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Structure-function analysis of human IL-6 receptor: dissociation of amino acid residues required for IL-6-binding and for IL-6 signal transduction through gp130

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Here, we report the analysis of the structure-function relationship of the extracellular region of human interleukin 6 receptor (IL-6R). Upon binding of IL-6, IL-6R becomes associated extracellularly with a non-IL-6-binding but signal transducing molecule, gp130, and the IL-6 signal is generated. In this region, the cytokine receptor family domain, but not the immunoglobulin-like domain, was responsible both for IL-6 binding and for signal transduction through gp130. Because a soluble, extracellular portion of IL-6R (sIL-6R) could bind IL-6 and mediate IL-6 functions through gp130, amino acid substitutions were introduced into sIL-6R by site-directed mutagenesis. The results, together with the previously proposed tertiary structure model, suggested that the amino acid residues critical for IL-6 binding have a tendency to be distributed to the hinge region between the two 'barrel'-like fibronectin type III modules and to the same side of these two 'barrels'. Amino acid residues, of which substitutions barely affected the IL-6-binding but did abolish the IL-6 signalling capability of sIL-6R, were identified and found to be located mainly in the membrane proximal half of the second barrel. sIL-6R mutants carrying such substitutions lacked the capacity to associate with gp130 in the presence of IL-6.

Key words: cytokine receptor family/IL-6 receptor/ligand binding/signal transduction/structure-function analysis

Introduction

Interleukin-6 (IL-6) is a typical example of cytokines with a variety of biological activities on a wide range of tissues (Kishimoto, 1989). The IL-6 signal is transduced through a cell surface receptor complex in a specific manner. We have demonstrated that two transmembrane molecules are involved in this step; an IL-6 receptor (IL-6R) molecule (Yamasaki *et al.*, 1988) and a non-IL-6-binding signal transducer, gp130 (Taga *et al.*, 1989; Hibi *et al.*, 1990). IL-6 triggers the association of IL-6R with gp130 at their extracellular regions, resulting in the formation of high-affinity IL-6 binding sites, and gp130 in turn generates the IL-6 signal. It has been demonstrated that genetically engineered soluble IL-6R (sIL-6R) lacking transmembrane and cytoplasmic regions retains IL-6-binding capability. sIL-6R enhances biological functions of IL-6 rather than inhibits them (Taga *et al.*, 1989; Yasukawa *et al.*, 1990) unlike sIL-1R and sIL-4R (Fanslow *et al.*, 1990; Mosley *et al.*, 1989), since sIL-6R associates with the cell surface gp130 in the presence of IL-6. Therefore, the extracellular region of IL-6R is responsible for two functions of IL-6R; (i) IL-6-binding and (ii) association with gp130 to generate the signal. The 339 amino acid extracellular region of human IL-6R is composed of an immunoglobulin (Ig)-like domain and a cytokine receptor family domain, the latter of which comprises two fibronectin type III modules (Yamasaki *et al.*, 1988; Bazan, 1990). Therefore, it is interesting to know which domain of the extracellular region of IL-6R is responsible for IL-6-binding and/or IL-6 signal transduction through gp130, and to identify amino acid residues critical for these two functions.

Here we describe the structure-function analysis of human IL-6R. We show that only the cytokine receptor family domain is responsible both for IL-6-binding and for IL-6 signal transduction through gp130. We further identify amino acid residues critical for IL-6-binding and/or IL-6 signal transduction by introducing site-directed amino acid substitutions into sIL-6R and discuss their spatial locations. We also describe that some mutants retain IL-6-binding capability but are inadequate to associate with gp130 in the presence of IL-6, resulting in the loss of the potential to mediate the IL-6 signal.

Results

Unnecessity of the immunoglobulin-like domain of IL-6R for IL-6 binding and IL-6 signal transduction

The extracellular region of human IL-6R, which is responsible both for IL-6-binding and for IL-6 signal transduction through gp130, can be divided into two domains; an Ig-like domain (positions ~20 to ~110; Yamasaki *et al.*, 1988) and a cytokine receptor family domain (~110 to ~330; Bazan, 1990). To determine which domain is responsible for binding the IL-6 molecule and/or mediating the IL-6 signal, two types of deletion mutants were constructed. One, IL-6RΔIg, lacks the Ig-like domain (Ala27-Thr105) and the other, IL-6RΔFam, lacks the cytokine receptor family domain (Lys124-Asn342). Each of these mutants as well as wild-type IL-6R was expressed in COP cells and stained with biotinylated IL-6 and FITC-conjugated avidin. As shown in Figure 1 (left and centre panels), the cells expressing IL-6RΔIg were stained comparably to the cells expressing wild-type IL-6R. The staining was IL-6-specific since it was completely inhibited by the addition of excess non-biotinylated IL-6 (unpublished data). However, the cells expressing IL-6RΔFam were not stained (Figure 1, right panel). The IL-6RΔFam molecule

