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A RECEPTOR-BINDING PEPTIDE FROM HUMAN INTERLEUKIN-6: ISOLATION AND A PROTON NUCLEAR MAGNETIC RESONANCE STUDY

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SUMMARY: In order to locate the receptor-binding region of human interleukin-6 (IL-6), twelve peptide fragments were prepared by digestion of IL-6 with lysylendopeptidase. A significant activity of the receptor-binding was observed only for a peptide Ile88-Lys121, although the activity was estimated at 10^4-fold less than that of intact IL-6. Solution structure of the peptide Ile88-Lys121 was analyzed by using two-dimensional nuclear magnetic resonance (NMR) spectroscopy. The results indicate the presence of α-helices in the regions Leu93-Phe106 and Glu110-Ser119. On the basis of the NMR data, we also prepared two peptides. Four-fold less binding activity than that of the peptide Ile88-Lys121 was observed for the peptide Ile88-Arg105, but no activity for the peptide Glu110-Lys121. These results suggest that the helical peptide Ile88-Arg105 composes a part of the receptor-binding region.

Interleukin-6 (IL-6) is a multifunctional cytokine which regulates the growth and differentiation of various cells (1). Complementary DNA encoding human IL-6 (Mr = 21,000) has been

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ABBREVIATIONS: IL-6, interleukin-6; E.coli, Escherichia coli; NMR, nuclear magnetic resonance; RP-HPLC, reverse phase-high performance liquid chromatography; TFE, trifluoroethanol; CD, circular dichroism; DQF-COSY, double quantum filtered correlated spectroscopy; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; NOE, nuclear Overhauser effect; NOESY, 2-dimensional nuclear Overhauser effect spectroscopy.

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isolated (2) and recombinant IL-6 has been expressed at a high level in Escherichia coli (E.coli) (3). The recombinant IL-6 was indistinguishable from natural IL-6 as assessed by their biological activities (3). Only after the binding of IL-6 to its receptor on the cell surface (4), the receptor can associate with a non-ligand signal transducer, gp130 (5).

It is important to design IL-6 antagonists, because the over-production of IL-6 is linked to a variety of diseases. By uncoupling the receptor-binding and biological activities, it should be possible, in principle, to design IL-6 antagonists. However, little information has so far been obtained concerning the structure function relationship of IL-6 (6,7,8,9). It is still unclear which segments of IL-6 are actually involved in the receptor-binding.

In a previous study, on the basis of chemical modification and ^{1}H-nuclear magnetic resonance (NMR) data, we have concluded that Trp158, Met162, His165, and Met185 comprise the receptor-binding region (10). We have prepared several IL-6 mutants and suggested that hydrophobic side chains of Leu and Met residues existing in the C-terminal region are significantly involved in the receptor-binding of IL-6 (11). In the present study, we have prepared the peptide segments by digesting IL-6 protein with lysylendo-peptidase, and shown that the peptide Ile88-Lys121 also has the significant activity of the receptor-binding. On the basis of NMR data, we have discussed the secondary and tertiary structure of the peptide which composes the receptor-binding region.

MATERIALS AND METHODS

Materials: Recombinant human IL-6 was produced in E. coli and purified as described by Yasukawa et al.(12). Purity of the final preparation was checked by reverse phase-high performance liquid chromatography (RP-HPLC) and SDS-polyacrylamide gel electrophoresis.

Separation of the fragments digested with lysylendopeptidase by RP-HPLC: IL-6 (3 mg) was digested with 10 μg of lysylendopeptidase (Wako Prue Chemical) in 2 ml of 20 mM Tris-HCl buffer (pH 8.5) in the presence of 2 M urea at 30°C for 5 h. The resultant segments were separated by HPLC on an ODS-A-312 column (6 x 150 mm) (Yamamura Chemical Laboratories) with a linear gradient of 5 - 75 % acetonitrile containing 0.1 % trifluoroacetic acid (flow rate 1 ml/min). The partial amino acid sequences of the separated segments were determined using an Applied Biosystems Model 470A gas-phase protein sequencer. Each fraction was lyophilized, and dissolved in 20 mM phosphate, 120 mM NaCl (pH 7.4) for testing the receptor-binding activity or the same buffer containing 50 % trifluoroethanol (TFE) for circular dichroism (CD) and NMR measurements.

Radioisotope immunosorbent assay for testing the receptor-binding activity of the peptide fragment: The 96-well microplates were coated with anti-(IL-6 receptor) antibody (400 ng)(13). The wells were blocked with 1 % bovine serum albumin for 2 h. Soluble IL-6 receptor (10 ng) (14) was then added. After a 2-h incubation, the wells were washed, and test samples containing ^{125}I-labeled IL-6 (3,000 cpm) were incubated for 2 h. The wells were washed and the radioactivity of each well was measured with a γ-counter.

