Carbohydrate Recognition Site of Interleukin-2 in Relation to Cell Proliferation*

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Interleukin-2 (IL-2) is a cytokine with important roles in the immune system. IL-2 initially binds a high mannose-type glycan and a specific peptide sequence of the IL-2 receptor α-subunit and subsequently forms a high affinity complex of IL-2-IL-2 receptor α-, β-, and γ-subunits. This formation induces cellular signaling and cell proliferation (Fukushima, K., and Yamashita, K. (2001) J. Biol. Chem. 276, 7351-7356). To determine the carbohydrate-binding site of IL-2, we prepared wild-type and point-mutated 35S-IL-2 by an in vitro transcription and translation method. We found that wild-type 35S-IL-2 tends to form a dimer spontaneously, and the dimeric form has both carbohydrate recognition activity and cell proliferation activity. Moreover, substitution of Asn-26 in IL-2 with Gln or Asp conserved the dimeric form and affected the carbohydrate recognition activities in correspondence with the cell proliferation activities, suggesting that Asn-26 in IL-2 is involved in the carbohydrate recognition site. These results suggest that the carbohydrate recognition of IL-2 dimer triggers formation of high affinity complex (IL-2-IL-2Rα-, β-, γ), and the hetero-octamer stimulates IL-2-dependent T-cell proliferation by intensifying cellular signaling.

Interleukin-2 (IL-2)† has been widely studied as a mediator of cellular signaling in the immune system. The receptor for IL-2 (IL-2R) consists of α-, β-, and γ-subunits (IL-2Rα, β, and γ), and the intracellular portions of the β- and γ-subunits are associated with a variety of cytoplasmic proteins including tyrosine kinases Jak1 and Jak3 (1, 2). The phosphorylated cytoplasmic domain of IL-2β especially plays a critical role in attracting downstream signaling molecules including the transcription factors STAT3 and STAT5 into the activated receptor complex (1, 3). IL-2Rα, β, and γ bind to distinct sites of IL-2, and these associations appear to occur in a stepwise manner (4–8). When IL-2Rα, β, or γ was independently expressed, This is an open access article under the CC BY license.

Materials and Chemicals—Redivue TM L-[35S]methionine (1175 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Ribonuclease B (type III-B from bovine pancreas), ribonuclease A, ovalbumin, thyroglobulin, and human serum albumin were obtained from Sigma. Endo-β-N-acetylglucosaminidase H (Endo H) was obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Blue dextran 2000, chymotrypsinogen A, ribonuclease A, and bovine serum albumin (Gen Filtration LMW Calibration kit) as molecular weight markers for Superose 12 column chromatography were purchased from Amersham Pharmacia Biotech.

Preparation of rhIL-2—cDNA encoding human IL-2 (R & D Systems Europe Ltd., Abingdon, UK) was used to produce rhIL-2 in E. coli. Plasmid pET3a (Novagen Inc., Madison, WI) was used as the T7 expression plasmid. An Ndel-HindIII fragment corresponding to a synthetic human IL-2 cDNA was inserted between the Ndel and HindIII sites of pET3a to produce the expression plasmid (pET3a-IL-2). The pET3a-IL-2 was expressed in E. coli strain BL21(DE3) under the control of the T7 promoter. A 15-ml culture of E. coli BL21(DE3) containing pET3a-IL-2 was grown overnight to stationary phase and was used to inoculate 500 ml of L broth containing 100 μg/ml ampicillin. After incubation for 2.5 h at 37 °C, cells were induced to produce rhIL-2 with
0.5 mM isopropyl-β-thiogalactoside and grown for 2.5 h. rhIL-2 was mainly incorporated in inclusion bodies. Therefore, the inclusion bodies were solubilized, and rhIL-2 was refolded as follows (14). The cells were collected by centrifugation and homogenized by lysozyme treatment and sonication at 4 °C. The lysate was centrifuged at 10,000 rpm for 10 min, and the precipitate was collected. The pellet was dissolved in 20 mM Tris-HCl buffer, pH 8.3, containing 10 mM EDTA and 6 mM guanidine hydrochloride. Then the solution was treated with 10 mM reduced glutathione and 1 mM oxidized glutathione in the presence of 2 mM guanidine hydrochloride at pH 5.0. The solution was allowed to stand for 16 h at room temperature and then dialyzed against PBS. An aliquot of rhIL-2 was subjected to SDS-PAGE using 12.5% polyacrylamide gels to check the molecular weight of the biological activity of the refolded rhIL-2 was determined in a proliferation assay using CTLL-2 cells. The unit of rhIL-2 purchased from Sigma was used as a standard. The protein concentration was determined by Bio-Rad Protein Assay dye reagent using bovine serum albumin as a standard. The unit of rhIL-2 used in this study was 1–10 units/ng.

