On average, the long-chain cytokine helices ($\sim$30 residues in length) are 10 amino acids longer than those in the short-chain class (10–20 residues). The helices in the long-chain class are more closely packed, falling into the "square" class of four helix bundles (all helices cross with $<40°$ angles) (Harris et al., 1994), than the short-chain cytokines, which resemble the "splinter" class of helix bundles where one of these helix crossing angles is $>40°$ (e.g., GM-CSF) (Harris et al., 1994). The most striking difference between the short- and long-chain classes occurs in the long AB- and CD-loops. In the short-chain class the AB-loop passes over helix D and under the CD-loop, forming a small segment of β-sheet. In the long-chain class (as seen in the structures of G-CSF, LIF, GH) the AB-loop descends from helix A, crosses helix D forming a small helix (AB-loop helix) and then passes along side the CD-loop (for references see Table 2). In CNTF, the AB-loop helix region is not seen in the electron density map (McDonald et al., 1995).

In the interferon-like class of cytokines (class 2), the helix lengths are similar to the short-chain group, but the helix packing is more like the long-chain cytokines. IFN-β has a fifth helix in the CD-loop (Senda et al., 1995), while IFN-γ and IL-10 have six helices which form an intercalating dimer, sharing the fifth and sixth helices across the dimer (Zdanov et al., 1996). IL-5, in the short-chain class, also forms an intercalating dimer that is disulfide-linked, sharing the second β-strand and helix D (Milburn et al., 1993). Macrophage colony-stimulating factor (M-CSF) is a disulfide-linked dimer where each monomer has a standard short-chain cytokine conformation (Pandit et al., 1992).

NMR studies have shown that both human and mouse IL-6 have a flexible N-terminal region, approximately 15 and 22 residues in length for hIL-6 (Proudfoot et al., 1993) and mIL-6 (Morton et al., 1995), respectively, with a compact, predominantly α-helical core. NMR studies on synthetic peptides have shown the presence of at

Table 2. *The four helix bundle cytokines and their receptors for which structures are known*

Cytokine or Receptor	Class[a]	Species	Method	PDB[b]	Reference
IL-2	Short chain	Human	X-ray 2.5 Å	3INK	McKay, 1992
			NMR	1IRL	Mott et al., 1995
IL-3	Short chain	Human	NMR	1JLI	Feng et al., 1996
IL-4	Short chain	Human	NMR	1ITM	Redfield et al., 1994
			NMR	1ITI	Powers et al., 1992, 1993
			NMR	2CYK	Müller et al., 1994
			X-ray 2.4 Å	2INT	Walter et al., 1992a
			X-ray 2.3 Å	1RCB	Wlodawer et al., 1992
			X-ray 2.6 Å	1HIJ	Müller et al., 1995
GM-CSF	Short chain	Human	X-ray 2.7 Å	1CSG	Walter et al., 1992b
			X-ray 2.4 Å	2GMF	Rozwarski et al., 1996
IL-5	Short chain	Human	X-ray 2.4 Å	1HUL	Milburn et al., 1993
M-CSF	Short chain	Human	X-ray 2.5 Å	1HMC	Pandit et al., 1992
G-CSF	Long chain	Human	X-ray 2.2 Å	1RHG	Hill et al., 1993
			NMR	1GNC	Zink et al., 1994
		Canine	X-ray 2.2 Å	1BGE	Lovejoy et al., 1993
			X-ray 2.3 Å	1BGD	Lovejoy et al., 1993
		Bovine	X-ray 1.7 Å	1BGC	Lovejoy et al., 1993
LIF	Long chain	Murine	X-ray 2.0 Å	1LKI	Robinson et al., 1994
GH	Long chain	Human	X-ray 2.8 Å	3HHR	de Vos et al., 1992
			X-ray 2.0 Å	1HUW	Ultsch et al., 1994
			X-ray 2.9 Å	—	Somers et al., 1994
			X-ray 2.9 Å	1HWH	Sundström et al., 1996
			X-ray 2.5 Å	1HWG	Sundström et al., 1996
			X-ray 2.5 Å	1HGU	Chantalat et al.[c]
		Porcine	X-ray 2.8 Å	—	Abdel-Meguid et al., 1987
CNTF	Long chain	Human	X-ray 2.4 Å	1CNT	McDonald et al., 1995
IL-10	IFN-like	Human	X-ray 2.0 Å	1INR	Walter & Nagabhushan, 1995
			X-ray 1.6 Å	2ILK	Zdanov et al., 1996
IFN-α2b	IFN-like	Human	X-ray 2.9 Å	1RH2	Radhakrishnan et al., 1996
IFN-β	IFN-like	Murine	X-ray 2.2 Å	1RMI	Senda et al., 1995
IFN-γ	IFN-like	Human	X-ray 2.8 Å	1HIG	Ealick et al., 1991
			X-ray 2.9 Å	—	Walter et al., 1995
		Rabbit	X-ray 2.3 Å	2RIG	Samudzi et al., 1991
		Bovine	X-ray 3.0 Å	1RFB	Samudzi & Rubin, 1993
EpoR	Class I	Human	X-ray 2.8 Å	1EBP	Livnah et al., 1996
GHR	Class I	Human	X-ray 2.8 Å	3HHR	de Vos et al., 1992
			X-ray 2.9 Å	1HWH	Sundström et al., 1996
			X-ray 2.5 Å	1HWG	Sundström et al., 1996
PRLR	Class I	Human	X-ray 2.9 Å	—	Somers et al., 1994
IFN-γR	Class II	Human	X-ray 2.9 Å	—	Walter et al., 1995
Tissue factor	Class II	Human	X-ray 2.2 Å	1BOY	Harlos et al., 1994
			X-ray 2.4 Å	2HFT	Muller et al., 1996
			X-ray 2.0 Å	—	Banner et al., 1996

[a]Cytokine or receptor class is shown along with the method of structure determination and resolution where appropriate.
[b]If coordinates have been deposited in the protein data bank, the PDB code is given (some structures are still "on hold" in the PDB).
[c]Deposited in PDB only.

least nascent α-helical structure in 12 of the C-terminal 19 residues of mIL-6 (Morton et al., 1994), and from residues Leu 92–Phe 105 and Glu 109–Ser 118 in hIL-6 (Ekida et al., 1992). A peptide encompassing residues Ile 87–Arg 104 of hIL-6 has residual binding to human cells (Ekida et al., 1992).

Through a combination of NMR, chemical modification, and receptor-binding studies Nishimura et al. (1990, 1991a) have assigned several aromatic, histidine, and methionine residues of hIL-6, establishing that His 15, Tyr 31, and Trp 157 are solvent-exposed while Met 161, Tyr 97, and Tyr 100 are buried, that Met 161, His 164, and Met 184 are in close spatial proximity, and that Trp 157 is close to the receptor-binding region (site III, cf. Determination of the active site, below). More recently, Nishimura et al. (1996) have assigned all 6 valine, 7 phenylalanine, and many of the leucine and isoleucine residues of hIL-6 using specific double heteronuclear labeling and site-directed mutagenesis strategies. The location of the helices and the observed NOE network from these studies indicate that the folding topology of hIL-6 follows the up-up-down-down topology as seen in the other helical cytokines.

IL-6 is more closely related to G-CSF than to other four-α-helix bundle proteins, as evidenced by similar genomic structures (Tanabe et al., 1988), approximately 20% amino acid sequence identity,

similar locations of the predicted amphipathic helices and interconnecting loops (Bazan, 1991), and two conserved disulfide bridges (Clogston et al., 1989). These similarities between IL-6 and G-CSF led three groups to use the structure of G-CSF (Hill et al., 1993) as a template to construct three-dimensional models of hIL-6 (Ehlers et al., 1994; Savino et al., 1994b) and mIL-6 (Hammacher et al., 1994). All these models show the expected four-helix bundle topology of the helical cytokines with a small helix in the AB-loop, though the model of Savino et al. (1994b) has a second helix in the AB-loop similar to that found in hGH (de Vos et al., 1992). Each group's alignments of the helices of G-CSF and IL-6 are identical except for helix D. Ehlers et al. (1994) align the C-terminii of the molecules while the other groups align Trp 157 (conserved in all IL-6 species) with the conserved phenylalanine seen in the "D1 motif" (Bazan, 1991) of the other members of the gp130 family of cytokines. This shifting of the register of helix D by three residues between the models will affect the packing of the helix bundle, and how helix D interacts with residues 52–62 in the putative AB-loop helix of IL-6 (Hammacher et al., 1994). The sequence of IL-6 aligned to G-CSF, as used by Savino et al. (1994b) and Hammacher et al. (1994), is shown in Figure 3 along with the structural alignment of G-CSF, LIF, CNTF, and GH.

The NMR sequential assignments, secondary structure, and topology of human IL-6 were recently published (Xu et al., 1996). This work confirms that IL-6 has a four-helix bundle structure consistent with the up-up-down-down topology of the helical cytokines. The four main helices are: Thr 20–Lys 46 (helix A), Glu 80–Asn 103 (helix B), Glu 109–Lys 129 (helix C), and Gln 156–Met 184 (helix D). In addition, Pro 141–Gln 152 make up a small helix in the CD-loop (Xu et al., 1996). The AB-loop appears to be disordered after a tight turn between Cys 44 and Cys 50. Contrary to the other long-chain cytokines—GH (de Vos et al., 1992), G-CSF (Hill et al., 1993), and LIF (Robinson et al., 1994)—no AB-loop helices were reported for IL-6 (Xu et al., 1996; Somers et al., 1997).

Structure of the interleukin-6 receptor and gp130

The biological activities of IL-6 are mediated by the IL-6 receptor-system, which comprises two membrane proteins, the ligand-binding α-subunit receptor (IL-6R) and the signal transducing β subunit, gp130. gp130 also forms part of the receptor-complexes of LIF, OSM, CNTF, IL-11 (Gearing et al., 1992; Ip et al., 1992; Liu et al., 1992; Taga et al., 1992; Yin et al., 1993; Fourcin et al., 1994), as well as CT-1 (Pennica et al., 1995). Targeted disruption of gp130 in mice is lethal during early embryogenesis due to impaired myocardial development and hematopoiesis (Yoshida et al., 1996), whereas continuous activation of gp130 in mice transgenic for both IL-6 and the IL-6R causes myocardial hypertrophy (Hirota et al., 1995).

Coexpression of IL-6R and gp130 results in both low- and high-affinity binding sites for IL-6, the relative amounts of the two subunits dictating the ratio between the two affinity states (Hibi et al., 1990). High-affinity binding is likely due to the ability of IL-6 to interact simultaneously with sites on the IL-6R and gp130. Typically, the difference in affinity between low and high affinity binding is ~100-fold; for example, on human myeloma U266

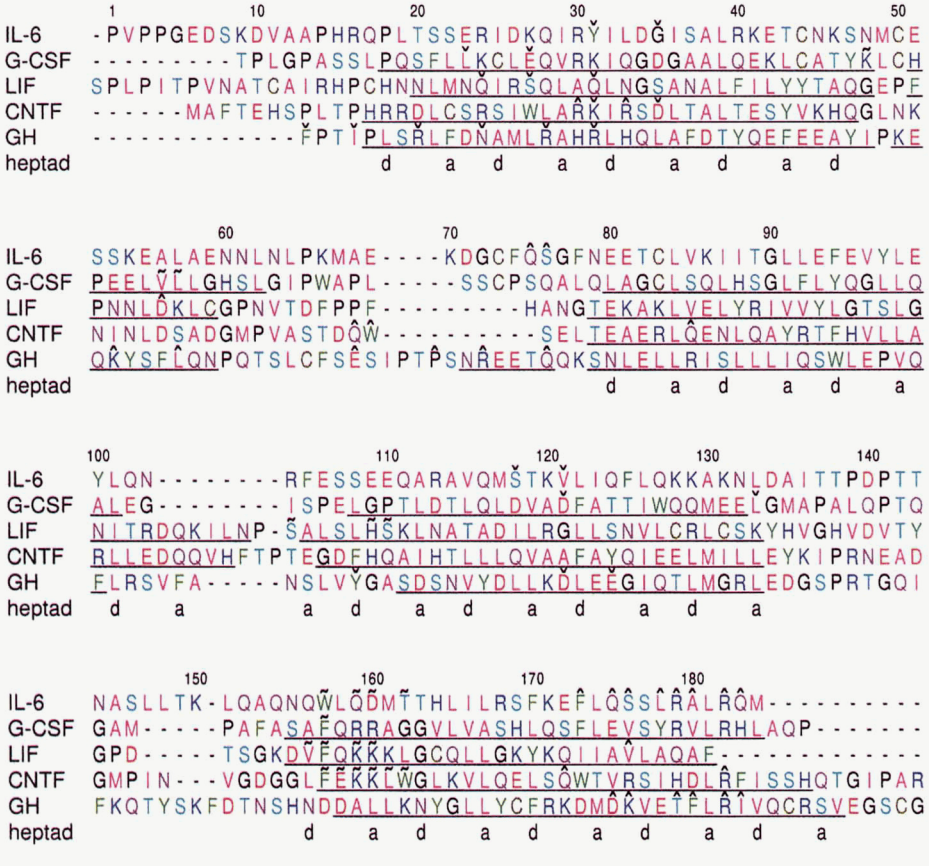

Fig. 3. See legend on facing page.