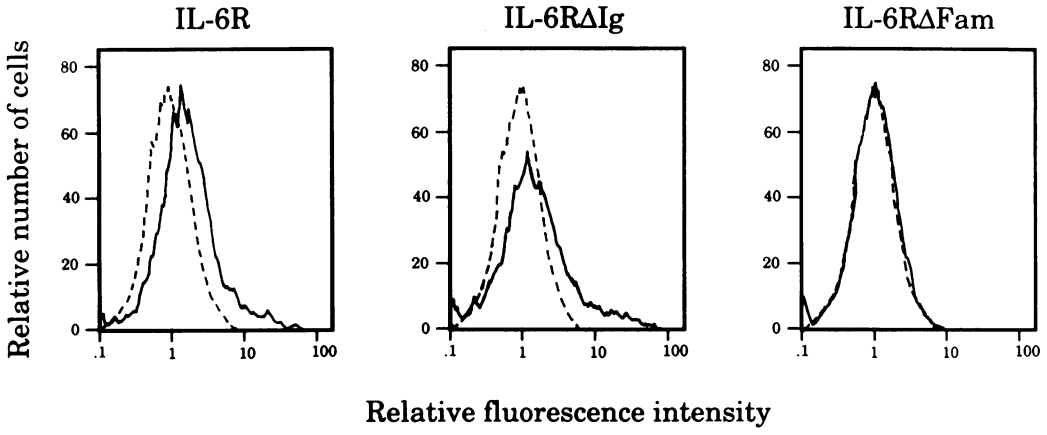

Fig. 1. IL-6-binding capability of IL-6R deletion mutants. Each solid line represents the staining pattern of COP cells expressing wild-type human IL-6R (**left**), IL-6RΔIg (**centre**) or IL-6RΔFam (**right**). Cells were stained with biotinylated IL-6 and FITC-conjugated avidin and analysed by a FACS. Dashed lines represent the staining with FITC-conjugated avidin alone.

was expressed on the cell surface, since this molecule on the COP cells was detected by FITC-conjugated anti-human IL-6R monoclonal antibody MT18. These results suggest that IL-6 binds to IL-6R at the cytokine receptor family domain.

We further examined whether the IL-6RΔIg molecule which retained IL-6-binding capability could normally mediate the IL-6 signal. Murine myeloid leukaemic M1 cells have been reported to respond to IL-6, resulting in the inhibition of growth, and in the differentiation into macrophages (Miyaura *et al.*, 1988). It was previously shown that M1IL-6R, the M1 transfectant carrying human IL-6R cDNA, acquired higher sensitivity to IL-6 than did the parental M1 in the growth inhibition assay (Taga *et al.*, 1989). IL-6RΔIg cDNA was introduced into M1 cells, producing the transfectant M1IL-6RΔIg. The numbers of IL-6 binding sites on M1IL-6R, M1IL-6RΔIg and M1 were 6700, 2600 and 50 per cell, respectively, as measured by Scatchard analysis. As shown in Figure 2, in association with the increase in the number of cell-surface-expressed IL-6-binding sites, M1IL-6R and M1IL-6RΔIg acquired ~36- and ~15-fold higher sensitivity to IL-6 respectively, than did M1, suggesting that mutant IL-6R lacking the Ig-like domain could mediate the IL-6 signal. Thus, the Ig-like domain in the IL-6R extracellular region was required neither for IL-6-binding nor for transducing the IL-6 signal. We have shown previously that recombinant sIL-6R with a termination codon at position 323 is able to bind IL-6 and to transduce the IL-6 signal by associating with the cell surface IL-6 signal transducer, gp130 (Taga *et al.*, 1989). Taken together, it is therefore suggested that the region of amino acids 106–322, which comprise the cytokine receptor family domain of IL-6R, is responsible both for IL-6-binding and for transducing the IL-6 signal through gp130.

Expression of sIL-6R mutants

To identify the amino acid residues in human IL-6R which are involved in binding IL-6 and/or in transducing the IL-6 signal, we introduced amino acid substitutions into the cytokine receptor family domain of sIL-6R by *in vitro* site-directed mutagenesis. All the mutants were prepared from sIL-6R with a termination codon at the position 345 which has previously been demonstrated to possess both IL-6-binding capability and the potential to mediate the IL-6

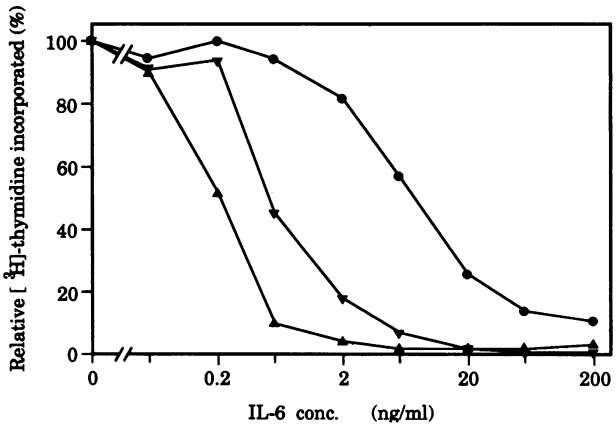

Fig. 2. Functional assay of the IL-6RΔIg mutant in M1 cells. M1 (●), M1IL-6R (▲) and M1IL-6RΔIg (▼) cells were cultured with the indicated concentrations of IL-6 and incorporated [^{3}H]thymidine was measured. Data represent the average of duplicate values.

signal through gp130 (Yasukawa *et al.*, 1990). Most of the sIL-6R mutants were designed to possess one or serial two substituted amino acid residue(s). Each sIL-6R mutant was expressed in COS-7 cells. To examine whether sIL-6R mutants are normally secreted from COS-7 cells, the cells transfected with each sIL-6R mutant cDNA were labelled with [^{35}S]methionine and the culture supernatant was immunoprecipitated with anti-human IL-6R monoclonal antibody MT18. By flow cytometric analysis, MT18 was shown to stain COP cells transfected with IL-6RΔFam cDNA but not those transfected with IL-6RΔIg cDNA (unpublished data), suggesting that this monoclonal antibody may recognize the Ig-like domain of human IL-6R. Therefore, it is unlikely that immunoprecipitation of sIL-6R mutants with MT18 would be affected by amino acid substitutions in the cytokine receptor family domain. As shown in Figure 3, the wild-type sIL-6R with a relative molecular mass of ~55 000 was precipitated (lane 2), but not from the supernatant of COS-7 cells transfected with pSVL vector without an insert (lane 1). All the sIL-6R mutants were immunoprecipitated with mobilities comparable to that of the wild-type sIL-6R (lanes 3–23; data of other mutants are not shown), showing that all sIL-6R mutants were secreted from the cells.