Preparation of 35S-rhIL-2—pET3a-IL-2 produced as described above was used as a template for in vitro transcription and translation in TnT®-coupled reticulocyte lysate systems (Promega Corp., Madison, WI) in the presence of [35S]methionine. To remove the endogenous mannose-binding lectins from the reagents, the reagents were mixed with a 20% slurry of ovalbumin-Sepharose (5 mg/ml) and centrifuged before the reagents were used. The in vitro transcription and translation was accomplished as described in the manufacturer’s instructions. The translation products were subjected to SDS-PAGE using 15% polyacrylamide gels and autoradiographed. An aliquot was also subjected to size-exclusion chromatography using a Superose 12 column (300 mm long, 10 mm inner diameter) and then eluted with PBS (flow rate, 0.5 ml/min) (Amersham Pharmacia Biotech). The Superose 12 column was pretreated with 100 μl of 3% human serum albumin in PBS to inhibit nonspecific adsorption. The remaining translation products were separated from free [35S]methionine using a PD-10 column (Amersham Pharmacia Biotech) with PBS and used immediately. One reaction using 1 μg of plasmid DNA template, 25 μl of lysate, and 20 μl of [35S]methionine provided reproducibly 33 ± 4.3 fmol of 35S-rhIL-2 (1.3 × 10^4 dpm/fmol). The unit of 35S-rhIL-2 was determined in a proliferation assay using CTLL-cells, as described above. The unit of 35S-rhIL-2 synthesized by in vitro transcription and translation was about 50 units/ng.

Solid-phase Binding Assay—The binding of 35S-rhIL-2 to ribonuclease B or ribonuclease A was measured by a solid-phase binding assay. Enzyme-linked immunosorbent assay plates (Corning Costar Japan, Tokyo) were coated with 100 μl of ribonuclease B or ribonuclease A at 20 μg/ml in PBS at 4 °C overnight. Ribonuclease B treated with Endo H was also used as a coated glycoprotein. For Endo H treatment, 10 μl of a reaction mixture containing 10 mg/ml ribonuclease B and 0.05 units of Endo H was mixed in 100 mM of phosphate-buffered saline, pH 5.0, was incubated at 37 °C for 18 h. The plates were washed with 0.05% Tween 20 in PBS and blocked with PBS containing 0.05% Tween 20 and 3% human serum albumin. Sequentially, the plates were treated with 2 × 10^4 dpm of 35S-rhIL-2, which corresponds to 15 fmol, in PBS containing 0.05% Tween 20 and 3% human serum albumin at 37 °C for 2 h. After washing with 0.05% Tween 20 in PBS, the bound 35S-rhIL-2 was released by treatment with 100 μl of 1% SDS. The radioactivity was measured by liquid scintillation counter.

Cell Culture—The mouse T-cell line CTLL-2 (RCB0637) was obtained from the RIKEN Cell Bank (Ibaraki, Japan). CTLL-2 cells were maintained in complete RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 100 unit/ml rhIL-2 at 37 °C under 5% CO2 atmosphere. rhIL-2 at 5 unit/ml final concentration was added every other day. Cells were cultured until the cell density reached 1.5 × 10^6 cells/ml, and the culture was then split.