cells, IL-6 first binds the IL-6R with an affinity of ~1 nM, and the IL-6/IL-6R complex then binds gp130 with a resulting affinity of ~10 pM (Yamasaki et al., 1988). After binding to the IL-6R, IL-6 is rapidly internalized ($t_{1/2}$ ~15–30 min) leading to receptor down-regulation (Nesbitt & Fuller, 1992; Zohlnhöfer et al., 1992). A region spanning amino acids 142–151 (TQPLLDSEER) and an upstream serine (Ser 139) in the cytoplasmic domain of gp130 were identified as being crucial for IL-6 internalization and down-regulation of the IL-6R (Dittrich et al., 1994, 1996). The presence in this region of a di-leucine motif, which has also been described as a lysosomal targeting signal (for a review see Trowbridge et al., 1993), suggests that the gp130-related receptors are endocytosed via clathrin-coated pits.

The IL-6 receptor

Binding of IL-6 has been demonstrated on a variety of human (Coulie et al., 1987; Taga et al., 1987) and mouse cells (Coulie et al., 1989). While hIL-6 binds to mouse receptors, the converse is not true (Coulie et al., 1989). Although the hIL-6R shows a broad distribution (e.g., activated B cells, resting T cells, B lymphoblastoid cell types, hepatoma lines, myeloma and monocyte cell lines), some cell types lack this receptor. Normal cells usually express between 10^2 and 10^3 receptors. Thus far, the largest number of receptors ($1–2 \times 10^4$ per cell) has been found on the human myeloma U266 and on EB virus-transformed CESS cells (Taga et al., 1987; Syners et al., 1989).

The cDNA of the hIL-6R encodes a protein of 468 amino acids, including a signal peptide of 19 amino acids, an extracellular region of 339 amino acids, a transmembrane domain of 28 amino acids, and a short cytoplasmic domain of 82 amino acids (Yamasaki et al., 1988) (see Fig. 4). There are six potential N-linked glycosylation sequons in the extracellular domain. The mature 80K IL-6R is a glycosylated form of the predicted 50K precursor (Hirata et al., 1989). The cDNAs of murine (Sugita et al., 1990) and rat (Baumann et al., 1990) IL-6R encode proteins that show 54% and 51% overall amino acid sequence identity with the hIL-6R (Table 3). When conservative substitutions are taken into account, the homology with the hIL-6R increases to 70% and 66% for mouse and rat IL-6R, respectively (Table 3).

The extracellular region of the IL-6R is highly modular, consisting of domains of approximately 100 amino acids (Fig. 4). There is an N-terminal domain characteristic of the Ig superfamily and a cytokine binding domain (CBD) that consists of two fibronectin type III-like (FN III) domains. The FN III domain, which is a subclass of the Ig-fold (Bork et al., 1994; Vaughn & Bjorkman, 1996), consists of seven β-strands that form a compressed

Fig. 3. (*Figure is on facing page.*) Alignment of the human IL-6, G-CSF, LIF, CNTF, and GH sequences. G-CSF (Hill et al., 1993), LIF (Robinson et al., 1994), CNTF (McDonald et al., 1995), and GH (de Vos et al., 1992) are aligned based on optimal superpositions of their respective crystal structures, and IL-6 is aligned to G-CSF as used in the modeling of Hammacher et al. (1994). The LIF secondary structure is taken from the crystal structure of murine LIF (Robinson et al., 1994), which is highly similar to that of human LIF (Hudson et al., 1996). In the alignment the helical segments of G-CSF, LIF, CNTF, and GH are underlined, and for the four main helices, the "a" and "d" positions of the heptad repeats are indicated. The residues that are in these positions can pack together to form the hydrophobic core of the four-helix bundle. However, as the ends of the helices may extend beyond the core of the helix bundle, hydrophilic residues may here be seen in the "a" and "d" positions. Also indicated on the alignment are the residues that are involved in receptor-binding as identified by mutagenesis studies (IL-6, G-CSF, LIF, CNTF, and GH) and from the crystal structure (GH). Site I residues are marked (^), site II (^{v}), and site III (~). Sites I and II in GH bind to the first and the second GHR, respectively. Site I residues that are marked on GH have an average energetic contribution to binding of >0.5 kcal/mol (Cunningham & Wells, 1993). Site II residues that are marked on GH are in the structural epitope (de Vos et al., 1992) and, when mutated to alanine, have EC50 values that are at least 1.7-fold higher than the EC50 of wild-type GH in a fluorescence homoquenching assay, suggesting that they are deficient in GHR dimerization (Cunningham et al., 1991). Tyr 103 in GH is included on sterical grounds, and buried residues Asn 12 and Arg 16 (de Vos et al., 1992), which showed little effect when mutated to alanine (Cunningham et al., 1991), are indicated as they might be expected to constrain the range of mutations available to the surrounding residues. In IL-6, site I binds to the IL-6R, while sites II and III are involved in IL-6R-dependent interactions with gp130. As residues Phe 173, Leu 178, Arg 179, and Arg 182 in IL-6 were assayed for receptor-binding using cell-based assays, it has not been conclusively shown that they are involved in IL-6R binding. However, as these residues localize to the C-terminal end of helix D in the homology models of IL-6 (Ehlers et al., 1994; Hammacher et al., 1994; Savino et al., 1994b), and residues in this region are implicated in site I binding (de Vos et al., 1992; Mott & Campbell, 1995), they are indicated as belonging to site I. The charged residues Lys 66, Glu 69 and Lys 70 as well as Gln 75 and Ser 76 (Toniatti et al., 1996), and the region encompassing Gly 77–Glu 95 (Ehlers et al., 1994) may form part of site I in IL-6. With the exception of Phe 78 (Ehlers et al., 1996), which is discussed in Determination of the active site, the individual residues in the latter region have not yet been identified and are therefore not marked. Residues in the AB-loop that have been implicated in site III binding in IL-6 have not been marked, as discussed in Determination of the active site and The ternary complex. Sites I, II, and III of GH and IL-6 are discussed in more detail in The complex of growth hormone and its receptor and Determination of the active site, respectively. Residues in G-CSF, LIF, and CNTF are marked as site I, II, or III according to their colocalization with the corresponding sites in GH or IL-6. For IL-6, LIF, CNTF, and GH these residues are marked in CPK form in Figure 6. Residues whose effect is believed to be on the structures of IL-6, G-CSF, LIF, CNTF, and GH, such as those in the heptad "a" or "d" positions, have not been marked except where those residues are outside the core of the helix bundle. For a comprehensive list of the residues identified as being implicated in receptor-binding in G-CSF, LIF, and CNTF, the quoted literature should be consulted. In G-CSF, Leu 15 and Glu 19 in helix A (Layton et al., 1993; Reidhaar-Olson et al., 1996), and Asp 112 and Leu 124 in helix C and the CD-loop may form part of site II (Reidhaar-Olson et al., 1996); Lys 40 in helix A, Val 48 and Leu 49 in the AB-loop, and Phe 144 in helix D may form part of site III (Reidhaar-Olson et al., 1996). The residues marked as being in site III in G-CSF colocalize with residues in both sites I and III in the other cytokines and might reflect a slightly different binding mode in G-CSF for the G-CSFR. Alternatively, Phe 144, Val 48, and Leu 49 may have a structural role, affecting the interactions between the D1 motif and the helix in the AB-loop (as discussed in The ternary complex), and therefore the stability of site III. Leu 124 may also affect the stability of the C-terminal end of helix C in G-CSF. Epitope mapping studies of neutralizing G-CSF mAbs have implicated residues 20–46 in binding to the G-CSFR (Layton et al., 1991). In LIF, Asp 57 in the AB-loop and Val 175 in helix D (Layton et al., 1994; Hudson et al., 1996) may form part of site I; Gln 25, Ser 28, and Gln 32 in helix A (Hudson et al., 1996) may form part of site II (gp130 site); Ser 107 in the BC-loop, His 112 and Ser 113 in helix C (Layton et al., 1994), and Val 155, Phe 156, Lys 158, and Lys 159 in the D1 motif (Layton et al., 1994; Hudson et al., 1996) may form part of site III (LIFR site). Ser 107, His 112, and Ser 113 (Layton et al., 1994) colocalize with the residues in the D1 motif and appear to extend the structural range of site III. Asp 57 (Layton et al., 1994; Hudson et al., 1996) is spatially separate from the other residues and colocalizes with site I residues in GH. In CNTF, Arg 25 and Arg 28 in helix A, Gln 63 and Trp 64 in the AB-loop, Gln 74 in helix B (Panayotatos et al., 1995), and Gln 167 and Arg 177 in helix D (Panayotatos et al., 1995; Saggio et al., 1995) may form part of site I (CNTFR site); Lys 26 and Asp 30 in helix A (Panayotatos et al., 1995) may form part of site II (gp130 site); and Phe 152, Glu 153, Lys 154, Lys 155, Leu 156, and Trp 157 in the D1 motif (Inoue et al., 1995; Krüttgen et al., 1995; Panayotatos et al., 1995; Thier et al., 1995; di Marco et al., 1996) may form part of site III (LIFR site).

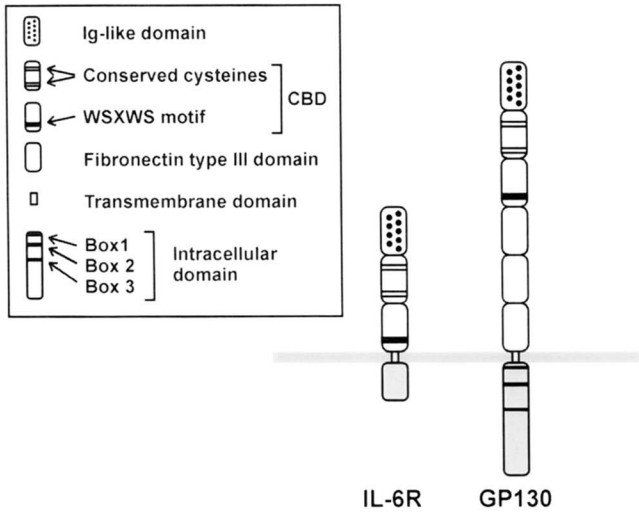

Fig. 4. Structure of IL-6R and gp130. Schematic representation of the IL-6R and gp130, as discussed in Structure of the interleukin-6 receptor and gp130. The shaded horizontal line represents a cell membrane.

β-sandwich built up from one three-stranded β-sheet packed against one four-stranded sheet. The CBD of the IL-6R is characteristic of the class I cytokine receptors (Bazan, 1990a, 1990b). Ligands that interact with the class I family of cytokine receptors include interleukins -2, -3, -4, -5, -6, -7, -9, -11, -13, LIF, OSM, Epo, CNTF, GH and prolactin (Thoreau et al., 1991; Gearing & Ziegler, 1993; Kishimoto et al., 1994). In common with other members of this receptor family, the N-terminal FN III domain of the CBD of the IL-6R has four conserved cysteines, while the C-terminal domain has a conserved sequence motif, the "WSXWS" motif. The Ig-like domain of the hIL-6R was deleted without major effects on IL-6 binding and signal transduction, suggesting that the CBD mediates binding to IL-6 and gp130 (Yawata et al., 1993). Residues in the two Ser-Ser-Phe-Tyr repeats in the CBD of the IL-6R were shown to form part of the binding site for IL-6 (Kalai et al., 1996). As seen in the crystal structure of the GH/GHR complex (de Vos et al., 1992), the corresponding regions in the GHR (loop regions EF and BC2) are implicated in GH recognition.