To estimate the expression level of each sIL-6R mutant,

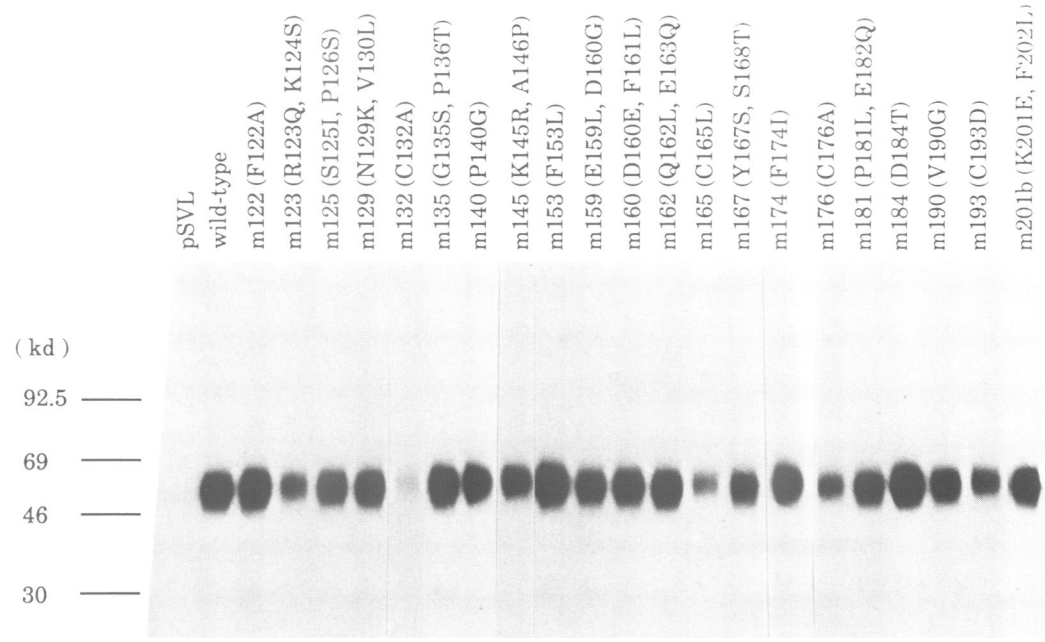

Fig. 3. Immunoprecipitation of sIL-6R mutants. COS-7 cells transfected with pSVL vector containing each sIL-6R mutant cDNA were cultured with [^{35}S]methionine and the supernatant was immunoprecipitated with the anti-human IL-6R monoclonal antibody MT18. In parentheses following the name of each sIL-6R mutant, the amino acid position number is indicated together with the original and changed amino acids (on the left and right of the number, respectively; amino acid residues are represented by one-letter symbols).

the radioactivity of each immunoprecipitated ^{35}S-labelled sIL-6R mutant in the SDS–polyacrylamide gel was measured. Values were obtained from more than two independent transfections. The expression levels of most of the sIL-6R mutants were comparable to that of wild-type sIL-6R. However, some amino acid substitutions resulted in the low expression levels of the mutants: m121a (15% of wild-type sIL-6R), m121b (14%), m132 (19%), m165 (36%), m236 (14%), m250 (30%), m254b (12%), m293a (32%), m293b (29%), m301 (17%), m303 (36%), m304 (26%) and m305 (31%). When these sIL-6R mutants were immunoprecipitated with guinea pig anti-human sIL-6R polyclonal antibody (Yasukawa *et al.*, 1992), almost consistent results were obtained (unpublished data). Amino acid residues substituted in such sIL-6R mutants might be important for the expression of sIL-6R. Interestingly, amino acids substituted in such mutants include some of highly conserved ones in the cytokine receptor family, e.g. Cys121, Cys132, Cys165, Trp303, Ser304, Trp306, Ser307.

IL-6-binding capability of sIL-6R mutants

As described above, monoclonal antibody MT18 could detect all of the sIL-6R mutants, and MT18 could not inhibit the binding of IL-6 to IL-6R (Hirata *et al.*, 1989). Thus, to examine the IL-6-binding capability of sIL-6R mutants, each sIL-6R mutant in the COS-7 culture supernatant was trapped on MT18-coated microtitre plates and the binding of ^{125}I-labelled IL-6 was measured. When the culture supernatant of COS-7 cells that had been transfected with wild-type sIL-6R cDNA was serially diluted down to 1/50 with the control supernatant and subjected to this assay, the radioactivity of the bound [^{125}I]IL-6 correlated with the dilution of sIL- 6R. So, the IL-6-binding capability of each sIL-6R mutant was evaluated as follows. The radioactivity of [^{125}I]IL-6 bound to each sIL-6R mutant was measured and compensated for its expression level as estimated in the previous section, and represented as the ratio (in %) to the value obtained with wild-type sIL-6R. Figure 4 summarizes the IL-6-binding capability of sIL-6R mutants. The results suggested that sIL-6R mutants can be roughly classified into two groups on the basis of their IL-6-binding capability. The sIL-6R mutants in one group possess IL-6-binding capability comparable to that of wild-type sIL-6R, while the IL-6-binding capability of the sIL-6R mutants in the other group was $< 10\%$ of that of wild-type sIL-6R (indicated by asterisks in Figure 4). Therefore, it was suggested that the amino acid residues substituted in sIL-6R mutants in the latter group are critical for the IL-6-binding capability of sIL-6R.