Bioassay of rhIL-2—For the bioassay, 2 days after the last addition of rhIL-2, the cells were washed three times in RPMI 1640 medium. They were then resuspended in complete medium and plated out in microtiter wells at a density of 10^4 cells/well with 100 μl added per well. A series of concentrations of rhIL-2 diluted 1:2 in complete RPMI medium was then added. After the cells had been incubated at 37 °C with 5% CO2 for 2 days, 20 μl of Cell Titer 96® Aqueous solution reagent was added to each well, and the absorbance at 525 nm was read after incubation for 2 h by a dual wavelength flying spot scanning densitometer CS-9300PC (Shimadzu Corp., Kyoto). The Cell Titer 96® Aqueous solution reagent used to measure cell proliferation activity was obtained from Promega Corp. The solution was composed of a tetrazolium compound and an electron coupling reagent, phenazine ethosulfate, in Dulbecco’s phosphate-buffered saline, pH 6.0.

Oligosaccharides—Man, GlcNAc, Asn (M5) and Man, GlcNAc, Asn (M6) were prepared by exhaustive Pronase digestion of ovalbumin followed by Dowex 50 × 2 (H+ form) column chromatography (20, 21). 300 μg of ovalbumin was digested with Pronase (10 mg/ml) for 2 h at 37 °C in 0.1 M phosphate-buffered saline, pH 6.0, and the digests were chromatographed on a DEAE-Ag 1-0.5 (Bio-Rad, USA) column (20, 21). The fractions containing M5 and M6 were collected. The carbohydrate-binding site in IL-2, we prepared wild-type and several mutant forms of 35S-rhIL-2, and in vitro transcription and translation. In this paper, rhIL-2 indicates IL-2 produced by E. coli, and 35S-rhIL-2 indicates 35S-IL-2 synthesized in vitro. First of all, we studied whether 35S-rhIL-2 can be used as a core to investigate the carbohydrate-binding site in IL-2, we prepared wild-type and several mutant forms of 35S-rhIL-2 by an in vitro transcription and translation method. The expression vectors pET3a-rhIL-2 were prepared by a two-step PCR method, as described by Mikaelian and Sergeant (19). The following primers were used: 5′-TAATACGACTCACTATAGGG (common I); 5′-GCTAGTTATGGCTCACGGG (common II); 5′-CTGACTCAGATCTCTCCACG (common III); 5′-CTGCAATTGGGACCATGCTG (for E15D); 5′-CTCATCTGGTGGACCATGCGATG (for L21D); 5′-ATGATTCTGGACGGCATCAAT (for N26D); 5′-ATGATTCTGGACGGCATCAAT (for G27A); 5′-GCCATCAATCAAGAAC (for N26Q); and 5′-AACGGCCGTCGAGCAATTT (for E67Q). Primers specific to each fragment containing the desired mutation. Two primers were used as a pair in one reaction, and common II and mutant-specific primer were used in a separate reaction in the first round of PCR. In the second round of PCR, the resulting two individual fragments were purified and mixed and then amplified using common I and common II primers. The fragments obtained were then digested with NdeI and HindIII and ligated into pET3a. The primary structure of all PCR-derived inserts was verified by DNA sequencing. After DNA sequencing of each mutant, [35S]methionine-labeled mutant IL-2 was prepared in the same way as wild-type IL-2.