The cytoplasmic segment of the IL-6R is relatively short (82 amino acids) and lacks intrinsic catalytic activity (Yamasaki et al., 1988). The transmembrane and cytoplasmic domains of the IL-6R are not necessary for signal transduction, as shown by the fact that the complex of IL-6 and extracellular "soluble" domain of the IL-6R (sIL-6R) induces signal transduction on cells expressing gp130 (Taga et al., 1989; Hibi et al., 1990; Yasukawa et al., 1990; Ward et al., 1994a). Furthermore, the sIL-6R increases the sensitivity to IL-6 of cells expressing both IL-6R and gp130 (Yasukawa et al., 1990; Mackiewicz et al., 1992). The ligand-dependent stimulatory role of the sIL-6R is similar to that reported for the soluble receptors for CNTF (Davis et al., 1993a) and IL-11 (Baumann et al., 1996b), which also use the β subunit receptor gp130, but differs from other known soluble receptors (e.g., tumor necrosis factor, IL-1, IL-2, IL-4, IFN-γ, nerve growth factor, LIF, G-CSF, GM-CSF). The latter soluble receptors act as inhibitors by competing for ligand-binding with cellular receptors (for reviews see Rose-John & Heinrich, 1994; Heaney & Golde, 1996). Ligand-bound sIL-6R and sCNTFR have an interesting parallel in IL-12, a cytokine that consists of two disulfide-linked modules, p40 and p35 (Wolf et al., 1991). With reference to the IL-6 system, Gearing & Cosman (1991) noted that p40 could be considered to be equivalent to a soluble receptor and p35 to its specific cytokine.

sIL-6R may be generated by proteolytic shedding of membrane-bound IL-6R (Müllberg et al., 1993), or from an alternatively spliced mRNA species (Lust et al., 1992; Horiuchi et al., 1994). A natural secreted soluble form of IL-6R that is capable of binding IL-6 has been detected in low abundance in human urine (Novick et al., 1989) and serum (Narazaki et al., 1993; Frieling et al., 1994) (see below). Recombinant sIL-6R purified from CHO cells (Ward et al., 1995a) and baculovirus-infected insect cells (Weiergräber et al., 1995) bind IL-6 with similar affinity (~20 nM). A non-glycosylated sIL-6R expressed in *E. coli*, which was refolded from inclusion bodies to its native conformation, exhibits biological activity comparable to the sIL-6R expressed in eukaryotic cells (Stoyan et al., 1993).

Table 3. *Percentage sequence identities (upper triangle) and similarities (lower triangle) for the IL-6 receptor and gp130*

IL-6R	Human	Mouse	Rat
Human		54	51
Mouse	70		83
Rat	66	88	

gp130	Human	Mouse	Rat
Human		77	77
Mouse	85		86
Rat	88	91	

The IL-6 signal transducer, gp130

The binding of IL-6 to IL-6R allows the association of this binary complex with gp130 to form the high-affinity ternary complex, IL-6/IL-6R/gp130 (Taga et al., 1989). gp130 has no intrinsic IL-6-binding nor IL-6R-binding capability (Hibi et al., 1990). In contrast to the IL-6R, gp130 is ubiquitously expressed (Hibi et al., 1990).

The cDNA of human gp130 encodes a protein consisting of 918 amino acids, which includes a leader sequence of 22 amino acids, an extracellular domain of 597 amino acids, a transmembrane domain of 22 amino acids, and a cytoplasmic domain of 277 amino acids (Hibi et al., 1990). There are 10 potential N-linked glycosylation sequons in the extracellular domain of the predicted M_r 101K protein (Hibi et al., 1990), suggesting that the mature 130K gp130 is highly glycosylated. Pulse-chase analysis in primary rat hepatocytes in the presence of tunicamycin has identified an incompletely glycosylated precursor of gp130 (Wang & Fuller, 1995).

The extracellular region of gp130 has the same modular structure as the G-CSF receptor (Fukunaga et al., 1990; Larsen et al., 1990) including an N-terminal Ig-like domain and a CBD, which is followed by three FN III domains (Fig. 4). The extracellular "soluble" domain of gp130, sgp130, as well as a truncated form of sgp130 lacking the three-membrane-proximal FN III domains, are able to bind the IL-6/sIL-6R complex (Yasukawa et al., 1992; Horsten et al., 1995). The primary structure of the extracellular

region of gp130 is more closely related to that of the G-CSFR than to the extracellular regions of the other members of the cytokine receptor family (Hibi et al., 1990). The amino acid identity between the full length gp130 and the G-CSFR is 27.3% (Mosley et al., 1996). The amino acid sequence of murine (Saito et al., 1992) and rat gp130 (Wang et al., 1992) share an overall sequence identity of 85% and 88%, respectively, with that of human gp130 (Table 3).

Although gp130 is the signaling subunit of the IL-6 receptor system, the cytoplasmic domain of gp130 has no intrinsic catalytic activity (Hibi et al., 1990). Upon stimulation with IL-6, IL-11, LIF, OSM and CNTF, gp130, and several other proteins become phosphorylated on tyrosine (Boulton et al., 1994; Yin & Yang, 1994). The tyrosine phosphorylation of at least some of these substrates is mediated by members of the Janus tyrosine kinase (JAK) family, which associate constitutively with gp130 (Lütticken et al., 1994; Stahl et al., 1994). Association and activation of the JAK kinases involves the juxtamembrane region of the cytoplasmic domain of gp130, which contains two motifs, referred to as box 1 and box 2, that show weak homology among some of the signaling cytokine receptors (Murakami et al., 1991). A third homology region, box 3 (Fig. 4), was initially identified as being unique to gp130 and the receptors for LIF and G-CSF (Baumann et al., 1994). The box 3 motif includes a tyrosine that becomes phosphorylated upon JAK activation. This phosphotyrosine forms part of the consensus sequence (Tyr-Xaa-Xaa-Gln) for activation of the signal transducer and activator of transcription STAT3/APRF (Stahl et al., 1995). Inspection of the six tyrosine residues in the cytoplasmic domain of gp130 reveals that not one, but four of these are located within the consensus sequence for STAT3 activation. It was recently shown that two of these latter residues are also important for activation of STAT1 (consensus sequence Tyr-Xaa-Pro-Gln) (Gerhartz et al., 1996). The box 3 motif is also found in the receptors for IL-7 (Goodwin et al., 1990), IL-9 (Renauld et al., 1992), IL-10 (Liu et al., 1994), and thrombopoietin (Vigon et al., 1992), in the OSM-specific receptor (OSMRβ) (Mosley et al., 1996), and in the leptin receptor (OB-R) (Tartaglia et al., 1995). The OSMRβ and the OB-R are related to gp130 in primary structure (23.3% and 20.7% overall amino acid identity, respectively) and in modular structure, featuring both Ig-like and FN III domains (Hibi et al., 1990; Tartaglia et al., 1995; Mosley et al., 1996). While the OB-R and gp130, at least in part, activate overlapping intracellular signaling molecules, gp130 does not form part of the receptor-complex of leptin (Baumann et al., 1996a).

It is thought that, upon tyrosine phosphorylation of gp130 by JAK kinases, SH2-containing molecules such as the STATs associate with gp130 and, in turn, become tyrosine-phosphorylated and activated. In addition to the JAKs and STATs, tyrosine kinases such as *Btk*, *Tec*, *Fes,* and *Hck*, and intracellular signaling molecules such as Ras and MAP kinase appear to be activated upon stimulation of gp130. The roles of these molecules in gp130-mediated intracellular signaling and their links with the JAK/STAT pathway remain to be determined. For recent reviews on JAK/STAT pathways and gp130-mediated signaling see Ihle, 1995; Schindler & Darnell, 1995; Taniguchi et al., 1995, Hibi et al., 1996, and Taga, 1996.

Soluble IL-6R and gp130: physiological roles?

While it is now well recognized that many membrane-bound receptors also exist as secreted soluble forms, their physiological importance in cytokine action is not well understood. For IL-6, a 50K sIL-6R that is able to bind IL-6 is found in normal human urine and serum (Novick et al., 1989; Narazaki, et al., 1993; Frieling et al., 1994). Constitutive levels of sIL-6R in serum from healthy individuals are ~80 ng/mL (Honda et al., 1992), while increased serum-levels of sIL-6R are found in certain disease states. For example, patients with HIV infection or multiple myeloma have ~170–200 ng/mL sIL-6R in their plasma (Honda et al., 1992; Gaillard et al., 1993). Similarly, in healthy individuals the serum levels of IL-6 are generally very low or undetectable (usually <10 pg/mL) (Akira et al., 1993), but increased levels (up to ng/mL) are seen in a significant number of patients suffering from, e.g., multiple myeloma (Bataille et al., 1989), rheumatoid arthritis (Houssiau et al., 1988), acute bacterial infection (Helfgott et al., 1989; Lu et al., 1993), and HIV infection (Breen et al., 1990; Honda et al., 1990). In normal human serum, the concentration of 90K and 110K forms of sgp130 has been reported to be ~390 ng/mL (Narazaki et al., 1993).

It has been suggested that part of the serum-derived IL-6 in patients with systemic juvenile rheumatoid arthritis circulates in a complex with sIL-6R, by contrast with sera from healthy individuals, wherein no such complexes were detected (de Benedetti et al., 1994). In the presence of IL-6, serum-derived sIL-6R has a stimulatory effect on cells expressing gp130 (Narazaki et al., 1993). Therefore, the IL-6/sIL-6R complex has been labeled as the real target to inhibit in vivo (Gaillard et al., 1996). Serum-derived sgp130 acts as an IL-6 antagonist in the presence of serum-derived sIL-6R and IL-6, and also inhibits the biological effects of LIF, OSM, and CNTF (Narazaki et al., 1993). The relative concentrations of sIL-6R, sgp130 and IL-6 in serum may thus, in part, determine their physiological effects. For a review of the pharmacokinetic characteristics in vivo of human cytokine-binding proteins see Klein & Brailly (1995).

The complex of growth hormone and its receptor

The current paradigm for the ternary complexes of helical cytokines and the extracellular domains of their receptors comes from the crystal structure of the complex of GH and its receptor (GHR) (de Vos et al., 1992). GH interacts with two identical receptors through two sites on the ligand that have been designated sites I and II (for recent reviews see Wells, 1996, and Wells & de Vos, 1996) (Fig. 5).

