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The Use of Proteolysis and Direct N-terminal Sequence Analysis to Study Human Interleukin-2/Receptor Interaction on Solid Support

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An immobilized interleukin-2 receptor which is capable of binding interleukin-2 and suitable for direct N-terminal sequence analysis was employed to study interleukin-2/receptor interactions. Sensitive tryptic sites on the immobilized receptor and its interleukin-2 complex were identified by sequence analyses and compared. The results have revealed that the N-terminal region of interleukin-2 is not involved in receptor binding and the peptide segment covering residues 36-39 in the receptor is probably near or involved in the interleukin-2 binding site. The rapidity and simplicity make this solid phase sequence approach a good method for analyzing interleukin-2/receptor interaction and may be suitable for studying other protein-ligand interactions.

The interaction of interleukin-2 (IL-2) and its receptor (IL-2R) is a key regulatory event during an immune response (1,2). An understanding of the molecular interaction between the two protein molecules should aid in designing an effective IL-2 antagonist. In this communication, we report the development of a novel approach to study this system. It was based on the finding that a covalently immobilized human recombinant IL-2R, which was prepared originally for purification of recombinant human IL-2, is also suitable for direct N-terminal sequence analysis. In conjunction with tryptic digestion, the unique properties of this immobilized protein (capable of binding IL-2 and suitable for direct sequence analysis) have allowed us to obtain some detailed structural information of the IL-2/IL-2R complex. This immobilized receptor was prepared by coupling a soluble

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Abbreviations:

IL-2, recombinant human interleukin-2; IL-2R, recombinant soluble human interleukin-2 receptor; SiIL-2R, covalently immobilized IL-2R on the derivatized silica gel; SiIL-2R·IL-2, binary complex of SiIL-2R and IL-2; T[SiIL-2R], tryptic digestion products of SiIL-2R; T[SiIL-2R·IL-2], tryptic digestion products of SiIL-2R·IL-2; QA, quarternary ammonium; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid.

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recombinant form of the Tac protein, a human IL-2 receptor, onto the N-hydroxysuccinimide derivative of poly-hydroxy silica gel (3). Sensitive tryptic cleavage sites on the immobilized IL-2R (SiIL-2R) and its IL-2 complex (SiIL-2R·IL-2) were identified by sequence analysis. By comparing the two cleavage patterns, we were able to deduce the regions that are possibly involved in the interaction between the two molecules, which are consistent with the results obtained from site-directed mutagenesis. This approach is rapid and direct, and may be useful for studying other protein-ligand interactions.

MATERIALS AND METHODS

Recombinant IL-2R was purified by previously published procedures (3). Covalently immobilized IL-2R was prepared by coupling purified IL-2R on NuGel P-AF Poly-N-hydroxysuccinimide (PNHS, 500 A, 50 um, Separation Industries, Metuchen, NJ) as previously described (4). Recombinant IL-2 was purified by either affinity chromatography (4) or by a conventional procedure using HPLC (F. R. Khan, et al., unpublished results).

To prepare SiIL-2R·IL-2, SiIL-2R was suspended in phosphate buffered saline (PBS) with agitation in the presence of a large excess of IL-2 at room temperature for 20-30 min. The supernatant containing unadsorbed IL-2 was then discarded, and the IL-2 saturated gel was rinsed extensively with PBS. Tryptic treatments of SiIL-2R and SiIL-2R·IL-2 were carried out in 0.2 M ammonium bicarbonate (pH 8) with an IL-2R to trypsin ratio of 100 to 1 (w/w) for 17 hours under constant agitation. At the end of digestion, the supernatant containing residual trypsin and released peptide fragments was discarded. After extensive wash with PBS, the digestion products retained on the silica support, designated as T[SiIL-2R] and T[SiIL-2R·IL-2], were stored at 4°C before subjecting to sequence analysis.