CD measurements: CD spectra were recorded at 25°C on a Jasco 600 spectrophotometer in a 0.1-cm cell. Samples were dissolved in 20 mM phosphate, 120 mM NaCl (pH 7.4) or the same buffer containing 50 % TFE. The spectra were obtained by employing blank samples containing all of the assay components. The content of the secondary structure was calculated by the method of Greenfield et al.(15).

NMR measurements: Two-dimensional ^{1}H-NMR spectra were recorded at 500 MHz on a Varian VXR-500S spectrometer with a probe temperature of 30°C in 20 mM phosphate, 120 mM NaCl (pH 7.4)/50 % H_2O containing 50 % CF_3CD_2OD. Double quantum filtered correlated spectroscopy (DQF-COSY)(16), homonuclear Hartmann-Hahn spectroscopy (HOHAHA)(17), and 2-dimensional nuclear Overhauser effect (NOE) spectroscopy (NOESY)(18) spectra were obtained in the phase-sensitive mode as described by States et al.(19). The water resonance was suppressed by selective irradiation during the relaxation delay. The 256 blocks were acquired with data points of 2048 and were zero-filled to yield a data matrix of 2K (F_1) x 2K (F_2) of the real data points. The 128 scans were accumulated for each t_1 with a relaxation delay of 1.5 s. HOHAHA and NOESY spectra were recorded with mixing times of 60 and 150 ms, respectively. For both DQF-COSY and HOHAHA specta, a phase-shifted sine bell function was applied for both t_1 and t_2 dimensions.

Synthesis of peptide fragments: Peptide fragments, Ile88-Arg105 and Glu110-Lys121, were synthesized using an Applied Biosystems Model 430A peptide synthesizer. Purification of the crude peptides was carried out by RP-HPLC. The amino acid composition of each peptide was checked. The whole amino acid sequence of each peptide was also confirmed by an Applied Biosystems Model 470A gas-phase sequencer.

RESULTS AND DISCUSSION

In order to show the receptor-binding region of human IL-6, the peptide fragments were prepared by digestion of IL-6 with

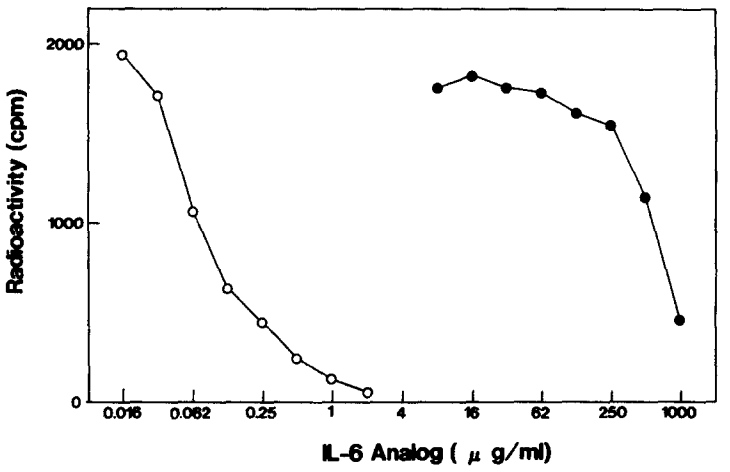

Fig. 1. The competitive binding activity to the soluble IL-6 receptor of native IL-6 (O) and the peptide Ile88-Lys121 (●). The immunosorbent assay was performed as described under MATERIALS AND METHODS. Radioactivities of ^{125}I-IL-6 binding to the soluble IL-6 receptor were measured by a γ-counter. Data represent means of triplicate assays.

lysylendopeptidase. After the separation of the peptide fragments by RP-HPLC, all 12 fragments, each of which was composed of more than five amino acid residues, were identified by peptide sequence analyses as described in a previous paper (20). The receptor-binding activities of all peptide fragments were measured by competitive inhibition of the binding of ^{125}I-IL-6 to the soluble IL-6 receptor (Fig. 1). A significant activity of the receptor-binding was observed for the peptide Ile88-Lys121, but not for the other peptide (data not shown). This result indicates that the peptide Ile88-Lys121 comprises a part of the receptor-binding region. The amount of the peptide Ile88-Lys121 required for 50 % displacement of ^{125}I-IL-6 (IC_{50}) was determined. The receptor-binding activity of the peptide Ile88-Lys121 was estimated at 10^4-fold less than that of intact IL-6, probably because the peptide fragment cannot take the same tertiary structure as the corresponding region which exists in intact IL-6.