In the GH/GHR complex the dimerization of the GH receptors, resulting from GH crosslinking two receptor molecules, follows a sequential pattern. One receptor binds first at site I, followed by the second receptor, which binds this complex at site II while also binding to the first receptor molecule (Cunningham et al. 1991; Fuh et al., 1992). The surface area of the contact of site I with its receptor (1230 Å^2) is considerably greater than that of site II (900 Å^2), but the complex is stabilized by an interaction (500 Å^2) between the two receptors (de Vos et al., 1992). This smaller contact area on site II and the contribution of the interaction between the two receptors may form the molecular basis for the inability of a receptor to bind at site II in the absence of site I binding (de Vos et al., 1992; Wells, 1996). Because of the sequential binding of the receptors, it is possible to construct GH antagonists by blocking binding at site II and/or enhancing binding at site I (Fuh et al., 1992). This pattern of sequential receptor oligomerization is emerging as a common trait of cytokine/receptor binding (for reviews see Mott & Campbell, 1995; Wells, 1996) and

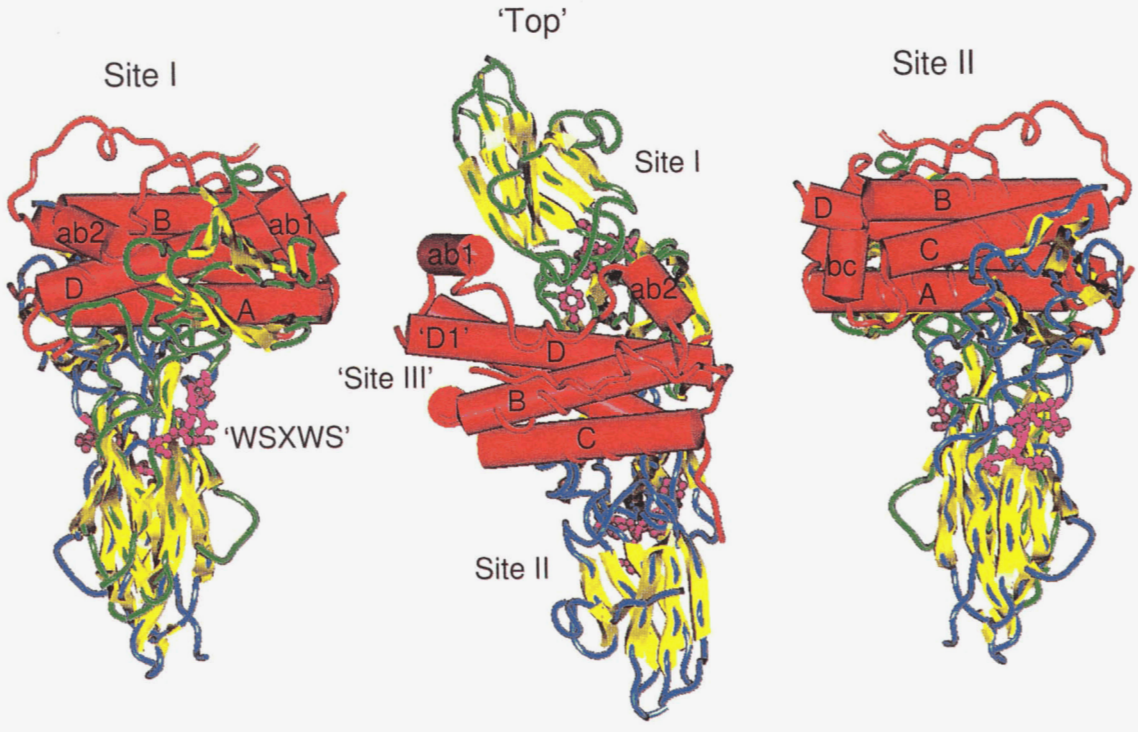

Fig. 5. Three views of the complex of growth hormone and its receptor. The illustrations are adapted from de Vos et al. (1992). These views were chosen to illustrate the interaction of the receptors with the ligand. The left view shows the site I interaction where the first binding GHR can be seen interacting with helices A and D and the AB-loop of GH. In the view on the right the GH/GHR complex has been rotated by 180° to show the site II interaction. Here the second GHR binds to helices A and C of GH. Both views show the receptors forming a contact dimer in their membrane proximal domains. The "WSXWS" equivalent motif is shown in magenta and it can be seen that it does not participate in ligand interactions. The central or top view of the receptor complex emphasizes the regions equivalent to the site III binding site in the gp130 family of cytokines. The analogue of the D1 motif (Bazan, 1991), which is implicated in site III binding interactions, is marked on helix D. This region corresponds to residues 155–163 in hIL-6 (see Fig. 3). It can be seen that a third receptor can bind at site III without steric clashes with the receptors at sites I and II if the receptors binding at those sites have structures similar to that of GHR.

antagonists of IL-6 have been constructed on the same principle (Brakenhoff et al., 1994; Savino et al., 1994a, 1994b).

More detailed analyses of the contribution of individual residues to the binding sites of both GH and its receptor have been undertaken (Cunningham et al., 1991; Cunningham & Wells, 1993; Clackson & Wells, 1995). In these studies, alanine scanning mutagenesis was performed on both GH and the GHR and their binding affinities, relative to wild-type, were calculated. It was found that only a subset of the residues involved in contacts in the crystal structure of the binary complex contributed to the energy of binding, showing, therefore, that the functional epitope is much smaller than the structural epitope (Cunningham & Wells, 1993). In site I on GH, 31 side chains are buried in the interface with the receptor, yet eight residues (Lys 41, Leu 45, Pro 61, Arg 64, Lys 172, Thr 175, Phe 176, and Arg 178) (cf. Fig. 3) contribute 85% of the binding energy (Cunningham & Wells, 1993). On the first receptor, nine of the 33 buried residues, predominantly in the loop regions, contribute almost all of the binding energy (Clackson & Wells, 1995). Among the important residues in the functional epitope there is also a considerable variation in the contribution of individual residues. In particular, Trp 104 and Trp 169 on the receptor make dominant contributions to the binding energy (Clackson & Wells, 1995) and the most important interactions for binding are hydrophobic in nature (Wells, 1996).

In Figure 5, views of binding sites I and II on GH are shown. The site I interface in the complex of GH with the first binding GHR encompasses residues in GH located in the C-terminal region of helix D, part of helix A (the residues in helix A do not make major energetic contributions to site I binding) and the AB-loop (de Vos et al., 1992; Cunningham & Wells, 1993) (cf. Fig. 3). Residues in GH involved in binding the second GHR (site II) are located in helices A and C (Cunningham et al., 1991; de Vos et al., 1992) (cf. Fig. 3). Using the same terminology, sites I and II on IL-6 interact with the IL-6R and gp130, respectively (cf. Determination of the active site and Fig. 3). However, IL-6 has an additional gp130-interaction site, site III (Brakenhoff et al., 1994), that has no equivalent in GH (cf. Determination of the active site and Fig. 3). The usage of site III for receptor-interaction has been shown for LIF (Layton et al., 1994; Hudson et al., 1996) and CNTF (Inoue et al., 1995; Krüttgen et al., 1995; Panayotatos et al., 1995; Thier et al., 1995; di Marco et al., 1996), and may be a general feature of the IL-6 family of cytokines (cf. The ternary complex).

The region equivalent to site III in IL-6, LIF and CNTF is shown in the "top" view of Fig. 5. This region encompasses residues in the "D1 motif" (Bazan, 1991) in IL-6 and CNTF (Brakenhoff et al., 1994; Inoue et al., 1995). In LIF, this region encompasses residues in the "D1 motif" (Layton et al., 1994; Hudson et al., 1996), the BC-loop and the N-terminus of helix C (Layton et al., 1994). It has been proposed that residues in the N-terminal part of the AB-loop also form part of site III in IL-6 (Ehlers et al., 1994; Ciapponi et al., 1995; de Hon et al., 1995a; Toniatti et al., 1996).

From the top view of Figure 5, it can be seen that it is possible for a third receptor to bind to site III without sterically clashing with the other two receptor sites. This assumes that the receptors follow the structural pattern that has been observed in the GHR, PRLR, and EpoR, the class 1 cytokine receptors for which structures are known (de Vos et al., 1992; Somers et al., 1994; Livnah et al., 1996). For IL-6 and CNTF, such a three-site cytokine/receptor binding model is consistent with mutagenesis data and the finding that the ternary receptor-complexes of IL-6 (Ward et al., 1994b; Paonessa et al., 1995) and CNTF (de Serio et al., 1995) are hexameric (cf. The ternary complex).

Determination of the active site

Based on the assumption that the complex formation of IL-6 with its cellular receptors can be mimicked by soluble forms of IL-6R and gp130, we (Ward et al., 1994b) and, more recently, Paonessa et al. (1995) showed that IL-6 mediates its actions on target cells through the formation of a hexameric complex comprising two molecules each of IL-6, IL-6R, and gp130. While there are models of the formation and topology of the hexameric complex (Paonessa et al., 1995; Ward et al., 1996), a detailed understanding of the interactions between IL-6 and its receptor molecules at a high-resolution structural level is lacking. Such an understanding may provide insights into how other members of the gp130 family of cytokines (CNTF, OSM, LIF, IL-11, CT-1) activate their receptors as well as information critical for the design of small molecules that will function either as antagonists or as agonists.

Two functionally distinct regions in IL-6

The notion of two functionally distinct regions of IL-6 was first indicated experimentally by mutagenesis studies combined with epitope mapping studies using IL-6-specific neutralizing monoclonal antibodies (mAbs) (Brakenhoff et al., 1990). On the basis of competition experiments using N- and C-terminal deletion mutants of IL-6, a set of neutralizing mAbs was divided into two groups (I and II), group I recognizing N- and C-terminal regions of IL-6, whereas group II mAbs recognized a second site involved in biological activity. Using another neutralizing mAb, Ida et al. (1989) identified a region encompassing residues Ala 153–Thr 163 as being important for bioactivity.

Importance of the N- and C-terminii

The first glimpse of where IL-6 interacted with its receptor complex was obtained by deletion mutagenesis. However, in these early studies IL-6 mutants were assayed on a variety of murine and human cell lines and loss of biological activity was not specifically assigned to interference with the IL-6R and/or gp130 interactions, often resulting in confusing terminology such as "receptor-binding" or "receptor-activation." Furthermore, with the usage of IL-6 chimeras (see below) it has become clear that biological activity on mouse cells does not necessarily correlate with the ability to bind to and activate human cells.

Several studies suggested that both the C- and N-terminal regions of IL-6 are important for biological activity. Hence, major decreases in hIL-6 activity on mouse hybridoma B9 cells occurred upon removal of the C-terminal residues Met 184 and Gln 183–Met 184 (Krüttgen et al., 1990a). Removal of the five C-terminal amino acids of hIL-6 (Brakenhoff et al., 1990; Krüttgen et al., 1990b) and mIL-6 (Ward et al., 1993a) resulted in ~1000-fold loss of activity on mouse hybridoma cells. In contrast, it has been shown that a C-terminal tripeptide extension to mIL-6 (Glu-Lys-Leu) did not affect biological activity (Danley et al., 1991). A similar finding has been made for a pentapeptide extension to mIL-6 (Hammacher et al., 1997).

Point mutations at Arg 182 and Met 184 (Lütticken et al., 1991; Li et al., 1993), Arg 179 (Fontaine et al., 1993; Li et al., 1993), and semi-random mutagenesis of the region Leu 181-Met 184 (Yasueda et al., 1992) suggested the importance of a positively charged residue at positions 179 and 182 for activity. In addition to alanine substitution mutants at Arg 179 or Arg 182, an IL-6 mutant with Phe 173 substituted for alanine was shown to have significantly reduced binding to human CESS cells (Li et al., 1993). Substitution of Phe 170 for leucine yielded an IL-6 mutant with increased binding to human myeloma U266 cells (Leebeek et al., 1992). An IL-6 mutant with Arg 168 substituted for methionine showed a significant loss of binding and bioactivity on human melanoma 375 cells (Fontaine et al., 1994). By creating a library of IL-6 variants with single amino acid substitutions in the C-terminal region it was shown that mutations at Leu 178, Arg 179, and Ala 180 resulted in loss of bioactivity and loss of binding to U266 cells (Leebeek et al., 1992). These authors concluded that the region Ser 176–Arg 182 may encompass a major receptor-binding site in IL-6. However, the NMR study by Xu et al. (1996) has shown NOE signals between Phe 173, Leu 178, Ala 180, and residues in adjacent helices, suggesting that the former may have a structural role in packing against the helix bundle. Substitution of residues 165–183 of hIL-6 with the corresponding residues of mouse or rat IL-6 resulted in low activity on human cells suggesting that the C-terminal region of IL-6 is important for species specificity (Leebeek & Fowlkes, 1992).

Based on epitope mapping of neutralizing anti-hIL-6 antibodies (Brakenhoff et al., 1990) and NMR studies of mIL-6 (Ward et al., 1993a), it was suggested that the N- and C-terminal regions of IL-6 are in close proximity. Whereas up to 28 N-terminal residues of IL-6 can be deleted without loss of biological activity (Brakenhoff et al., 1989; Snouwaert et al., 1991a; Krakauer et al., 1992), removal of amino acids Ile 29–Arg 30 or Ile 29–Asp 34 influenced the biological activity on mouse hybridoma cells (~50-fold decrease and virtual abolishment, respectively) (Brakenhoff et al., 1989). Similarly, deletion mutagenesis in Ile 29–Glu 42 of IL-6 showed that Ile 29–Ala 38 are important for activity on human and mouse cells and, using conformation-specific mAbs, that deletions in this region have conformational effects (Arcone et al., 1991b). Deletion of residues Ser 21–Glu 51 of IL-6 resulted in structural changes in the molecule, over 10,000-fold loss of activity on mouse hybridoma cells, and loss of binding to human CESS cells (Fontaine et al., 1991). The 22 N-terminal residues of mouse IL-6 have also been deleted without loss of biological activity (Ward et al., 1993a). As yet, it is unclear whether the N-terminal residues 29–34 are directly involved in binding the IL-6R. It is therefore possible that they act merely as a structural support for residues in the C-terminal region that interact with the IL-6R. Iodination of Tyr 31 does not interfere with the ability of IL-6 to bind to the sIL-6R (Nishimura et al., 1990). Results from site directed mutagenesis studies (see below) suggest that Tyr 31 and Gly 35 participate in IL-6R-dependent interaction with gp130 (Savino et al., 1994b).