Sequence analysis was performed using an Applied Biosystems gas phase sequencer (ABI, model 470A) (5). Each silica gel sample (10-20 microliters) containing 200 - 300 pmoles of SiIL-2R was loaded onto a quaternary ammonium (QA) treated glass fiber filter (6). Before sequencing, the gel was washed extensively with 0.2 M ammonium bicarbonate solution which was absorbed by a piece of tissue paper placed beneath the filter. The original sequencer program supplied by the manufacturer was used without modification. Since the primary structures of IL-2R and IL-2 are known, no efforts were made in this study to improve the sequencer efficiency. N-terminal sequence analyses were merely used for identification of newly generated cleavage sites. Phenylthiohydantoin (PTH) amino acid derivatives were identified "on-line" with an ABI model 120A PTH analyzer. Chromatograms of PTH amino acid analyses were collected, stored, and analyzed with an IBM computer using TURBOCHROM software (PE-Nelson, Cupertino, CA). Multiple sequences were assigned by comparing with the known sequences of IL-2 and IL-2R and confirmed when necessary by using the ISEARCH AMB program to search the sequences in the protein data base of the protein identification resource (PIR) (7).

RESULTS AND DISCUSSION

Suitability of immobilized IL-2R for sequence analysis: The first ten PTH amino acid chromatograms obtained from the sequence analyses of SiIL-2R and SiIL-2R·IL-2 are given in Fig. 1. The sequence results are also summarized in the first two columns in Table 1. The expected single sequence of IL-2R

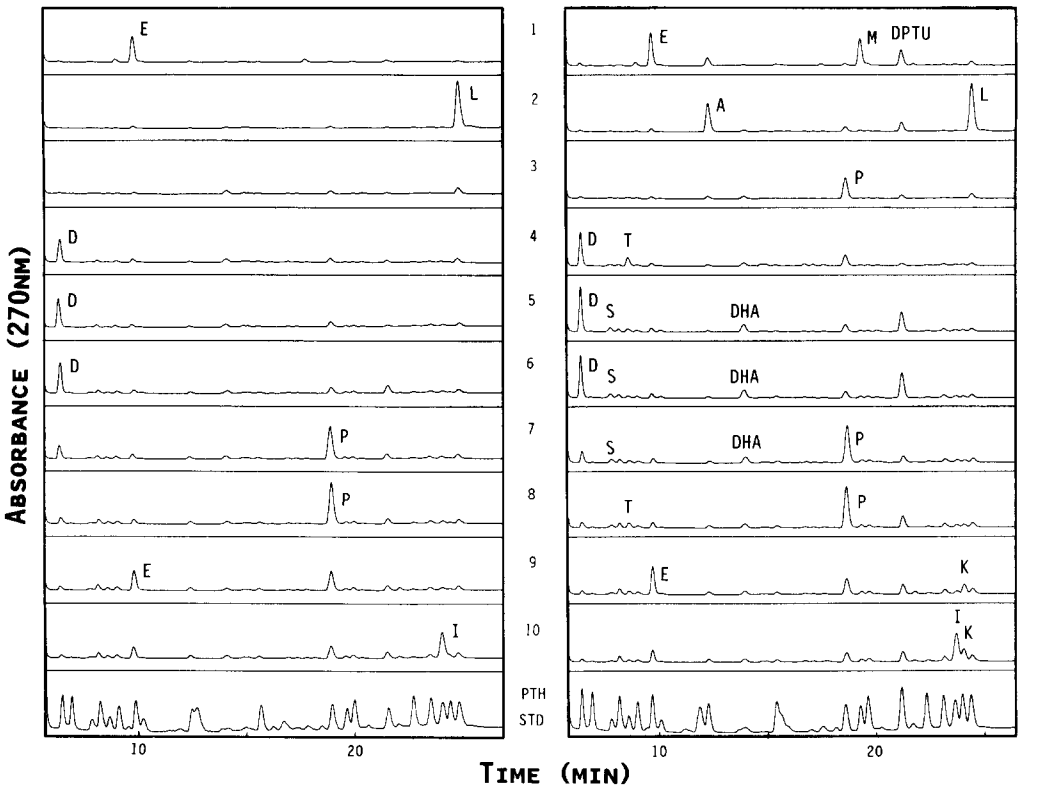

Fig. 1. Sequence analyses of SiIL-2R (left) and SiIL-2R·IL-2 complex (right). The first ten chromatograms are shown with the identified PTH amino acids marked. The sequence yields of various PTH amino acids are summarized in Table 1.