RESULTS

Molecular Forms of rhIL-2—In order to investigate the carbohydrate-binding site in IL-2, we prepared wild-type and several mutant forms of 35S-rhIL-2 by an in vitro transcription and translation method. In this paper, rhIL-2 indicates IL-2 produced in E. coli, and 35S-rhIL-2 indicates 35S-IL-2 synthesized in vitro. First of all, we studied whether 35S-rhIL-2 can be used to measure the carbohydrate binding activity and CTLL-2 cell proliferation activity in comparison with rhIL-2. The molecular forms of rhIL-2 having cell proliferating activity were separated by Superose 12 column chromatography. The elution patterns of rhIL-2 (4 unit/ng) showed several molecular forms (Fig. 1A). Since the ratio of each molecular form was different in each sample, we showed one typical pattern. However, the CTLL-2 proliferation activities in the respective fractions of rhIL-2 were detected at the elution position corresponding to 30 kDa (Fig. 1B). On the other hand, when 35S-rhIL-2 was immediately size-fractionated by Superose 12 column chromatography after the excess [35S]methionine had been removed on a PD-10 column; ~80% of 35S-rhIL-2 was reproducibly eluted at the elution volume corresponding to 30 kDa, and the remaining 20% was eluted at the elution volume corresponding to 60 kDa (Fig. 1C). However, the 35S-rhIL-2 corresponding to 60 kDa increased with time (data not shown). Only the fraction corresponding to 30 kDa showed cell proliferation activity (Fig. 1D), and the specific activity was 50 units/ng (see “Experimental Procedures”). Since the molecular mass of 35S-rhIL-2 is...
calculated as 15,030 Da on the basis of the amino acid sequence (20). 35S-rhIL-2 spontaneously forms a dimeric structure in the PBS buffer. These results showed that this in vitro translated method is useful for studying the relationship between carbohydrate recognition activity and the cell proliferation activity of IL-2 muteins.

Conditions for the Solid-phase Binding Assay of 35S-rhIL-2—We established a direct analytical method to investigate the precise carbohydrate binding specificity of IL-2, because the inhibitory concentrations of carbohydrates determined by solid-phase enzyme-linked immunosorbent assay method was dependent on the activities of the antibodies used (data not shown). 35S-rhIL-2 was translated in vitro in the presence of [35S]methionine and then was separated from excess [35S]methionine by PD-10 column chromatography (see "Experimental Procedures"). After the preparation, 35S-rhIL-2 was immediately used for the direct solid-phase binding assay. To select the glycoprotein to be immobilized on the plates, a preliminary experiment was performed using ovalbumin (15), porcine tryptase (25 kDa) (7), chymotrypsinogen (22), ovalbumin (43 kDa) (21), and bovine serum albumin (67 kDa) (22), with each well and allowed to stand for 2 h at 37°C. The plates were then washed with 0.05% Tween 20 in PBS, and the radioactivity of bound 35S-rhIL-2 was measured after being released with 1% SDS. As shown in Fig. 2A ( ), the binding of 35S-rhIL-2 to ribonuclease B-coated plates was concentration-dependent up to 4 × 10^5 dpm/ml (30 pM). In contrast, 35S-rhIL-2 did not bind to ribonuclease A-coated plates, although ribonuclease A has the same amino acid sequence as ribonuclease B without any N-glycan (Fig. 2A, ). The same results were obtained using Endo H-treated ribonuclease B-coated plates (Fig. 2A, ). These results suggested that the binding of 35S-rhIL-2 to immobilized ribonuclease B occurs via Man6GlcNAc2 or Man9GlcNAc2, which are linked to ribonuclease B (22).
Inhibitory Effects of Various High Mannose-type Glycans on the Carbohydrate Binding Activity of 35S-rhIL-2—It had been already reported that IL-2 interacts with Man$_n$GlcNAc$_2$ and Man$_n$GlcNAc$_2$Asn (13). However, it has remained unclear how the sugar chain structures of the reducing terminal side affect the carbohydrate-binding ability. We examined the inhibitory effects of various high mannose-type glycans (see Table I) on the binding of 35S-rhIL-2 to ribonuclease B-coated plates. As shown in Fig. 2B, M5 and M6 showed inhibitory effects, whereas M7, M8, M9, and M3 did not. As summarized in Table I, in the case of M5 or M6, the 50% inhibition concentration was estimated to be 0.2 μM, whereas in the case of M7, M8, M9, or M3, it was greater than 1 mM. Since Man$_n$GlcNAc$_2$Asn, Man$_n$GlcNAc$_2$, and Man$_n$GlcNAc showed the same inhibitory effects, with respect to the structural requirement of the reducing terminal end, neither asparagine nor N, N'-diacetylcysteine structure was required, but Man$_n$GlcNAc$_2$OH did not show any inhibitory effect. These results indicate that two non-substituted α-mannosyl residues linked to the α₁-δ side of the tri-mannosyl core and reducing terminal 4-O-β-mannosyl)-N-acetylglucosaminyl pyranoside are required for the inhibitory activity.