IL-6 residues implicated in binding the IL-6R: site I

By analogy with GH, where site I corresponds to residues interacting with the first GHR, site I in IL-6 is defined as those residues

that interact with the IL-6R. Site-directed mutagenesis of hydrophobic residues in the C-terminal region of IL-6 (Met 161 and Leu 158, 165, 167, 174, and 181) yielded several mutants that lack bioactivity as well as the ability to bind to the sIL-6R (Nishimura et al., 1991b, 1992). Since the majority of these residues are conserved between IL-6 species (Fig. 2) and, based on the G-CSF structure (Hill et al., 1993), Leu 165 is in the interface between the AB-loop and helix D and Leu 174 and Leu 181 are buried (Fig. 3), it is likely that the mutated residues are important for structural reasons rather than being involved in IL-6R binding. Using a saturation mutagenesis approach in the region encompassing Gln 175-Leu 181, Savino et al. (1993) identified a mutant, (Ala 180 Arg)IL-6, that had essentially no activity on human hepatoma Hep3B cells, and lacked the ability to bind to the sIL-6R. By contrast, another mutant, (Ser 176 Arg)IL-6, exhibited over threefold increased biological activity and affinity for sIL-6R (Savino et al., 1993).

Cabibbo et al. (1995) used a monovalent phage display approach to investigate the involvement of residues Gln 175, Ser 177, Leu 181, and Gln 183 in IL-6R binding. This technique, where mutants are expressed as fusion proteins with the phage coat protein pIII on the surface of M13 phage, had been previously used to obtain variants of hGH that bound with high affinity to the soluble hGHR (Lowman & Wells, 1993). The library with the IL-6 mutants was subjected to affinity selection using both sIL-6R and sgp130, to counterselect for mutations that directly or indirectly affected binding to gp130, resulting in the identification of one mutant (Gln 175 Ile, Gln 183 Ala)IL-6 that, when expressed as a soluble protein, had a ~2-fold increased affinity for the sIL-6R relative to IL-6 (Cabibbo et al., 1995).

Although there is a relatively high degree of amino acid sequence identity (42%) between human and mouse IL-6, there is limited biological crossreactivity (Coulie et al., 1989). While hIL-6 binds to both human and mIL-6R, mIL-6 binds only to mIL-6R. When the mIL-6R is introduced into human hepatoma cells expressing gp130, they become responsive to mIL-6 (Fiorillo et al., 1992b). Hence, the inability of mIL-6 to act on human cells is due to its inability to bind to the hIL-6R, as distinct from its inability to facilitate IL-6R-dependent activation of human gp130. Taking advantage of the limited cross-reactivity of m- and hIL-6, several studies used human/mouse IL-6 chimeras to elucidate regions of IL-6 interacting with the human IL-6R. In addition to both the N- and C-terminal regions of IL-6 being required for efficient binding to sIL-6R (Fiorillo et al., 1992a; van Dam et al., 1993; Hammacher et al., 1994), a region comprising part of the AB-loop through to the beginning of helix C (Leu 62–Arg 113) (Hammacher et al., 1994) and a region encompassing Lys 41–Glu 95 (van Dam et al., 1993) of IL-6 were identified as critical for the interaction with the IL-6R. Ehlers et al. (1994) further narrowed down this region to residues Gly 77–Glu 95 in the AB-loop/helix B, and recently suggested that Phe 78 is involved in binding to the IL-6R (Ehlers et al., 1996). Although the equivalent residue is not seen in the crystal structure of G-CSF (Hill et al., 1993), it is possible that Phe 78 stabilizes the orientation of the end of the AB-loop in IL-6, in a similar way to Leu 67 in CNTF (McDonald et al., 1995).

Using affinity maturation by monovalent phage display, residues in the AB-loop of IL-6 were assessed for their contribution in binding to the IL-6R (Toniatti et al., 1996). The mutagenesis was performed in the region of Ala 56–Ser 76 on a backbone of (Gln 175 Ile, Ser 176 Arg, Gln 183 Ala)IL-6, a D-helix mutant with ~5-fold increased affinity for the sIL-6R (Cabibbo et al., 1995), and the obtained variants were selected for binding to the sIL-6R (Toniatti et al., 1996). The substitution of Gln 75 for tyrosine and Ser 76 for lysine in (Gln 175 Ile, Ser 176 Arg, Gln 183 Ala)IL-6 resulted in a "superbinder" with a ~42-fold increase in affinity for sIL-6R, and further substitution of four residues (Lys 66 Val, Ala 68 Arg, Glu 69 Met, and Lys 70 Glu) increased the affinity to ~70-fold compared to wild-type IL-6 (Toniatti et al., 1996). In the absence of the mutations in helix D, the latter IL-6 mutant showed ~14-fold increased affinity for the sIL-6R, suggesting that the AB-loop and helix D mutations are functionally independent (Toniatti et al., 1996).

Taken together, the available mutagenesis data on IL-6 suggest that site I comprises the C-terminal end of helix D, and a region encompassing the C-terminal part of the AB-loop/N-terminal part of helix B (cf. Fig. 3).

IL-6 residues implicated in IL-6R-dependent interaction with gp130: site II

Crosslinking of ^{125}I-IL-6 to several cell lines expressing both IL-6R and gp130 suggested that IL-6 directly associates with a 130K membrane glycoprotein (D'Alessandro et al., 1993). The analogy with the GH/GHR complex and the available data on IL-6 and IL-6 analogs suggest that IL-6 indeed binds to gp130, but only in the presence of the IL-6R. Site II in IL-6, therefore, is defined as those residues that interact with gp130, in a manner similar to that of site II in GH.

Savino et al. (1994b) superimposed a homology model of IL-6 based on bovine G-CSF onto GH in the complex with the GHR (de Vos et al., 1992) and identified four amino acids in helix A (Arg 24, Lys 27, Gln 28, and Tyr 31) and two residues in helix C (Val 121 and Phe 125) of IL-6 that overlapped buried residues in site II of GH; these residues were predicted as contact points between IL-6 and gp130. Subsequent site-directed mutagenesis studies resulted in a mutant, (Tyr 31 Asp, Gly 35 Phe)IL-6, which, in the presence of sIL-6R, failed to associate with sgp130 and acted as a partial IL-6 antagonist on human hepatoma Hep3B cells (Savino et al., 1994b). By combining the mutations in helix A with mutations in helix C (Ser 118 Arg and Val 121 Asp), and substituting three residues (Gln 175 Ile, Ser 176 Arg, Gln 183 Ala) to increase the affinity for the IL-6R, a super-antagonist was created (Savino et al., 1994a). This molecule has ~4-fold improved binding to the IL-6R, no bioactivity on human hepatoma Hep3B cells, and, on certain cell types, exhibits half maximal inhibition of IL-6 activity at ~5-fold molar excess (Savino et al., 1994a).

The available data suggest that, similar to the second GHR binding site on GH, site II on IL-6 is composed of residues in helix A and helix C (cf. Fig. 3).

IL-6 residues implicated in IL-6R-dependent interaction with gp130: site III

The existence of a second site in IL-6 important for interaction with gp130 (site III) was recognized independently through antibody epitope-mapping (Brakenhoff et al., 1994), rather than by inspection of the GH/GHR complex. The epitope of group II neutralizing mAbs directed against IL-6 was found to contain Trp 157 in the N-terminal part of helix D (Brakenhoff et al., 1994). Extensive mutagenesis of the residues surrounding Trp 157 (Gln 152–Thr 162) was performed, based on the hypothesis that group II antibodies may interfere with the IL-6R-dependent inter-

action of IL-6 and gp130, and that IL-6 mutants which bind IL-6R, but not gp130, would be potential IL-6 antagonists (Brakenhoff et al., 1994). These studies resulted in the identification of a mutant, (Gln 159 Glu, Thr 162 Pro)IL-6, which could bind with low, but not high affinity to human CESS cells, suggesting that the complex of (Gln 159 Glu, Thr 162 Pro)IL-6 with the IL-6R could not associate with gp130 (Brakenhoff et al., 1994). Furthermore, (Gln 159 Glu, Thr 162 Pro)IL-6 acted as an IL-6 antagonist on CESS and human hepatoma HepG2 cells, although at high concentrations it had residual activity on the latter. (Gln 159 Glu, Thr 162 Pro)IL-6 also showed residual activity on human XG-1 cells (de Hon et al., 1995b). Compared to IL-6, this molecule had a similar capacity (two- to four-fold reduced) to bind to the IL-6R, as shown by competition binding with ^{125}I-IL-6 to cellular and soluble IL-6R (Brakenhoff et al., 1994) and by biosensor studies using sIL-6R (Hammacher et al., 1996). It was later reported that the complex of (Gln 159 Glu, Thr 162 Pro)IL-6 with sIL-6R had residual affinity for sgp130 (de Hon et al., 1994; Hammacher et al., 1996) but could not induce the formation of a stable hexameric receptor-complex (Hammacher et al., 1996). Another IL-6 mutant, (Trp 157 Arg, Thr 162 Pro)IL-6, which had similar bioactivities to (Gln 159 Glu, Thr 162 Pro)IL-6 but an increased affinity for the sIL-6R, was identified by further mutating the epitope of group II neutralizing mAbs (de Hon et al., 1995b). A similar mutant, (Trp 157 Arg, Asp 160 Arg)IL-6 had a significantly reduced ability to participate in hexameric receptor-complex formation (Paonessa et al., 1995).

Using human/mouse IL-6 chimeras, residues Lys 41–Ala 56 in helix A/AB-loop had been identified as another region of IL-6 involved in the IL-6R-dependent binding to gp130 (Ehlers et al., 1994). Homology models of IL-6 based on the structure of G-CSF (Ehlers et al., 1994; Hammacher et al., 1994) and in vitro ternary complex formation studies (Ciapponi et al., 1995) suggested that this region is localized in close proximity to residues Gln 159 and Thr 162 in the CD-loop/helix D and forms a single binding determinant with the latter. Because (Gln 159 Glu, Thr 162 Pro)IL-6, in addition to its activity on HepG2 cells (Brakenhoff et al. 1994), also showed activity on human myeloma XG-1 and erythroleukemia TF-1 cells, Lys 41–Ala 56 in this molecule were substituted for the corresponding mouse residues in an attempt to fully abolish interaction with gp130 (de Hon et al., 1994). By further introducing two additional substitutions (Phe 170 Leu and Ser 176 Arg) to increase the affinity of IL-6 for the IL-6R, the mutant was rendered capable of completely inhibiting IL-6 activity on XG-1 cells. This mutant is specific for IL-6 since it antagonizes the activity of IL-6, but not that of LIF, OSM, or GM-CSF on TF-1 cells (de Hon et al., 1994). A similar mutant, (Lys 54 Asp, Phe 170 Leu, Ser 176 Arg, Gln 159 Glu, Thr 162 Pro)IL-6, was also shown to act as an IL-6 antagonist on XG-1 cells (Ehlers et al., 1996).

Ehlers et al. (1995) narrowed down the region in helix A/AB-loop by showing that substitution of residues Cys 50–Asp 55 for the corresponding mouse residues on a backbone of (Gln 159 Glu, Thr 162 Pro, Phe 170 Leu, Ser 176 Arg)IL-6 yielded similar antagonist activity. Substitution of individual residues Glu 51–Glu 59 for alanine yielded only one mutant, (Leu 57 Ala)IL-6, which showed a reduced bioactivity and a reduced capacity to trigger IL-6R-dependent binding to gp130 (de Hon et al., 1995a). However, since these activities were only approximately five-fold reduced compared to wild-type IL-6, we speculate that residues Glu 51–Glu 59 in the AB-loop region stabilize the conformation of the gp130-determinant in helix D, but do not participate directly in binding gp130. More recently, a non-conservative substitution for Leu 57, Leu 57 Asp, on a backbone of (Gln 175 Ile, Ser 176 Arg, Gln 183 Ala)IL-6 to increase the affinity for the sIL-6R, was shown to greatly reduce the biological activity of IL-6 (Toniatti et al., 1996). If Leu 57 is involved in hydrophobic interactions to stabilize part of helix D, this mutation would be expected to be disruptive (cf. The ternary complex).