The cytokine receptor family domain comprises two fibronectin type III modules. Each of the two modules consists of seven predicted β-strands and the loops connecting them, and could form a 'barrel'-like globular protein fold (Bazan, 1990). The 'double-barrel' structure model of the cytokine receptor family domain was proposed on the basis of the crystallographically determined fold of *Escherichia coli* PapD protein which also has a tandem of spheres, each comprising seven predicted β-strands (Holmgren and Bränden, 1989; Bazan, 1990). Figure 5 schematically illustrates the 'double-barrel' structural model of the cytokine receptor family domain of human IL-6R based on Bazan's model. The positions of amino acid residues critical for IL-6-binding are indicated in this figure. 101 amino acid residues were substituted in total, 39 of which were revealed to be critical for IL-6-binding. Of these 39 residues, 23 were located in or near the hinge region between the two 'barrel'-like protein folds (Figure 5). Seventeen of the residues critical for IL-6-binding are predicted to be on the loops, and it is notable that all except one are located in the hinge region. These include a Trp303-Ser304-Glu305-Trp306-Ser307 motif which is highly conserved in the cytokine receptor family. Of the 22 amino acid residues critical for IL-6-binding that are on the β-strands, 17 (77%) were considered to be located on the same side of each 'barrel'-like type III module (at the front in Figure 5). These

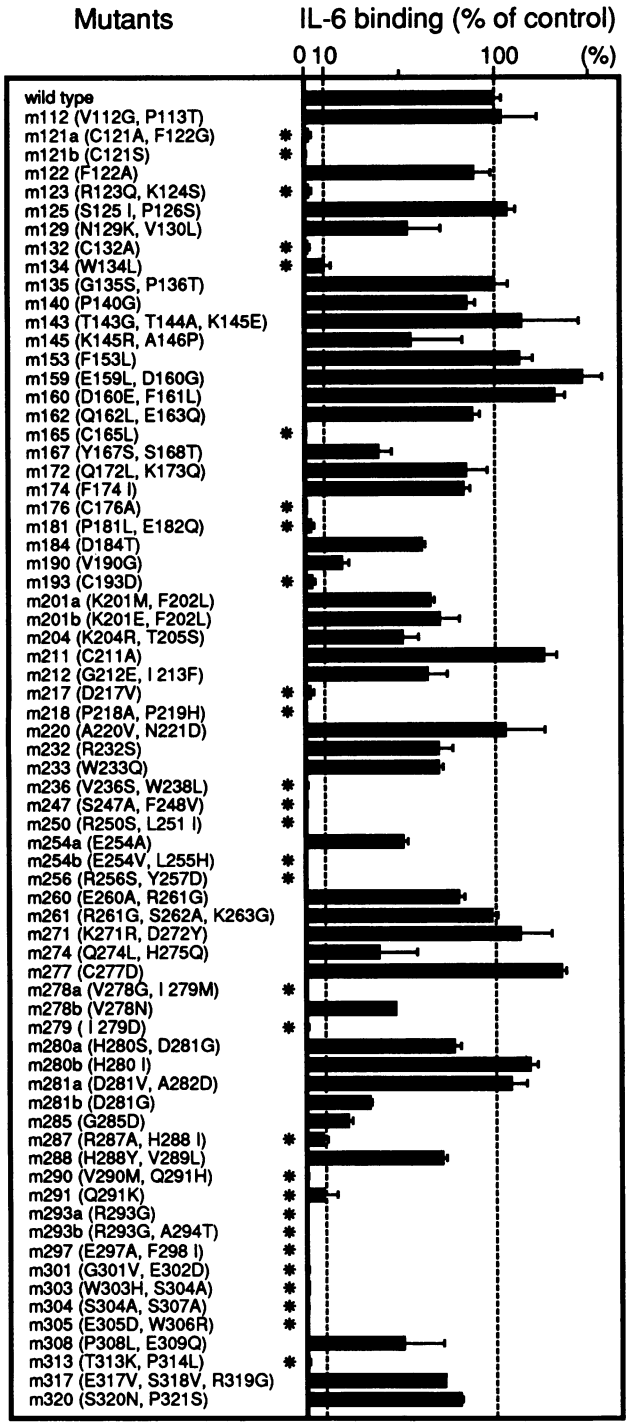

Fig. 4. IL-6-binding capability of sIL-6R mutants. The value obtained with each sIL-6R mutant is compensated for its expression level and represented as the ratio (in %) to that obtained with wild-type sIL-6R. Data represent the average of two or more independent experiments with standard deviations. Asterisks indicate sIL-6R mutants of which IL-6-binding capability was abolished. Mutant names and amino acid positions are indicated as in Figure 3.

include three of the four Cys residues (Cys121, Cys132 and Cys165) which are highly conserved in the cytokine receptor family.

Potential of sIL-6R mutants to mediate the IL-6 signal

NFS60 is a murine myeloid leukaemic cell line which responds to several cytokines including IL-6 (Weinstein