and double sequences of IL-2R and IL-2 were identified from the two samples, respectively. During sequencing IL-2 was dissociated from SiIL-2R·IL-2 when exposed to the cleavage reagent, trifluoroacetic acid (TFA). However, the disassociated IL-2 was retained by the QA-glass fiber filter. SiIL-2R was sequenced with a repetitive yield of 97% (for Glu^1 - Glu^9) and IL-2 at 90% (for Thr^3 - Thr^7). The fact that no interference in sequencer operation was encountered and no additional background peaks were detected in the PTH amino acid chromatograms suggested that the silica beads can tolerate the reagents and solvents used in the gas-phase sequencer.

The advantages of using covalently immobilized protein to study protein-protein (or ligand) interactions are its rapidity and simplicity. After extensive washing to remove unbound protein and peptide species, the complex retained by the silica support is ready for analysis without any further manipulation. An added advantage of this solid-phase approach is that the immobilized complex and its tryptic products (see below) are stable at alkaline pH when stored at 4°C.

Table 1. N-Terminal Sequence Analyses of SiIL-2R and SiIL-2R·IL-2 with and without Tryptic Treatment

N-terminal Sequences[+]	Tryptic Sites[++]	Sequence Yields (pmol)[+++]			
		SiIL-2R	SiIL-2R·IL-2	T[SiIL-2R]	T[SiIL-2R·IL-2]
From IL-2R					
E^{1}-L-C-D-D-D-		100-273-?-91-118-127-	100-215- ?-101-137-129-	100-267 - ? -110 -126 -116-	100 -307 - ? -98 -103
I^{37}-K-S-G-S-L-	R^{36}			59- 32 - ? - 40 - ? - 40-	
S^{39}-G-S-L-Y-M-	K^{38}			?- 86 - ? -110 - 50 - 85-	
Q^{72}-V-T-P-Q-P-	K^{71}			15- 19 -145*- 48 - 51*- 48-	18 - 6 -132*-51 -72*
K^{84}-T-T-E-M-Q-	R^{83}			100-130*- * -117*- 96 - 66-	137*-137*- * -92*-69
T^{85}-T-E-M-Q-S-	K^{84}			21- * - 28 - 28 - * - ?-	56*- * - 35 -22 - *
G^{141}-P-A-E-S-V-	R^{140}			48- 31 - 51 - * - ? - 20-	37 - 46 - 54*- * - ?
From IL-2					
M^{0}-A-P-T-S-S-			103-133-92- 63- ? - ? -		34 - 49 - * -33 - ?
K^{9}-T-Q-L-Q-L-	K^{8}				* - * - 32 -55 - *
T^{10}-Q-L-Q-L-E-	K^{9}				* - 29 - 58 -35 -53

[+] The positions of the first amino acid residues in different sequences are indicated by superscripts.

[++] The cleavages occured at the C-terminal sides of amino acid lysine or arginine

[+++] The sequence yields of different amino acids are reported in pmoles after normalization of PTH Glu^{1} in SiIL-2R to 100 pmol. Since the sequence yields vary among different PTH amino acids, the yields presented in this table do not represent the absolute cleavage efficiencies.

? The yields of PTH-Cys and PTH-Ser could not be calculated quantitatively.

* When a given amino acid was detected from more than one peptide segment in the same sequence cycle, the individual yield could not be distinguished. It is reported with the residue which appears first in the table.