The available mutagenesis data thus suggest that site III in IL-6 mainly comprises residues in the CD-loop/N-terminal end of helix D (cf. Fig. 3). These residues localize to the D1 motif identified by Bazan (1991), a region of significant similarity between IL-6, IL-11, LIF, OSM, and CNTF. However, by analogy with site III in LIF, which also contains residues from the BC-loop and the N-terminus of helix C (Layton et al., 1994), it is likely that other, as yet unidentified regions, also form part of site III in IL-6. A model of IL-6 depicting receptor binding sites I, II, and III, together with ribbon diagrams of the X-ray structures of GH, LIF, and CNTF showing the corresponding sites in these molecules, is given in Figure 6 (cf. Fig. 3).

Antagonists of IL-6

There are several ways in which the actions of IL-6 on target cells can be antagonized in vitro, some being more specific than others. gp130 binders such as OSM (Sporeno et al., 1994) and neutralizing gp130 mAbs (Hibi et al., 1990; Taga et al., 1992; Saito et al., 1993; Chevalier et al., 1996), as well as soluble forms of gp130 (Narazaki et al., 1993) may be less specific due to their potential interference with the activities of the other members of the IL-6 family of cytokines. Activin A, a stroma-derived antagonist of IL-6 and IL-11, may act by interfering with intracellular signaling induced via gp130 (Brosh et al., 1995).

Specific antagonists include neutralizing IL-6 mAbs (Ida et al., 1989; Brakenhoff et al., 1990; Shimamura et al., 1991; Wijdenes et al., 1991), antisense oligonucleotides to IL-6 mRNA (Levy et al., 1991), neutralizing IL-6R mAbs (Hirata et al., 1989; Novick et al., 1991; Liautard et al., 1994), sIL-6R mutants that bind IL-6 but cannot associate with gp130 (Salvati et al., 1995), and site II and III mutants of IL-6 (Brakenhoff et al., 1994; de Hon et al., 1994; Savino et al., 1994a, 1994b). The super-antagonist Sant7, a combined site II (Tyr 31 Asp, Gly 35 Phe, Ser 118 Arg, Val 121 Asp) and III (Leu 57 Asp, Glu 59 Phe, Asn 60 Trp) IL-6 mutant with four substitutions in site I (Gln 75 Tyr, Ser 76 Lys, Gln 175 Ile, Ser 176 Arg) to increase its affinity for the IL-6R ~70-fold, can no longer associate with gp130 in an sIL-6R-dependent manner and, on certain cell types, exhibits half maximal inhibition of IL-6 activity at approximately equimolar concentration (Sporeno et al., 1996).

In an attempt to generate a specific IL-6 antagonist de novo, Martin et al. (1996) made use of a "minibody," a 61-residue β-protein designed to act as a small stable scaffold, to enable the conformationally constrained display, in either or both of its loops, of large peptide libraries of minimal discontinuous epitopes, on the surface of filamentous phage. After selection for IL-6 binding, one minibody, MBk, was identified and shown to specifically bind to the C-terminus of helix D, in the region corresponding to site I in IL-6 (Martin et al., 1996). MBk has both of its loops substituted for foreign peptides (of seven and six residues, respectively), binds IL-6 with micromolar affinity, and acts as a hIL-6 antagonist (Martin et al., 1996).

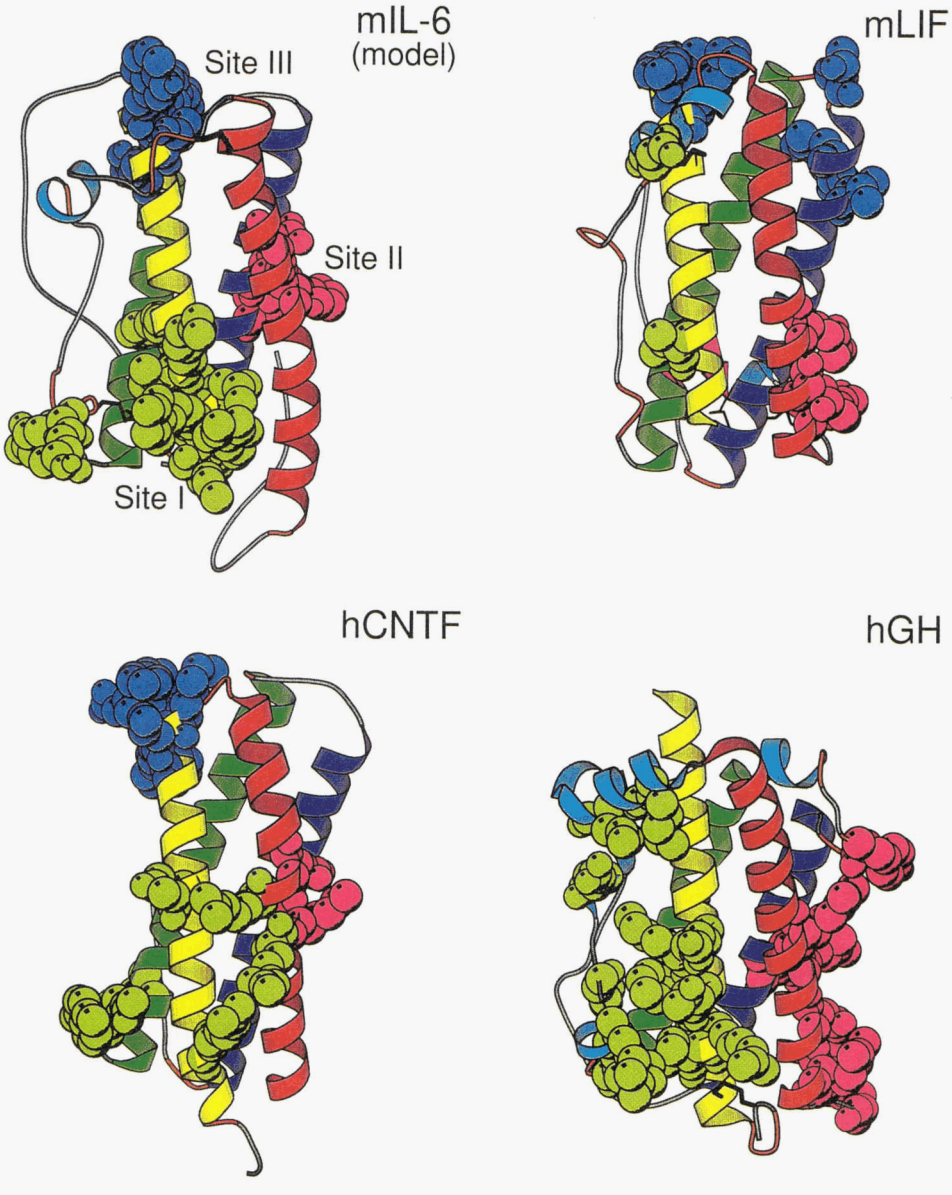

Fig. 6. Comparison of IL-6, LIF, CNTF and GH structures. Ribbon representation of a model of mIL-6 (Hammacher et al., 1994) based on the crystal structure of human G-CSF (Hill et al., 1993), mouse LIF at 2.0 Å resolution (Robinson et al., 1994), human CNTF at 2.4 Å resolution (McDonald et al., 1995), and human GH at 2.8 Å resolution (de Vos et al., 1992). Drawings were made with MOLSCRIPT (Kraulis, 1991). Helix A is shown in red, helix B in green, helix C in dark blue, helix D in yellow, nonhelical regions (including the connecting loops between the helices) in gray, and the loop helices in mIL-6, mLIF, and hGH in light blue. Hydrogen-bonded turns, as defined by Kabsch & Sander (1983), are indicated in orange in the loop regions of the structures. Residues identified in receptor interactions (see text) are shown in CPK form. For IL-6 and LIF, residues in the murine sequence that are equivalent to those implicated in receptor-binding in the human molecule are shown. The pattern and distribution of the residues involved in binding at sites I (lime), II (magenta) and III (light blue) are highlighted, and are the same as those marked in Figure 3 (see The complex of growth hormone and its receptor and Determination of the active site for references on GH and IL-6, respectively, and the legend to Fig. 3 for references on LIF and CNTF).

Although the number of components that can act as IL-6 antagonists in vitro is steadily increasing, their usefulness in the clinic cannot be extrapolated from in vitro studies. For example, neutralizing IL-6 mAbs, when injected into a patient with terminal disease, initially inhibited the myeloma cell proliferation, but ultimately led to increased levels of circulating IL-6 (Klein et al., 1991). The loss of effectiveness of the immunotherapy was due to the formation of immune complexes of IL-6 with the mAbs (Lu et al., 1992).

The ternary complex

Given that a common mechanism of activation of transmembrane receptors involves dimerization of their cytoplasmic domains (reviewed by Ullrich & Schlessinger, 1990; Heldin, 1995), and that the transmembrane and cytoplasmic domains of the IL-6R are not necessary for signaling (Taga et al., 1989; Hibi et al., 1990), intracellular signaling induced by IL-6 was postulated to involve gp130 dimerization (Ip et al., 1992). It was later shown that bind-

ing of IL-6 to the IL-6R induced gp130 homodimerization, which correlated with tyrosine kinase activation (Davis et al., 1993b; Murakami et al., 1993). Unexpectedly, it was also found that these gp130 dimers are covalently linked through an intermolecular disulfide bridge (Davis et al., 1993b; Murakami et al., 1993). Similarly, disulfide-linked heterodimers of gp130 and the LIFR are formed upon activation of target cells with LIF or CNTF (Davis et al., 1993b), while OSM (Gearing et al., 1992), CT-1 (Pennica et al., 1995), and possibly IL-11 (Neddermann et al., 1996) also signal via heterodimerization of gp130. The activation of gp130 (and LIFR) by the IL-6-related cytokines thus conforms to the model of ligand-induced dimerization, reminiscent of the mechanism proposed for the activation of tyrosine kinase receptors (Ullrich & Schlessinger, 1990) (Fig. 7). As discussed below, IL-6 (Ward et al., 1994b; Paonessa et al., 1995) and CNTF (de Serio et al., 1995) form a hexameric receptor-complex consisting of two ligands, two ligand-binding receptors, and two signaling receptors.

The high affinity ternary IL-6 receptor-complex is a hexamer

Similar to the GH/GHR complex, a ternary complex (M_r ~368K) of IL-6 with the sIL-6R and sgp130 can be reconstituted in vitro and isolated by size-exclusion chromatography (Ward et al., 1994b). When analysed by sedimentation equilibrium ultracentrifugation, this ternary complex had a calculated M_r ~320K, as compared to M_r ~305K, which is the calculated sum of two molecules of each component (Ward et al., 1994b). As the sIL-6R was shown to interact with IL-6 in a 1:1 stoichiometry, both the size-exclusion and the analytical ultracentrifugation studies support a model where the ternary complex consists of two molecules of each of IL-6, IL-6R, and gp130 (Ward et al., 1994b). This finding was confirmed by careful immunoprecipitation studies using differentially tagged wild-type and mutated IL-6 and sIL-6R as well as sgp130 (Paonessa et al., 1995). The model proposed by Paonessa et al. (1995), shown in Figure 8A, suggests that several interaction surfaces hold the complex together.

In this model, the interactions in the hexamer are thought to be more complex than the juxtaposition of two GH/GHR-like trimers. A consequence of this model is that the CBD of each gp130 has two sites of interaction with IL-6 and two with the IL-6R (Fig. 8A). Thus, each gp130 molecule is proposed to bind IL-6 at site II and dimerise with IL-6R in the GHR trimeric style while also binding to site III on a second IL-6 molecule and to an as yet unidentified site on a second IL-6R molecule. The model shown in Figure 8A also suggests that IL-6 dimerization may play a role in the linking and stabilizing of the hexameric complex, though, to date, it has not been shown that an IL-6 dimer is involved in the hexameric complex.