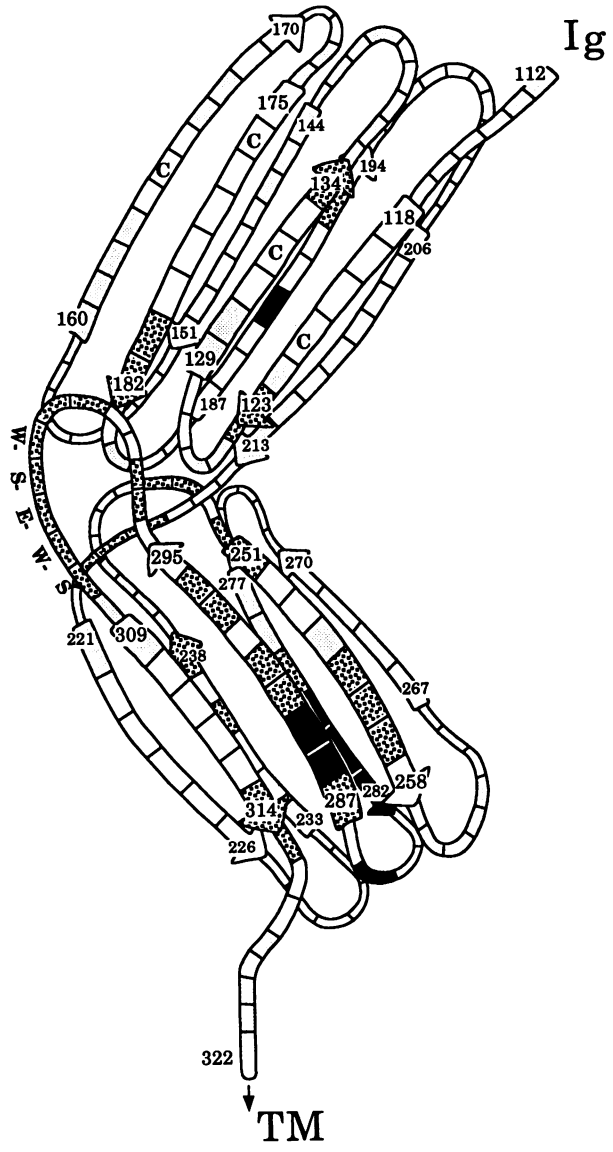

Fig. 5. Three-dimensional structural model of the cytokine receptor family domain of human IL-6R. The wide arrows represent the presumed β-strands, and narrow strands represent the presumed loops as predicted by Bazan (1990). The numbers indicate the positions of some amino acid residues. Small-dotted patches, amino acid residues showing no influences after substitution; large-dotted patches, amino acid residues required for binding IL-6; closed patches, amino acid residues critical for mediating the IL-6 signal but not for binding IL-6 (see Figure 6). The letters 'C' represent four Cys residues conserved in the cytokine receptor family, of which substitutions abolish IL-6-binding capability. The Trp-Ser-Glu-Trp-Ser motif is indicated in the one-letter code. Ig, Ig-like domain; TM, transmembrane region.

et al., 1986). Human gp130 cDNA was introduced into NFS60 and the resulting transfectant, NFS130, was obtained. The cell-surface expression of human gp130 molecules on NFS130 cells was confirmed by staining with anti-gp130 monoclonal antibody AM64 (Hibi *et al.*, 1990). As shown in Figure 6, in the presence of IL-6, NFS130 and NFS60 cells responded to sIL-6R in a dose-dependent manner. However, NFS130 cells were much more sensitive to sIL-6R than were parental NFS60 cells. We therefore used NFS130 cells in the biological assay of sIL-6R mutants. The extent of DNA synthesis initiated by the addition of sIL-6R could represent a potential of the sIL-6R to mediate the IL-6 signal through cell surface gp130. In addition to recombinant

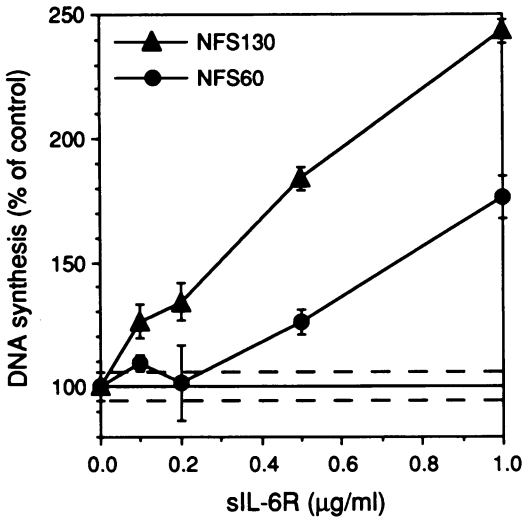

Fig. 6. Responsiveness of NFS130 and NFS60 cells to sIL-6R. NFS130 or NFS60 cells were cultured as described in Materials and methods with various concentrations of sIL-6R in the presence of 400 ng/ml IL-6. The responsiveness to each concentration of sIL-6R is represented as the ratio (in %) of radioactivity of incorporated [^{3}H]thymidine to the value obtained in the absence of sIL-6R. Vertical bars represent SD. Horizontal solid and broken lines indicate the average ± SD, respectively, obtained from the culture without sIL-6R.

sIL-6R, culture supernatant of COS-7 cells transfected with wild-type sIL-6R cDNA (25% v/v) enhanced the DNA synthesis in NFS130 cells up to ~200% of control, suggesting that this assay system could be used to evaluate the potential of sIL-6R mutants to mediate the IL-6 signal. None of sIL-6R mutants nor the wild-type sIL-6R could initiate DNA synthesis in NFS130 cells in the absence of IL-6. By the use of the above biological assay in the presence of 400 ng/ml of IL-6, the enhancement of DNA synthesis induced by the addition of each sIL-6R mutant was measured and compensated for the radioactivity of [^{125}I]IL-6 bound to each sIL-6R mutant as measured in the previous section. The compensated value obtained from each sIL-6R mutant was represented as the ratio (in %) to that obtained from wild-type sIL-6R and summarized in Figure 7. sIL-6R mutants that lacked the IL-6-binding capability as revealed in Figure 4 were omitted from Figure 7, because none of them possessed the potential to mediate the IL-6 signal. As shown in Figure 7, there were seven sIL-6R mutants which could hardly or no longer transduce the IL-6 signal (indicated by arrowheads). This result suggests that at least seven amino acid residues in sIL-6R are critical for mediating the IL-6 signal but not for IL-6-binding. The locations of these residues are illustrated in Figure 5. Six of these seven residues (His280, Asp281, Ala282, Gly285, His288 and Val289) were clustered in a membrane proximal half of the second 'barrel' in this figure.