Identification of sensitive tryptic sites on SiIL-2R and SiIL-2R·IL-2 by N-terminal sequencing and the localization of potential interaction sites in IL-2R/IL-2 complex: Limited proteolysis has been used to monitor conformational changes in various proteins (8). Potential cleavage sites in the extended polypeptide chain which normally are available to proteolytic attack, may be protected when involved in higher-order structure and/or in its ligand complex. In conjunction with sequence analysis for the identification of sensitive cleavage sites, this approach was extended to study the interaction between SiIL-2R and IL-2. The binary complex of SiIL-2R and IL-2 (SiIL-2R·IL-2) was stable during tryptic digestion, with the exception of several sensitive sites which were identified by direct N-terminal sequence anlaysis. For comparison, tryptic treated SiIL-2R (T[SiIL-2R]) was also subjected to sequence analysis.

The first six PTH amino acid chromatograms obtained from the sequence analyses of T[SiIL-2R·IL-2] and T[SiIL-2R] are shown in Fig. 2. The results are summarized in Table 1, and the new sites detected on

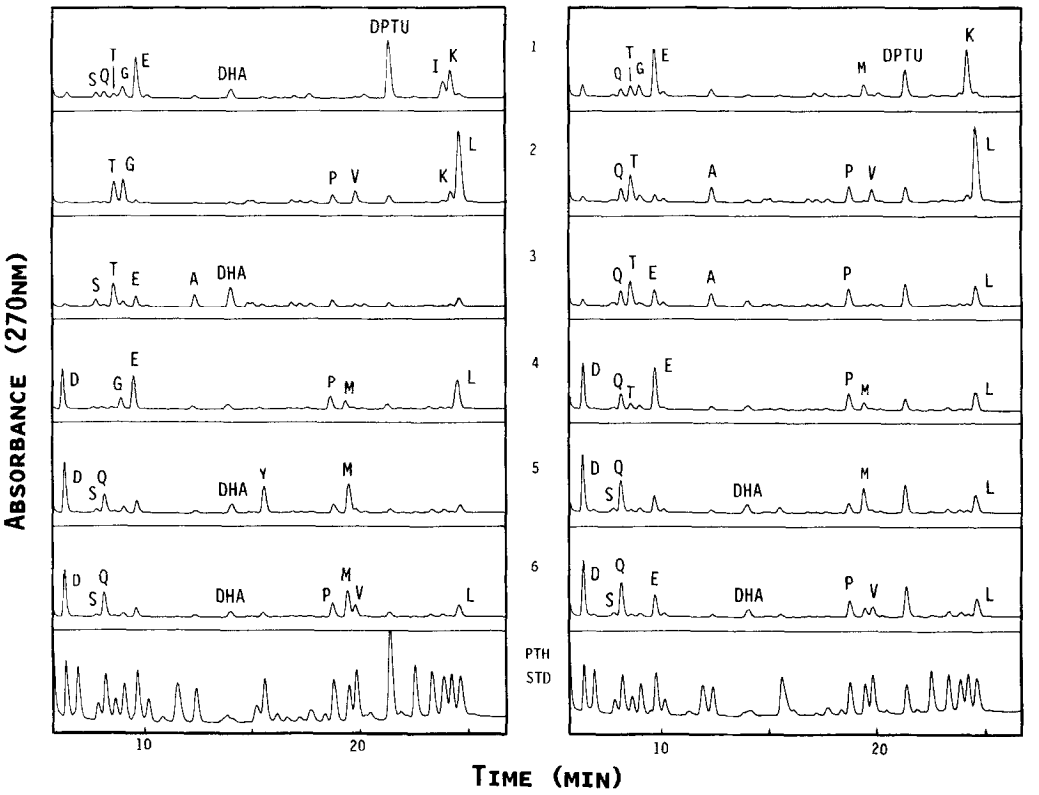

Fig. 2. Sequence analyses of T[SiIL-2R] (left) and T[SiIL-2R·IL-2] (right). The first six PTH chromatograms are shown with the identified PTH amino acids marked. The sequence yields of various PTH amino acids are summarized in Table 1.