Whereas the studies by Paonessa et al. (1995) showed for the first time that sites II and III on IL-6 interact independently with two different gp130 molecules, their model of the hexameric complex (Fig. 8A) requires interactions between trimers that might be unlikely on steric grounds. The structures of the class I cytokine receptors solved to date, GHR (de Vos et al., 1992), PRLR (Somers et al., 1994), and EpoR (Livnah et al., 1996) all show an angle of ~90° between the two FN III domains of the CBD. Although there is a slight variation in the orientation of the two FN III domains in the CBDs relative to one another (a translation of 2–5 Å and a rotation of 8–12°) (Livnah et al., 1996; Wells and de Vos, 1996), the receptors bind their ligands through the same loop regions (Livnah et al., 1996). There is a similar conservation of the angle between the two FN III domains of the binding domain in the

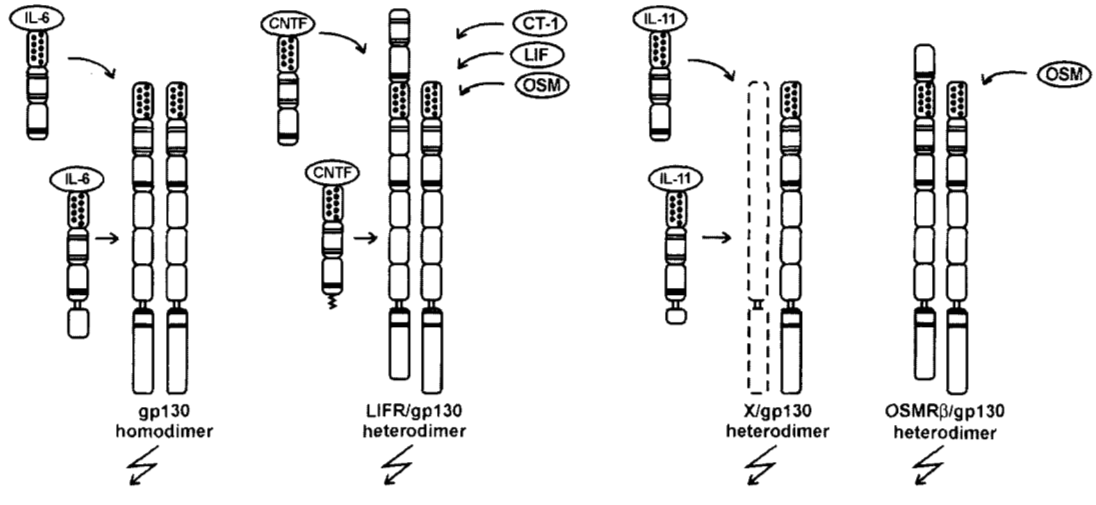

Fig. 7. Dimerization of gp130 induces intracellular signaling. Schematic representation of gp130 (Hibi et al., 1990), the LIFR (Gearing et al., 1991), the OSMRβ (Mosley et al., 1996), and the membrane bound receptors for IL-6 (Yamasaki et al., 1988), IL-11 (Chérel et al., 1995), and CNTF (Davis et al., 1991). The glycosyl-phosphatidylinositol cell membrane anchor of the CNTFR is indicated by a zig-zag. The stippled transmembrane protein (denoted "X") represents a putative component of the IL-11 receptor-complex. The other symbols are as in Figure 4. Soluble receptors that have a stimulatory role when bound to the corresponding ligand include the receptors for IL-6 (Yasukawa et al. 1990), CNTF (Davis et al., 1993a), and IL-11 (Baumann et al., 1996b). Homodimerization of gp130 leading to intracellular signaling is induced by the IL-6/IL-6R complex (Davis et al., 1993b; Murakami et al., 1993) and by mAbs B-S12 and B-P8 (Wijdenes et al., 1995; Fourcin et al., 1996) (not in figure). Heterodimerization of gp130 with the LIFR leading to intracellular signaling is induced by LIF and OSM (Gearing et al., 1992), CT-1 (Pennica et al., 1995) and the CNTF/CNTFR complex (Davis et al., 1993b). There is evidence for a third component in the receptor-complex of CT-1 (Robledo et al., 1996) (not in figure). IL-11-induced intracellular signaling may involve heterodimerization of gp130 with an as yet unidentified receptor (Neddermann et al., 1996). Heterodimerization of gp130 with the OSMRβ leading to intracellular signaling is induced by OSM (Mosley et al., 1996).

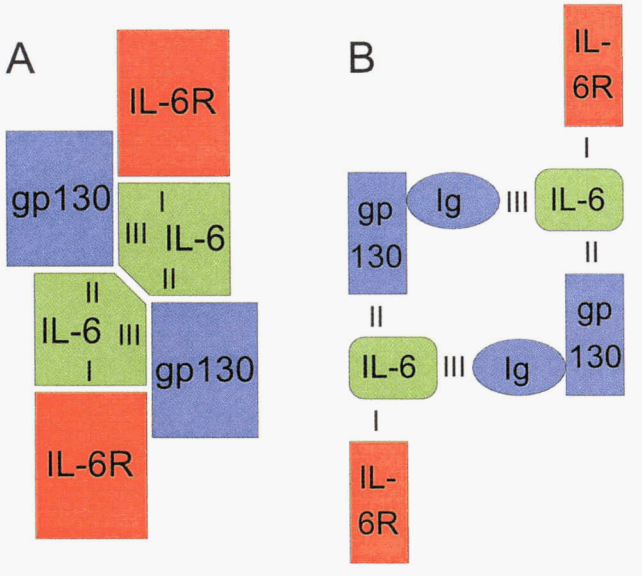

Fig. 8. Proposed models of the hexameric IL-6 receptor-complex. The schematic models are in top views and are arranged, for ease of comparison, in the same way as the top view of the GH/GHR trimeric complex in Figure 5. Boxes depict the CBD of the IL-6R and gp130. sites I, II and III on IL-6 are indicated in Arabic numerals. **A:** Model proposed by, and adapted from Paonessa et al. (1995). **B:** Model proposed by authors, postulating the involvement of the Ig-like domain of gp130. See The ternary complex for details.

known structures of the class II cytokine receptors. These are the ligand-bound IFN-γ receptor (Walter et al., 1995) and the ligand-bound (Banner et al., 1996) and soluble form (Harlos et al., 1994; Muller et al., 1996) of tissue factor. This general conservation of the structural form of the receptors within each class makes it seem likely that the structure of other receptors will be similar to the currently known members of each class. In the case of the receptor-complex of IL-6, the top view of Figure 5 suggests that, unless the receptor molecules have a different orientation than those mentioned above, a model such as that in Figure 8A might cause steric clashes between the receptor molecules.

In Figure 8B, therefore, we propose a different model to explain the interactions in the hexamer. Our model arose from a consideration of how one gp130 molecule might interact with two distinct sites on different IL-6 molecules, as well as the nature of the site III interaction. We have taken into account that the extracellular region of gp130 has the same modular structure as the G-CSFR (Fukunaga et al., 1990; Hibi et al., 1990; Larsen et al., 1990), including an Ig-like domain that is lacking in the GHR (Leung et al., 1987). Both the Ig-like domain and the CBD of the G-CSFR are required for ternary complex formation with G-CSF (Hiraoka et al., 1995). In agreement with our model, we have recently identified neutralizing mAbs that recognize the Ig-like domain of gp130 (A. Hammacher & R.J. Simpson, in preparation) and interfere with the biological activity of IL-6 (Saito et al., 1993; Wijdenes et al., 1995; Chevalier et al., 1996).

In contrast to the model of Paonessa et al. (1995) (Fig. 8A), our model is biased towards the association of two GH/GHR-like trimers. The Ig-like domain of gp130 links the IL-6/IL-6R/gp130 trimers by binding to site III on IL-6 in the other trimer, thereby forming the hexamer. As seen in the GH/GHR complex (de Vos et al., 1992) and the complex of GH with the PRL receptor (Somers et al., 1994), the interactions of the receptors with the ligands are mostly mediated via the loop regions of the receptors. This is also the pattern seen in immunoglobulin domains (Wilson & Stanfield, 1993). To satisfy the model of Figure 8A, interactions from the β-sheet domain framework of the receptors with IL-6 would probably be required. In our model (Fig. 8B) the loops in the vicinity of the N-terminus of the Ig-like domain are postulated to interact with site III on IL-6. The possibility that loops in the junction between the Ig-like domain and the CBD of gp130 might interact with site III would require a very tightly packed hexameric complex, which could result in steric clashes. The involvement of the Ig-like domain of gp130 in hexamer complex formation, as proposed in Figure 8B, would allow interactions via loop regions in the vicinity of the N-terminus, resulting in a sterically favourable positioning of the two IL-6/IL-6R/gp130 trimers. To achieve this, the Ig-like domain would need to form an angle of ~90° to the CBD, similar to the angle between the two FN III domains of the CBD.

An important consideration in hexamer formation is that gp130 binding at sites II and III on IL-6 is IL-6R-dependent. Paonessa et al. (1995) favor the notion that, for both sites, this is due to additional stabilizing interactions between the IL-6R and gp130, rather than by conformational changes induced in IL-6 upon binding to the IL-6R. By mutating the IL-6R in the region corresponding to the GHR dimerization interface, Salvati et al. (1995) have identified one gp130-interaction site in the IL-6R. This site in the IL-6R was shown to bind the same gp130 molecule that binds to site II on IL-6 (Paonessa et al., 1995). We suggest that each IL-6R in the hexameric complex has only one site of interaction with gp130, which would localize to the site identified by Salvati et al. (1995). Based on the available mutagenesis data on IL-6 (see Determination of the active site and below), we postulate that binding of the IL-6R to IL-6 induces a conformational change in site III, which allows binding of gp130 to this site. The binding of the IL-6R would "activate" site III by stabilizing structural interactions between residues in the AB-loop and the site III residues in the D1 motif. The N-terminal part of the AB-loop in IL-6, which has been suggested to be in close proximity to the D1 motif (Ehlers et al., 1994; Hammacher et al., 1994; Ciapponi et al., 1995), appears to be disordered based on the recently published NMR sequential assignments and topology of hIL-6 (Xu et al., 1996). It is thus possible that IL-6R binding to IL-6 provides a structural stabilization to this part of the AB-loop, thereby favoring its interactions with the D1 motif and allowing binding of gp130 to site III.

Since a complete description of the functional and structural epitopes for the IL-6/IL-6R interaction is not yet available, an interaction of the IL-6R with residues Glu 51–Glu 59 in the AB-loop, as is seen in the binding of site I on GH to the GHR (cf. Fig. 3), is possible. The notion that the residues in the AB-loop of IL-6 are functionally distinct from the residues in the D1 motif is supported by data from de Hon et al. (1995b). In this study, two neutralizing anti-IL-6R mAbs were shown to differentially inhibit the bioactivity of (Gln 159 Glu, Thr 162 Pro)IL-6, a D1 motif mutant, and of an AB-loop mutant with residues Lys 41–Ala 56 substituted for the corresponding mIL-6 residues.

Mutagenesis studies of the residues in the AB-loop proposed to be in site III and of the D1 motif in IL-6 provide evidence for the importance of a structural interaction between these regions. Substitution of the mouse residues Leu 43–Asp 55 for the human residues showed an effect on gp130 binding and dimerization (Ehlers et al., 1994; Ciapponi et al., 1995). This can be explained by the

altered packing interactions failing to stabilize the D1 motif, particularly as an alanine scan of residues Glu 51–Glu 55 showed no effect on gp130 binding (de Hon et al., 1995a). Leu 57, though, was shown to be important for the sIL-6R-dependent interaction of IL-6 with gp130 (de Hon et al., 1995a). This residue is solvent exposed in the model of Ehlers et al. (1994) but is directed towards helix D in the model of Hammacher et al. (1994), which uses a different alignment of the D helix. It is likely that Leu 57 is important for packing interactions with helix D. A triple mutant, (Leu 57 Asp, Glu 59 Phe, Asn 60 Trp)IL-6, was essentially unable to induce sIL-6R-dependent dimerization of sgp130, with consequent loss of bioactivity (Toniatti et al., 1996). The substitution of a hydrophilic, charged residue for Leu 57 would cause greater disruption to any hydrophobic interactions involving Leu 57 (for example in the suggested interaction with the D1 motif) than the relatively conservative substitution to alanine, and could explain the greater loss of bioactivity of the latter mutant compared to (Leu 57 Ala)IL-6. Similarly, the substitution of Lys 54 for aspartic acid (Ehlers et al., 1996) but not for alanine (de Hon et al., 1995a) was found to negatively affect IL-6R-dependent gp130 binding of IL-6. By similarity to the AB-loop helix in G-CSF (Hill et al., 1993), it is likely that Lys 54 and Glu 51 participate in a stabilizing interaction on the putative AB-loop helix of IL-6. The mutation Lys 54 Asp would then cause an unfavorable charge interaction, destabilizing the putative AB-loop helix and its interaction with the D1 motif.