It was then examined whether these seven sIL-6R mutants lost the capability to associate with gp130 in the presence of IL-6. The recombinant extracellular soluble human gp130 (Yasukawa *et al.*, 1992) was labelled with ^{125}I and mixed with IL-6 together with the 30-fold concentrated COS-7 culture supernatant containing wild-type or sIL-6R mutants. After a 30 min incubation, immunoprecipitation was performed with anti-IL-6R monoclonal antibody MT18. As shown in Figure 8, ^{125}I-labelled soluble gp130 was co-precipitated with wild-type sIL-6R, suggesting their physical interaction. However, when the seven sIL-6R mutants which

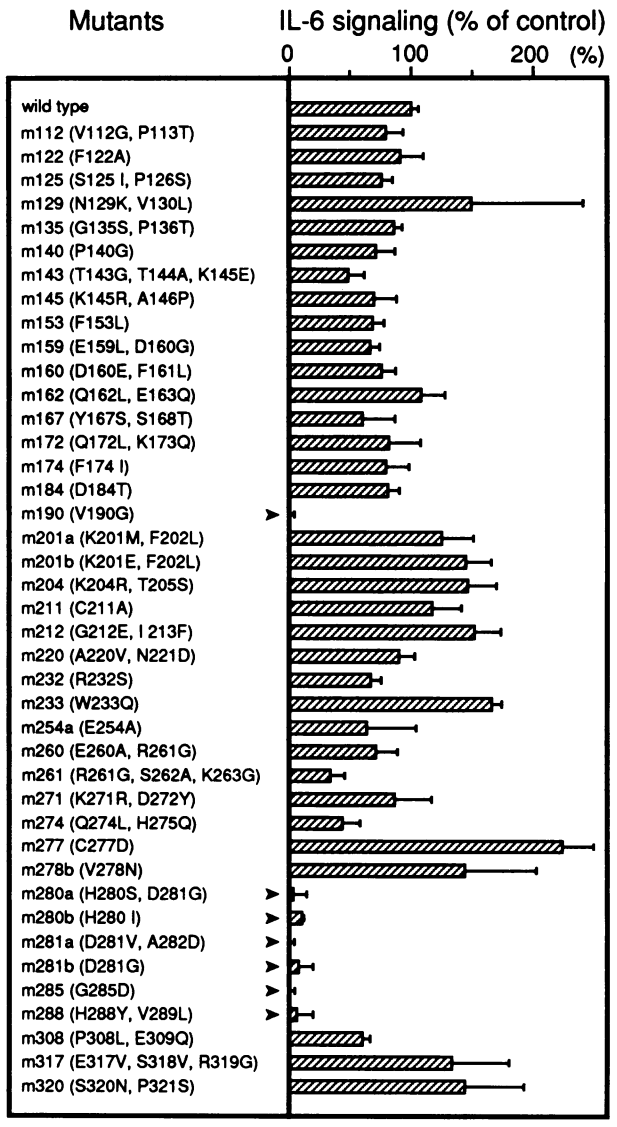

Fig. 7. Potential of sIL-6R mutants that retain IL-6-binding capability to mediate the IL-6 signal. The value obtained with each sIL-6R mutant is compensated for its IL-6-binding capability and represented as the ratio (in %) to that obtained with wild-type sIL-6R. Data represent the average of at least two independent experiments with standard deviations. Arrowheads indicate sIL-6R mutants of which potential to mediate the IL-6 signal was abolished.

could bind IL-6 but could hardly or no longer mediate the IL-6 signal were used in this assay, ^{125}I-soluble gp130 was barely co-precipitated (Figure 8). These results suggest that the amino acid residues substituted in these mutants are critical for the interaction with gp130 and thus important for mediating the IL-6 signal.

Discussion

IL-6 triggers the association of IL-6R with a non-IL-6-binding cell surface molecule, gp130, at their extracellular regions and gp130 in turn generates the IL-6 signal (Taga *et al.*, 1989; Hibi *et al.*, 1990). Therefore, the extracellular region of IL-6R possesses two functions: IL-6-binding and association with gp130 to generate the IL-6 signal. In the present study, we demonstrate that the cytokine receptor family domain of IL-6R is responsible for both of the above functions and that the Ig-like domain is required

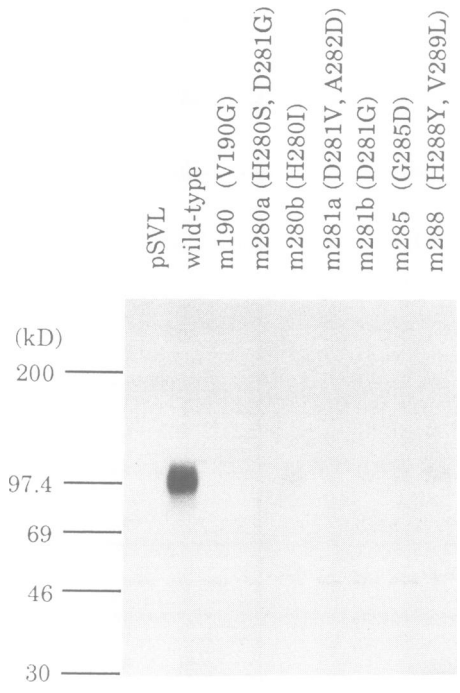

Fig. 8. *In vitro* association of sIL-6R mutants with soluble gp130. Concentrated COS-7 culture supernatants containing wild-type sIL-6R or sIL-6R mutants lacking potential to mediate the IL-6 signal were mixed with ^{125}I-labelled soluble gp130 and IL-6, and immunoprecipitation was performed with the anti-human IL-6R monoclonal antibody MT18.

neither for IL-6-binding nor for mediating the IL-6 signal. It has been reported that the NH_2-terminal Ig-like domain is not required for ligand-binding and signalling in the case of FGF-R and GCSF-R (Johnson *et al.*, 1990; Fukunaga *et al.*, 1991).