The CD spectra of the peptide Ile88-Lys121 were measured in 20 mM phosphate, 120 mM NaCl (pH 7.4) or the same buffer containing

50 % TFE, which provides the peptide with a less polar environment. A large negative ellipticity at 208 and 222 nm was observed for the latter solution system, whereas only a small negative ellipticity was observed for the former solution (data not shown). The helical content of the peptide Ile88-Lys121 in the former and the latter buffers was estimated at about 19 and 62 %, respectively. These results strongly suggest that the hydrophobic environment provided by TFE induces the peptide to take the intact structure which exists in IL-6 molecule.

On the basis of the results of CD spectra, 2D-NMR spectra of the peptide Ile88-Lys121 were measured in 20 mM phosphate, 120 mM NaCl (pH 7.4)/50 % H_2O containing 50 % CF_3CD_2OD. Sequence-specific resonance assignments were made according to the method established by Wüthrich et al.(21). Briefly, resonances were assigned to the spin systems of specific amino acid residues using DQF-COSY and 2D-HOHAHA data. The spin systems were then aligned along the primary structure on the basis of the distance

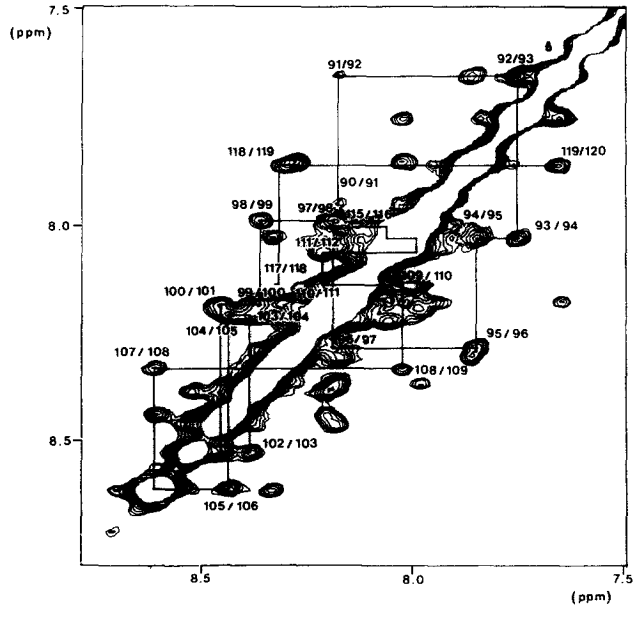

Fig. 2. d_{NN} connectivities in the NOESY spectrum of the peptide Ile88-Lys121 observed at 30°C in 20 mM phosphate, 120 mM NaCl (pH 7.4)/50 % H_2O containing 50 % CF_3CD_2OD. Recorded with a mixing time of 150 ms.

Table 1. Chemical shifts of the proton resonances of the peptide Ile88-Lys121 observed at 30°C