An IL-6 mutant, in which both Trp 157 and Leu 158 in the D1 motif were mutated to alanine, showed a strongly reduced response in all biological assays tested (de Hon et al., 1995b). In the model of mIL-6 (Hammacher et al., 1994), there are hydrophobic contacts between Trp 157 and Met 51, and between Leu 158 and Leu 57, suggesting that mutation of Trp 157 and Leu 158 affects the interactions between the residues in the AB-loop and the D1 motif. Further evidence for the importance of these packing interactions comes from the crystal structures of LIF (Robinson et al., 1994) and G-CSF (Hill et al., 1993), which show hydrophobic interactions from the D1 motif to the equivalent region in the AB-loop, which is helical in these structures. CNTF also shows a similar pattern of hydrophobic residues in the D1 motif, though the AB-loop is not seen in the crystal structure (McDonald et al., 1995). Solvent-exposed residues in the D1 motif that are directed away from the residues in the AB-loop have been shown to be important for receptor binding in LIF (Layton et al., 1994; Hudson et al., 1996) and CNTF (Inoue et al., 1995; Krüttgen et al., 1995; Panayotatos et al., 1995; Thier et al., 1995; di Marco et al., 1996). In the mIL-6 model of Hammacher et al. (1994), Arg 159 is directed away from the residues in the AB-loop and is solvent exposed. The equivalent residue in human IL-6, Gln 159, has been shown to be involved in gp130 interaction (Brakenhoff et al., 1994).

In summary, the model shown in Figure 8B is based on two GH/GHR-like trimer complexes where IL-6 binds the IL-6R at site I, and subsequently gp130 binds at site II and also to the IL-6R. The binding of the IL-6R to IL-6 stabilizes the D1 motif, allowing the Ig-like domain of gp130 in one trimer to bind to site III of the IL-6 molecule in the other trimer, thus forming a hexameric complex. This model favors the association of two trimers but does not preclude other means of association; the relative affinities of gp130 for sites II and III in an IL-6/IL-6R complex might govern this association. An interaction between the two IL-6 molecules in this model is unlikely. A further contribution to the stability of the hexameric complex may come from interactions between the three membrane proximal FN III domains of each of the gp130 molecules, as evidenced by the fact that this region is recognized by a neutralizing anti-gp130 mAb (A. Hammacher, unpublished observation). Interactions between the extracellular membrane proximal domains of two gp130 molecules could be envisaged to result in dimerization of the cytoplasmic domains and, thereby, induction of intracellular signaling.

Formation of an intermediate trimeric ternary complex?

There is evidence that a trimeric intermediate consisting of one molecule of each IL-6, sIL-6R and sgp130 precedes hexamer formation. Trimer, but not hexamer, formation is observed if either of the two gp130 sites in IL-6 is mutated or blocked by mAbs, showing that gp130 is able to associate with either site II or site III in an IL-6/IL-6R complex (Paonessa et al., 1995). The binary IL-6/sIL-6R complex has a lower affinity for gp130 in the trimeric complex than in the hexamer, as implied from both immunoprecipitation (Paonessa et al., 1995) and biosensor studies (Hammacher et al., 1996; Ward et al., 1996). A trimeric intermediate consisting of one molecule of each CNTF, sCNTFR, and sLIFR may also precede hexamer formation in the CNTF system, as shown by immunoprecipitation (de Serio et al., 1995). It does not appear that dimerization of the IL-6/IL-6R binary complex precedes hexamer formation (Ward et al., 1994b, 1996).

The ternary complexes of the IL-6-related cytokines

Mutagenesis studies on IL-6, LIF and CNTF suggest that receptor-interaction via site III is a general feature of the IL-6 family of cytokines. Depending on the cytokine, site III may associate with either gp130 or the LIFR. Site III/gp130 interactions have been shown for IL-6 (Brakenhoff et al., 1994; Paonessa et al., 1995) and may occur on CNTF (de Serio et al., 1995). Immunoprecipitation studies showed that two CNTF/CNTFR complexes can form an intermediate pentameric complex with one gp130 molecule, suggesting that gp130 interacts simultaneously with sites II and III in CNTF in the hexameric CNTF receptor-complex (de Serio et al., 1995). By contrast to the hexameric complex of IL-6, which contains two gp130 molecules (Fig. 8), the hexameric complex of CNTF is asymmetric, containing one LIFR and one gp130. The LIFR binds to site III on CNTF (di Marco et al., 1996), but the involvement of site II in LIFR binding has not been ruled out. As is the case for IL-6, site I in CNTF binds to the α-receptor (CNTFR), whereas site II binds to the β-receptor gp130 (Panayotatos et al., 1995) in a GH/GHR-like manner. It is therefore plausible that the topologies of the hexameric complexes of IL-6 and CNTF are similar. We propose that the Ig-like domain of gp130 may be involved in the formation of the CNTF hexameric receptor-complex, by binding to site III on CNTF in the opposite trimer. As shown in our model of IL-6 (Fig. 8B), such an arrangement could be envisaged to limit any steric clashes between the components of the hexameric CNTF receptor-complex.

The available structure-function data on IL-11 suggest that IL-11 is a four-helix bundle protein closely related to IL-6 and G-CSF (Boulay & Paul, 1993; Sprang & Bazan, 1993; Czupryn et al., 1995). Coexpression of the IL-11R and gp130, but not the IL-11R and the LIFR, was shown to mediate high affinity binding and signaling of IL-11 (Hilton et al., 1994). From the similarities of the IL-11R with the nonsignaling IL-6R and CNTFR (Hilton et al.,

1994; Chérel et al., 1995; Baumann et al., 1996b), it may be deduced that the ternary receptor-complex of IL-11 is hexameric. However, immunoprecipitation studies with IL-11, the sIL-11R, and sgp130 failed to detect the formation of a hexameric complex (Neddermann et al., 1996). Instead, two IL-11/sIL-11R complexes were found to form an intermediate pentameric complex with one gp130 molecule, suggesting that IL-11-induced intracellular signaling involves dimerization of gp130 with an as yet unidentified receptor (Neddermann et al., 1996). In the putative hexameric IL-11 receptor-complex, site I on each IL-11 molecule would bind to one IL-11R and, similar to CNTF (de Serio et al., 1995), the sole gp130 molecule would interact with site II on one IL-11 molecule (forming a GH/GHR-like trimer). We postulate that the Ig-like domain of gp130 interacts with the putative site III on IL-11, similar to IL-6 in Figure 8B.

Heterodimerization of gp130 with the LIFR is induced by LIF and CT-1, which first bind to the LIFR (Gearing et al., 1991; Pennica et al., 1995), and by OSM, which first binds to gp130 (Gearing et al., 1992). OSM also induces heterodimerization of gp130 with the OSMRβ, an OSM-specific signaling receptor, (Mosley et al., 1996). In the case of LIF and OSM, a specific non-signaling receptor, the equivalent of the IL-6R and the CNTFR, has not been identified. Therefore, LIF (N.A. Nicola, pers. comm.) and OSM are thought to form trimeric rather than hexameric signaling receptor-complexes consisting of one ligand, one gp130 molecule, and one LIFR or OSMRβ. As evidenced by studies on LIF where site III has been shown to interact with the LIFR (Layton et al., 1994; Hudson et al., 1996), a trimeric complex does not preclude the existence of site III/receptor interactions. Interestingly, the LIFR has two CBDs (Gearing et al., 1991) (cf. Fig. 7). It remains to be determined whether both CBDs are involved in ligand-binding, and whether CT-1 and LIF bind to the same region on the LIFR.

Recent evidence suggests that, in addition to the LIFR and gp130, the receptor-complex of CT-1 contains a third component of apparent M_r 80K (Robledo et al., 1996). Removal of N-linked carbohydrates from the 80K component resulted in a molecule of apparent M_r 45K (Robledo et al., 1996). The role of this receptor in CT-1 binding and signaling is as yet unknown, but its M_r compares well with that of the predicted protein backbone of the IL-6R (50K) (Yamasaki et al., 1988), and to the IL-11R (Chérel et al., 1995) and the CNTFR (Davis et al., 1991), suggesting that it may be a nonsignaling receptor. It is tempting to speculate that the 80K component is equivalent to the IL-6R and the CNTFR and, therefore, that the receptor-complex of CT-1 is hexameric. As in our proposed models for the receptor-complexes of IL-6, CNTF, and IL-11, this suggests that the Ig-like domain of gp130 is involved in the ternary receptor-complex of CT-1 by binding to the putative site III on CT-1. Alternatively, similar to OSM and the OSMRβ (Mosley et al., 1996), the 80K component may be a second CT-1-specific signaling receptor. Based on phenotypic differences in the cardiac development of mice with targeted disruptions in the genes for gp130 (Yoshida et al., 1996) or the LIFR (Ware et al., 1995), it is possible that the LIFR is not the exclusive dimerizing partner of gp130 in the receptor-complex of CT-1.

IL-6 and CNTF bind to specific nonsignaling receptors and form hexameric receptor-complexes with gp130. By contrast, LIF and OSM directly bind to signaling receptors and may form trimeric ternary complexes with gp130. The receptor-complexes of IL-11 and CT-1 may be hexameric. The involvement of the Ig-like domain of gp130 in ternary complex formation is postulated to correlate with the presence of two ligands in the signaling receptor-complex.

Summary

Identifying the sites at which growth factors interact with their receptors is a critical first step in their rational manipulation for therapeutic benefits. Over the last decade, our understanding of the mechanism by which IL-6 activates the components of its receptor-system has increased significantly. Structure–function studies have identified several receptor-interactive residues, clustered in three regions on the molecule (sites I, II, and III), necessary for low-affinity binding to the specific IL-6R, and for IL-6R-dependent binding to the high-affinity converter and signaling molecule, gp130. Similar to the case of GH, site I on IL-6, which is involved in the binding to the IL-6R, involves the C-terminal end of helix D and a region encompassing the C-terminal part of the AB-loop/N-terminal part of helix B, while site II (one of the two sites on IL-6 involved in gp130 binding) involves residues in helix A and helix C. In contrast to GH, IL-6 contains a third receptor interaction region, site III. This site, the second gp130 binding site, comprises residues in the CD-loop/N-terminal end of helix D; these residues are in the D1 motif, a region which exhibits sequence similarity with other members of the IL-6 family of cytokines (LIF, IL-11, CNTF, CT-1, OSM) that signal through gp130. A region encompassing residues in the N-terminal part of the AB-loop may stabilize the interactions of site III with gp130 but not itself be directly involved in gp130-binding.

The high affinity ternary complex of IL-6 is hexameric consisting of two molecules each of IL-6, IL-6R, and gp130. Several IL-6 antagonists have been generated by mutating IL-6 in sites II and/or III. Such antagonists remain able to bind to the IL-6R but are impeded in their ability to interact with gp130 and to participate in hexamer-complex formation. To understand the topology of the hexameric complex of IL-6 further investigations are needed. A model of the complex is presented which postulates the involvement of the Ig-like domain of gp130.

To progress further, it is clear that a detailed structural analysis of IL-6 and its associated receptor-complex(es), of the type undertaken for GH, is a necessary foundation for the rational design of small molecule therapeutics, which may act as IL-6 agonists or antagonists. In this regard, IL-6 serves as a prototype for the family of hematopoietin receptor cytokines that share gp130 as a signaling subunit.

Note added in proof

A 1.9 Å crystal structure of hIL-6 has been recently reported (Somers W et al., 1997, *EMBO J 16*:989–997).

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