Effects of Point Mutations of Asn-26 on the Carbohydrate Recognition Activity of IL-2—We tried to produce mutant forms of in vitro translated 35S-rhIL-2 with different levels of carbohydrate binding activities to directly determine whether the carbohydrate recognition activity of IL-2 is co-related to IL-2-induced cell proliferation activity. It has been reported by Sherblom et al. (13) that the amino-terminal portion of IL-2 exhibits a limited degree of sequence homology with human mannose-binding protein, MBP(H) (23), and two rat liver mannose-binding proteins, MBP(A) and MBP(C) (24). Glu-15, Asn-26, Gly-27, Asn-30, Cys-58, and Glu-67 are the amino acids conserved among MBP(H), MBP(A), MBP(C), and IL-2. His-16 and Leu-19 are common between MBP(H) and IL-2, whereas Leu-21 is conserved among MBP(C), MBP(H), and IL-2 (24). Moreover, it is confirmed that Asp-20 is the binding site for IL-2Rγ; Glu-126 is the binding site for IL-2Rγ, and Lys-35, Arg-38, Phe-42, and Lys-43 are the binding sites for IL-2Ra (8). On the basis of these data, we introduced point mutations at Glu-15, Leu-19, Leu-21, Asn-26, Gly-27, Asn-30, Cys-58, and Glu-67 into IL-2 cDNA by the PCR method (see “Experimental Procedures”), and we synthesized 35S-rhIL-2 muteins in vitro in the presence of 35S-methionine. When the respective products were developed on SDS-PAGE and visualized by autoradiography, all of them showed single bands corresponding to 16 kDa (data not shown), and the wild type of 35S-rhIL-2 and eight mutant forms of 35S-rhIL-2 were equally labeled with five 35S-methionine residues per molecule. Among these eight mutant forms, three 35S-labeled mutants showed similar yields of dimeric forms as the wild type on Superose 12 column chromatography, as summarized in Fig. 3 and Table II. IL-2 analogues E15D, L19D, L21D, G27A, and N30D which mostly could not construct dimeric forms had no carbohydrate recognition activities and cell proliferation activities up to 50 μM (Fig. 4, A, *, B, †, C, ‡, and E). The carbohydrate recognition activities of these IL-2 analogues with conserved dimeric forms were assayed by a ribonuclease B-coated plate method. Although mutated E67Q showed the same binding activity as the wild type (Fig. 4, A, ‡), mutated N26D showed higher carbohydrate-binding ability (Fig. 4A, ◊) compared with wild-type IL-2 (Fig. 4A, †), whereas mutated N26D showed lower carbohydrate binding activity (Fig. 4A, †). The carbohydrate binding specificities of N26D and N26Q seemed not to be altered in comparison with that of wild-type IL-2, because the binding activities of N26D and N26Q activity on the ribonuclease B-coated plates were inhibited by M5 or M6, whereas M7, M8, or M9 did not show inhibitory effect as summarized in Table III. The 50% inhibition concentration with M5 or M6 was 0.2 μM, whereas those of M7, M8, or M9 were greater than 1 mM. Since the side chain of Asn-26 is directed toward the outside of the α-helix structure as calculated on the basis of x-ray crystallography of IL-2 (25) (Fig. 5A), even if Asn-26 is replaced by Asp or Glu as a result of a point mutation, it is thought that the three-dimensional structure of these IL-2 molecules would not be affected. These results suggested that Asn-26 is involved in the carbohydrate binding of IL-2.