Residue	NH	$C_\alpha H$	$C_\beta H$	Others					
Ile88	N.D.	3.66	1.99	$C_\gamma H$	1.53 1.27	$C_\gamma H_3$	1.01	$C_\delta H_3$	0.94
Ile89	8.08	4.32	1.94	$C_\gamma H$	1.57 1.23	$C_\gamma H_3$	0.98	$C_\delta H_3$	0.90
Thr90	7.94	4.26	4.27	$C_\gamma H_3$	1.24				
Gly91	8.17	3.98 4.06							
Leu92	7.65	4.37	1.75 1.75	$C_\gamma H$	1.66	$C_\delta H_3$	1.02 0.93		
Leu93	7.75	4.21	1.70 1.70	$C_\gamma H$	1.74	$C_\delta H_3$	0.97 0.90		
Glu94	8.03	3.96	2.12 2.12	$C_\gamma H$	2.45 2.45				
Phe95	7.85	4.46	3.31 3.31	C26	7.22	C35	7.34	C4	7.32
Glu96	8.27	3.96	2.35 2.35	$C_\gamma H$	2.68 2.68				
Val97	8.19	3.68	2.19	$C_\gamma H_3$	1.08 0.96				
Tyr98	7.98	4.35	3.22 3.22	C26	7.05	C35	6.79		
Leu99	8.36	3.86	1.77 1.77	$C_\gamma H$	1.54	$C_\delta H_3$	0.78 0.75		
Glu100	8.17	3.97	2.30 2.22	$C_\gamma H$	2.54 2.54				
Tyr101	8.46	4.22	3.35 3.20	C26	7.04	C35	6.50		
Leu102	8.52	3.70	1.76 1.39	$C_\gamma H$	1.53	$C_\delta H_3$	0.80 0.76		
Gln103	8.37	4.02	2.20 2.20	$C_\gamma H$	2.53 2.34	$N_\epsilon H$	6.86 6.48		
Asn104	8.20	4.47	2.93 2.74	$N_\delta H$	7.43 6.71				
Arg105	8.43	3.98	1.74 1.74	$C_\gamma H$	1.42 1.42	$C_\delta H$	3.06 2.95	$N_\epsilon H$	6.99
Phe106	8.60	4.34	3.23 3.23	C26	7.22	C35	7.47	C4	7.38
Glu107	8.60	4.06	2.27 2.27	$C_\gamma H$	2.71 2.64				
Ser108	8.32	4.27	4.06 3.91						
Ser109	8.03	4.31	4.07 3.97						
Glu110	8.12	4.09	2.06 2.06	$C_\gamma H$	2.30 2.30				
Glu111	8.22	4.05	2.24 2.24	$C_\gamma H$	2.62 2.46				
Gln112	8.04	4.06	2.17 2.17	$C_\gamma H$	2.52 2.34	$N_\epsilon H$	6.96 6.50		
Ala113	7.99	4.08	1.50						
Arg114	8.04	4.04	1.95 1.95	$C_\gamma H$	1.81 1.69	$C_\delta H$	3.22 3.22	$N_\epsilon H$	7.20
Ala115	7.99	4.10	1.55						
Val116	8.13	3.71	2.23	$C_\gamma H_3$	1.10 0.97				
Gln117	8.18	4.05	2.30 2.30	$C_\gamma H$	2.63 2.63	$N_\epsilon H$	7.58 7.42		
Met118	8.30	4.39	2.19 2.19	$C_\gamma H$	2.74 2.64	$C_\epsilon H_3$	2.08		
Ser119	7.85	4.47	4.06 4.03						
Thr120	7.65	4.35	4.39	$C_\gamma H_3$	1.29				
Lys121	7.73	4.34	1.93 1.85	$C_\gamma H$	1.49 1.49	$C_\delta H$	1.73 1.73	$C_\epsilon H$	3.03 3.03

Chemical shifts expressed in ppm relative to DSS.
N.D., not detectable.

information obtained from the NOESY spectrum. Fig. 2 indicates the NH-NH region of the NOESY spectrum containing sequential $d_{NN}(i,i+1)$ connectivities. The $d_{NN}(i,i+1)$ NOE cross peaks were found in Thr90-Val116 and Val117-Lys121. On the basis of the $d_{\alpha N}(i,i+1)$, $d_{NN}(i,i+1)$, and $d_{\beta N}(i,i+1)$ NOE connectivities, the sequential assignments were established. The spectral assignments for the proton resonances of the peptide Ile88-Lys121 are summarized in Table 1. The interresidue NOE connectivities, $d_{\alpha N}(i,i+1)$, $d_{\beta N}(i,i+1)$, $d_{NN}(i,i+1)$, $d_{\alpha N}(i,i+3)$, $d_{\alpha\beta}(i,i+3)$, observed for the peptide Ile88-Lys121 are summarized in Fig. 3. The continuous $d_{\alpha N}(i,i+3)$ and $d_{\alpha\beta}(i,i+3)$ NOE connectivities were observed in the regions Leu93-Phe106 and Glu110-Ser119. The continuous $d_{NN}(i,i+1)$ connectivities were also observed in the same region. These results indicate the presence of α-helices in the region Leu93-Phe106 and Glu110-Ser119.

Long-range NOEs between Gln103 $C_\gamma H$ and Glu110 $C_\beta H$, between Asn104 $C_\beta H$ and Glu111 $C_\gamma H$, between Glu107 $C_\beta H$ and Glu111 $C_\gamma H$, and between Ser108 $C_\alpha H$ and Gln112 $C_\beta H$ were observed. These NOE data suggest that the peptide Ile88-Lys121 forms two α-helical rods with a kink in the region of Glu107-Ser109. The global fold of the peptide Ile88-Lys121 deduced on the basis of these results is