The cytokine receptor family domain has been predicted to form the 'double-barrel' structure (Bazan, 1990). A crystallographical study has shown that the extracellular domain of human growth hormone receptor (GH-R), which belongs to the cytokine receptor family, consists of two 'barrel'-like domains and that each of these domains contains seven β-strands as predicted by Bazan (De Vos *et al.*, 1992). So, the cytokine receptor family domain of IL-6R presumably forms the 'double-barrel' structure, where each 'barrel' consists of seven β-strands and the loops connecting them. All but one of the critical residues located on the loops were found in the hinge region between two 'barrels' (Figure 5). This result is consistent with that from a mutational study of human GH-R in which most of the residues critical for GH-binding could be located in or near the hinge region (Bass *et al.*, 1991). The involvement of some amino acid residues in the hinge region of GH-R has been demonstrated by a crystallographical study of the GH−$(GH\text{-}R)_2$ complex (De Vos *et al.*, 1992). In the case of the IL-2R β-chain and IL-3R, three or four amino acid residues on the loop which is located in the hinge region and which connects the second and the third β-strands in the second barrel are critical for ligand-binding (Imler *et al.*, 1992; Wang *et al.*, 1992). These findings are similar to ours, in which three amino acid residues (i.e. Ser247, Phe248 and Arg250) on the loop equivalent to the one mentioned above are critical for IL-6-binding (Figures 4 and 5). The Trp-Ser-X-Trp-Ser motif conserved in the cytokine receptor family is presumably located on the loop in the hinge region (Bazan, 1990; Figure 5). sIL-6R mutants containing substitutions of at least one of the amino acid residues in this motif showed no IL-6-binding capability (Figures 4 and 5). Some amino acid substitutions in the hinge region, including the ones in the Trp-Ser-X-Trp-Ser motif, made the expression levels of sIL-6R mutants relatively low (m254b, m301, m303, m304 and m305; 12, 17, 36, 26 and 31% of the wild-type sIL-6R, respectively), suggesting that the amino acid residues substituted in these sIL-6R mutants might be important for protein folding or for the stability of sIL-6R molecule. As for the IL-2R β-chain, some amino acid substitutions within the Trp-Ser-X-Trp-Ser motif affected its expression or IL-2-binding (Miyazaki *et al.*, 1991). It is not yet clear, because of the lack of crystallographical information, whether the hinge region in the IL-6R is involved in the direct interaction with the IL-6 molecule or whether it is important for fixing the angle formed by the two 'barrels' so that IL-6R could be properly expressed and possess IL-6-binding capability.

As for the β-strands in the double 'barrels' of sIL-6R, four Cys residues conserved among the members of the cytokine receptor family are predicted to be located on such strands in the NH_2-terminal 'barrel' (Bazan, 1990; see Figure 5). All the sIL-6R mutants containing substitutions in either of these four Cys residues lacked IL-6-binding capability (Figure 4). Of these five sIL-6R mutants, m121b and m132 showed very low expression levels (15 and 14% of the wild-type sIL-6R, respectively, as estimated in the Results section). So, substitutions of the conserved Cys residues may disturb overall conformation probably by preventing the formation of disulfide bonds between them, and may make the mutant sIL-6R unstable. Most (13 out of 17) of the other amino acid residues on the predicted β-strands in the mutants showing reduced IL-6-binding, are considered to be located on the same side of each 'barrel' (at the front of Figure 5). This result argues that such amino acid residues are involved in the direct interaction with IL-6 molecule. This could support the model proposed by Bazan (1990) in which the generic binding trough within the cytokine receptor molecule formed by two 'barrels' recognizes its ligand. At present, we have no structural information about the IL-6/IL-6R complex determined by X-ray crystallography to confirm this possibility. Therefore, we cannot exclude another possible explanation that overall structural changes may have occurred in such IL-6-binding reduced mutants. At least by the Chou−Fasman method, it does not seem to be predicted that severe structural disturbance in β-strands has occurred in such mutants except for mutants m256, m278a and m290, which are predicted to have some disturbance in the β-strand structure formation.

The present study revealed that some amino acid residues in sIL-6R are critical for the potential to mediate the IL-6 signal through gp130 but not for IL-6-binding, and that these residues are important for the association between IL-6R and gp130 in the presence of IL-6. All such residues except one are located in the membrane proximal half of the second 'barrel'. It is interesting to note that in the case of human GH-R, amino acid residues in the membrane proximal half of the COOH-terminal 'barrel' of the GH-R molecule interact with those of another GH-R molecule (De Vos *et al.*, 1992). Thus, it is possible that the residues in the membrane proximal half of the second 'barrel' of IL-6R may be

involved in the direct interaction between IL-6R and gp130 in the presence of IL-6. There are other possible mechanisms of the association of gp130 with the complex of IL-6 and IL-6R: (i) after binding of IL-6, such residues in IL-6R may cause the IL-6 molecule to be recognized by gp130, or (ii) gp130 may recognize both IL-6 and IL-6R only in a complexed form. To elucidate more exactly the molecular mechanism of the interaction of IL-6, IL-6R and gp130, it will be necessary to know the three-dimensional structures of each of the three molecules and of the complex of IL-6/IL-6R and IL-6/IL-6R/gp130.

It is known that aberrant expression of IL-6 coincides with several diseases (Kishimoto, 1989; Hirano *et al.*, 1990). In human multiple myelomas, IL-6 acts as a growth factor for myeloma cells, and addition of anti-IL-6 monoclonal antibody inhibited the growth of these cells *in vitro* and *in vivo* (Kawano *et al.*, 1988; Klein *et al.*, 1991). Therefore, if an efficient inhibitor of IL-6 is developed, it may be applied for clinical treatments of diseases caused by aberrant expression of IL-6 such as multiple myelomas. Indeed, anti-IL-6 monoclonal antibody was able to inhibit the growth of myeloma cells in a patient with multiple myeloma (Klein *et al.*, 1990, 1991). A sIL-6R mutant that retained IL-6-binding capability but does not have the potential to mediate the IL-6 signal, such as the sIL-6R mutants obtained in the present study, might be another candidate for this purpose.