Effects of Point Mutation of Asn-26 on the Induction of IL-2-dependent Cell Proliferation—We also investigated whether the mutated forms of IL-2 showed any change in effectiveness to induce T-cell proliferation, as compared with wild-type IL-2. As shown in a previous paper (12), CTLL-2 cells, a mouse T-cell line, proliferate in a manner dependent on IL-2. Upon incubation (1 × 10$^4$ cells/well) in the presence of in vitro translated 35S-rhIL-2 for 48 h, the cells showed a proliferative response that was dependent on the concentration of 35S-rhIL-2. The extent of cell proliferation was determined colorimetrically (see “Experimental Procedures”). The mutated E67Q had the same effect as the wild type of IL-2 on the T-cell proliferation activity (Fig. 4B, ‡), whereas the mutated N26Q showed higher effectiveness in inducing T-cell proliferation, as well as higher carbohydrate binding activity. The concentration at which 50% of the maximal proliferative response was observed in the case of the mutated N26Q (0.8 μM) (Fig. 4B, ◊) was one-tenth that of the wild-type (7 μM) (Fig. 4B, †). In contrast, the mutated N26D showed lower effectiveness in inducing T-cell prolifera-
tion, as well as lower carbohydrate binding activity. The concentration at which 50% of the maximal proliferative response was observed (21 pM) (Fig. 4B) was three times that of wild-type IL-2. Furthermore, N26D- or N26Q-inducible cell proliferation activity was inhibited by the preincubation with M5 (data not shown). These results suggest that Asn-26 is located within the carbohydrate recognition site of IL-2, and Asn-26 and the specific amino acids Lys-35, Arg-38, Phe-42, and Lys-43 in IL-2 as shown in Fig. 5A bind both Man5GlcNAc2 and a specific peptide in IL-2R/H9251, respectively.

**DISCUSSION**

We reported in a previous paper that the carbohydrate recognition of IL-2 is essential for expression of its physiological function (12). In this study, we prepared several mutant forms of IL-2 based on the homology with three mannose-binding proteins (13), and we compared the proliferation activities and carbohydrate recognition activities among them. We clearly demonstrated that the carbohydrate recognition site in IL-2 involves Asn-26. Before accomplishing this study, we needed to characterize the molecular forms of IL-2, which were synthesized by an in vitro translation method. As shown in Fig. 1, rhIL-2 produced in E. coli showed several molecular forms from polymer to monomer on Superose 12 column chromatography, although the dimeric form of IL-2 exclusively has the cell proliferation activity. Since rhIL-2 produced in E. coli is incorporated into inclusion bodies, the denaturing and refolding is necessary. These treatments might cause the oligomerization of IL-2. Freezing and thawing also caused oligomerization (data not shown). In contrast, 35S-labeled rhIL-2 prepared by an in vitro transcription and translation method in the presence of [35S]methionine reproducibly yielded the dimeric form, which has the same cell proliferation activity as the dimeric

![Fig. 3. Size fractionation of wild-type and mutant forms of 35S-rhIL-2.](image)

**TABLE II**

| Protein | Yield of dimer | Ratioa |
|---------|---------------|--------|
|         | (dpm)         | %      |
| IL-2    | 1.21 × 10^6   | 100    |
| E15D    | 2.12 × 10^6   | 17.0   |
| L19D    | 9.50 × 10^4   | 7.9    |
| L21D    | 0             | 0      |
| N26D    | 1.25 × 10^6   | 103    |
| N26Q    | 1.19 × 10^6   | 98     |
| G27A    | 0             | 0      |
| N30D    | 1.81 × 10^6   | 14.9   |
| E67Q    | 1.30 × 10^6   | 107    |

a% ratio was calculated as (dimer of mutated IL-2 (dpm))/dimer of wild-type IL-2 (dpm).

b Standard deviations were calculated from five independent experiments.