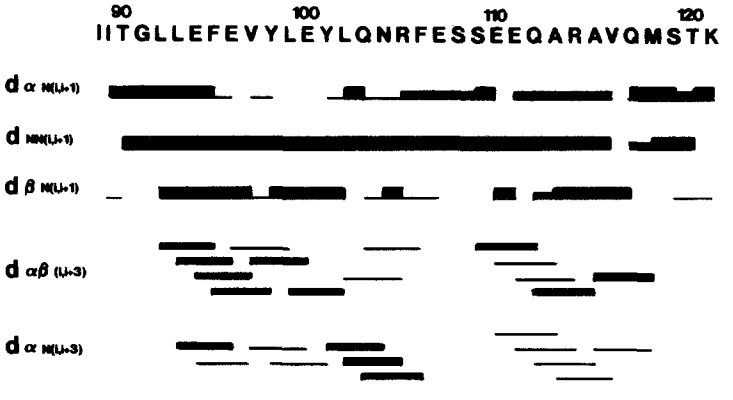

Fig. 3. The amino acid sequence of the peptide Ile88-Lys121 and summary of all the short-range NOEs. The intensities of NOEs are classified as strong, medium, and weak by the height of the bars.

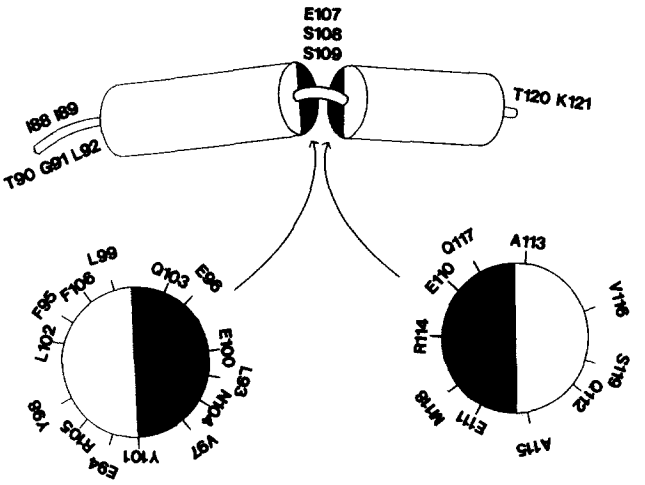

Fig. 4. Schematic drawing of the global fold of the peptide Ile88-Lys121. Tubes indicate the two helices.

schematically drawn in Fig. 4. The tertiary structure of the peptide was not determined by computer calculation, because of the small number of observed long-range NOEs.

On the basis of the NMR data, we have concluded that the peptide Ile88-Lys121 comprises two segments Leu93-Phe106 and Glu110-Ser119 which take the α-helical structure. We have

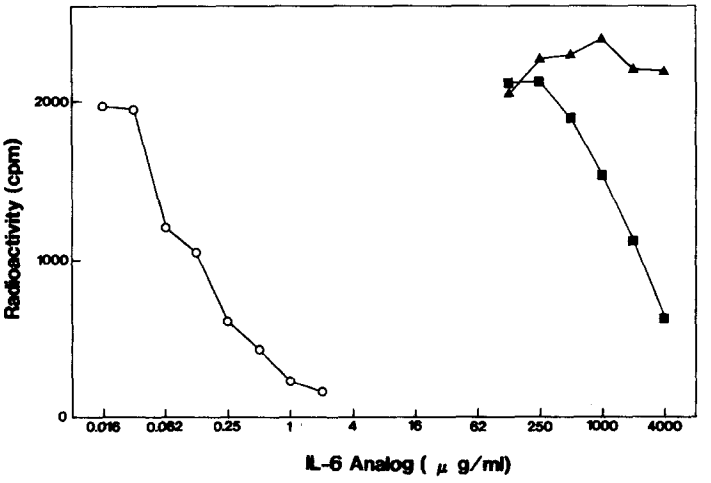

Fig. 5. The competitive binding activity to the soluble IL-6 receptor of native IL-6 (O), the peptide Ile88-Arg105 (■), and the peptide Glu110-Lys121 (▲). The immunosorbent assay was performed as described under MATERIALS AND METHODS. Radioactivities of ^{125}I-IL-6 binding to the soluble IL-6 receptor were measured by a γ-counter. Data represent means of triplicate assays.

prepared two peptide fragments, Ile88-Arg105 and Glu110-Lys121, corresponding to the two helical segments, and examined their receptor-binding activities (Fig. 5). A significant activity of the receptor-binding was observed for the peptide Ile88-Arg105. On the basis of the IC_{50} value, the activity of the peptide Ile88-Arg105 was estimated at 4-fold less than that of the peptide Ile88-Lys121. No receptor-binding activity was observed for the peptide Glu110-Lys121. These results suggest that the peptide Ile88-Arg105, which take a helical structure, comprises a part of the receptor-binding region.

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