Materials and methods

Deletion and site-directed mutagenesis of sIL-6R

To obtain IL-6RΔIg cDNA, pBSF2R.236 (Yamasaki *et al.*, 1988) was digested with *Apa*LI followed by digestion with S1 nuclease, and further with *Bam*HI. The resulting 2.5 kbp fragment was ligated to another 4.5 kbp fragment which has been obtained by *Fsp*I and *Bam*HI digestion of pBSF2R.236. To construct IL-6RΔFam cDNA, pBSF2R.236 was digested with *Acc*III, treated with Klenow fragment, and then digested with *Not*I. The resulting 5.7 kbp fragment was ligated to another 0.9 kbp fragment resulting from *Not*I and *Ssp*I digestion of pBSF2R.236. To construct sIL-6R mutants, sIL-6R cDNA was subcloned into M13mp18 vector from pSVL344 (Yasukawa *et al.*, 1990) and the resulting recombinant M13 single-stranded DNA was used as a template. Site-directed mutagenesis was performed using an oligonucleotide-directed *in vitro* mutagenesis system (Amersham) and synthetic oligonucleotides as primers. The sequences of these primers are available on request. The resulting sIL-6R mutant cDNAs were inserted into COS-7 expression vector pSVL (Pharmacia), and sequences of all the sIL-6R mutant cDNAs were confirmed by sequencing using Sequenase sequencing kit (USB).

Expression of IL-6R deletion mutants

COP cells were transfected with CDM8 vectors containing IL-6R, IL-6RΔIg or IL-6RΔFam cDNAs by the DEAE–dextran method as described by Yamasaki *et al.* (1988). Three days after transfection, the cells were stained with biotinylated IL-6 and FITC-conjugated avidin and analysed for transiently expressed IL-6R mutants by a FACS as described by Yamasaki *et al.* (1988). For permanent expression, 10^7 cells of murine myeloid leukaemic cell line M1 were transfected with 10 μg of CDM8 vector containing IL-6RΔIg cDNA and 1 μg of pSV2neo by electroporation and, after selection with G418, a transfectant M1IL-6RΔIg was obtained as described by Taga *et al.* (1989). M1, M1IL-6R and M1IL-6RΔIg cells (2×10^4 cells/well, 0.2 ml/well) were cultured with various concentrations of IL-6 for 64 h, then pulse-labelled with [^{3}H]thymidine (1 μCi/well, Amersham) for 8 h. The incorporated radioactivity was measured as described by Taga *et al.* (1989). The numbers of IL-6 binding sites on M1, M1IL-6R and M1IL-6RΔIg cells were estimated by Scatchard analysis as described by Taga *et al.* (1987, 1989).

Immunoprecipitation of sIL-6R mutants

COS-7 cells were transfected with the pSVL vectors containing sIL-6R mutant cDNAs as described above. Three days after transfection, the cells were labelled with [^{35}S]methionine (NEN) and each supernatant was immunoprecipitated with anti-human IL-6R receptor monoclonal antibody MT18 (Hirata *et al.*, 1989) and protein A–Sepharose 4 Fast Flow (Pharmacia). The Sepharose beads were washed with RIPA buffer (Harlow and Lane, 1988) and the immunoprecipitates were analysed by SDS–PAGE followed by autoradiography. The amounts of immunoprecipitated sIL-6R mutants were estimated by cutting out the portion of polyacrylamide gel containing each sIL-6R mutant identified in the autoradiogram and by subsequent measurement of its radioactivity with a liquid-scintillation counter (Beckman).

IL-6-binding assay

Recombinant human IL-6 (Yasukawa and Saito, 1990) was ^{125}I-labelled using Bolton–Hunter reagent (NEN) as described by Taga *et al.* (1987). The IL-6 binding capability of sIL-6R mutants was estimated using monoclonal antibody MT18-coated microtitre plates (Sumitomo) and ^{125}I-labelled IL-6 as described by Yasukawa *et al.* (1990).

NFS130 cells

NFS130 cells were obtained by transfecting murine myeloid leukaemic NFS60 cells (Weinstein *et al.*, 1986) with pZip130 (Hibi *et al.*, 1990) and by subsequent selection with G418. Expression of the human gp130 molecule on the cell surface of NFS130 cells was confirmed by FACS analysis with the anti-gp130 monoclonal antibody AM64 (Hibi *et al.*, 1990) and FITC-conjugated anti-mouse IgG polyclonal antibody. Responsiveness of NFS130 cells to sIL-6R was examined as follows. NFS130 cells (5×10^4 cells/well) were cultured in Iscove's modified Dulbecco's medium (Sigma) supplemented with fetal calf serum (10%) and various concentrations of wild-type sIL-6R in the presence of 400 ng/ml Il-6 for 15 h, then pulse-labelled with [^{3}H]thymidine (1 μCi/well) for 6 h. Cells were harvested and the incorporated radioactivity was measured with a liquid-scintillation counter (Beckman).

Biological assay of sIL-6R

The potential of wild-type or each mutant sIL-6R to mediate the IL-6 signal was assayed using NFS130 cells. 5×10^4 cells/well NFS130 cells were cultured as described above with COS-7 culture supernatant (25% v/v) containing wild-type sIL-6R or each sIL-6R mutant in the presence or absence of 400 ng/ml of IL-6 for 15 h. [^{3}H]thymidine incorporation was measured as described above.

In vitro association of sIL-6R with soluble gp130

COS-7 culture supernatant containing wild-type sIL-6R or each of some sIL-6R mutants was 30-fold concentrated using Centriprep-10 (Amicon). Partially purified soluble gp130 was prepared and ^{125}-labelled with Bolton–Hunter reagent as described by Yasukawa *et al.* (1992). ^{125}I-labelled soluble gp130 was mixed with the 30-fold concentrated COS-7 culture supernatant and 1 μg/ml of IL-6, incubated for 30 min at 37°C and immunoprecipitated with monoclonal antibody MT18 and protein A–Sepharose 4 Fast Flow (Pharmacia). The Sepharose was washed four times with 10 mM triethanolamine buffer (pH 7.5) containing 1% digitonin and 0.15 M NaCl at 4°C as described by Taga *et al.* (1989) and immunoprecipitated materials were analysed by SDS–PAGE followed by autoradiography.

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