![Fig. 4. Comparison between wild-type and mutant forms of IL-2 with respect to binding to ribonuclease B-coated plates (A) and inducing CTLL-2 cell proliferation (B).](image)
As reported previously (13), IL-2 is a helical cytokine (26), it should hold a helical structure to retain its physiological activity. However, most of the muteins could not conserve the dimeric form which exclusively has the cell proliferation activity. Interestingly, although the muteins at Asn-26 could retain the dimeric form, their carbohydrate binding activities were affected and that of N26Q was increased, whereas that of N26D was decreased. Accordingly, Asn-26 was considered to be involved in the carbohydrate recognition site. When a theoretical model of IL-2 attached to IL-2Rα, -β, and -γ was constructed on the basis of the structural model in the Rutgers University Protein Data base (27), which was obtained by both x-ray crystallographic and NMR studies, the position of Asn-26 and the positions of the residues involved in binding each of the receptor subunits were found to be close but not to be overlapped (Fig. 5B). This model also supports the view that IL-2 recognizes both the IL-2 receptor subunits and high mannose-type glycans and that binding to these ligands is necessary for expression of IL-2-induced cellular signal transduction. When the high affinity complex of IL-2Rα, -β, and -γ was formed via the IL-2 carbohydrate recognition site including Asn-26, intracellular signal transduction might be stimulated. IL-2Rα of mouse CTLL-2 cells has three potential N-glycosylation sites, Asn-33, Asn-43, and Asn-200 (28), and human IL-2Rα has two potential N-glycosylation sites, Asn-70 and Asn-89 (29), where high mannose-type glycan also binds. The question which carbohydrate at Asn-33, Asn-43, and/or Asn-200 in murine IL-2Rα contributes to the binding of IL-2 should be resolved in the near future.

We showed in a previous paper (12) that the formation of IL-2-IL-2Rα complex triggers the formation of a high affinity complex that consists of IL-2, IL-2Rα, -β, -γ, and other tyrosine kinases. The cell proliferation activity and carbohydrate recognition activity of IL-2 could be seen mostly in the dimeric form, which was separated on Superose 12 column chromatography. Since co-crystallized IL-2 and IL-2Rα were constructed by a ratio of 1:1 by x-ray crystallography (30), it was considered that as soon as the hetero-octamer consisting of (IL-2-IL-2Rα)-β, -γ was tightly formed, all the tyrosine kinases linked to the cytoplasmic domains of IL-2Rβ and -γ are immediately phosphorylated, and cellular signaling might be effectively intensified.

Several types of the binding mechanisms for cytokines and their receptors have been reported. First, growth hormone (31) and erythropoietin (32) were considered to form a 1:2 heterotrimeric complex with their receptors based on x-ray crystallographic analysis. Second, granulocyte colony-stimulating factor (33), basic fibroblast growth factor (34), and midkine (35) form a 2:2 heterotetramer complex with their receptors, and IL-6, IL-6 receptor α-subunit, and gp130 form a 2:2:2 hetero-

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**TABLE III**

Inhibition of mutated 35S-rhIL-2 binding to plates coated with ribonuclease B by oligomannosyl derivatives

| Compounds | Concentration for 50% inhibition |
|-----------|----------------------------------|
|           | N26Q | N26D |
| M5        | 0.2 μM | 0.2 μM |
| M6        | >1 mM | >1 mM |
| M7        | >1 mM | >1 mM |
| M8        | >1 mM | >1 mM |
| M9        | >1 mM | >1 mM |

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hexamer complex (36). It should be further confirmed by x-ray crystallography or NMR analysis whether IL-2 and IL-2Rα-β, and γ form a 2:2:2:2 hetero-octamer complex as suggested in this study.

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