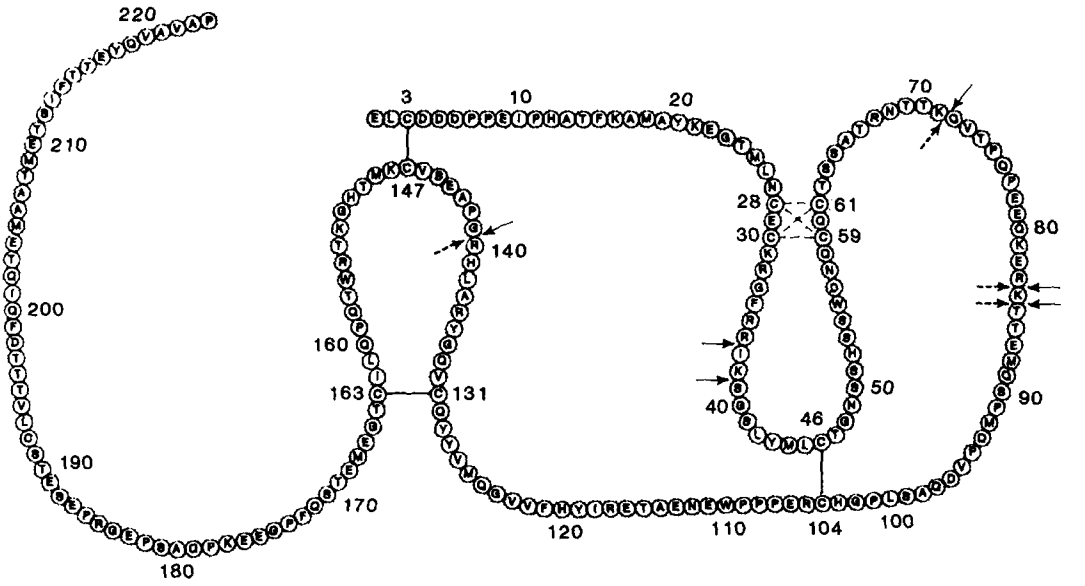

Fig. 3. Structural model of IL-2R and the locations of the sensitive tryptic cleavage sites in T[SiIL-2R] (⟶) and T[SiIL-2R·IL-2] (- - ➝).

SiIL-2R·IL-2 and SiIL-2R are also marked on the structural model of IL-2R in Fig. 3. IL-2 in SiIL-2R·IL-2 was found to be extremely stable against tryptic digestion as compared with the same treatment of free IL-2 in solution. Only two tryptic cleavage sites (Lys^8 and Lys^9) were detected from IL-2 in the complex after overnight digestion (Table 1). As a result, the sequence yield of $Met^{0,1}$ in T[SiIL-2R·IL-2] was found to be only 25% of that found in the SiIL-2R·IL-2. These results revealed that the first 2 tryptic peptides (covering residues 0-8 and 0-9) in IL-2 can be released preferentially from the complex without effecting IL-2 binding. This observation supports the results obtained by site-directed mutagenesis of IL-2 that the segment covering the first ten N-terminal amino acid residues in IL-2 is not essential for IL-2R binding (9).

Four out of six sensitive sites located in T[SiIL-2R] were also identified in T[SiIL-2R·IL-2], and two sites, Arg^{36} and Lys^{38}, were unique in T[SiIL-2R]. The identification of the four common sites with nearly identical sequence yields (Table 1) suggested that they were not protected by IL-2 in the complex and therefore were not involved in or near the interaction site between the two molecules. On the other hand, Arg^{36} and Lys^{38} in T[SiIL-2R·IL-2] were protected from being cleaved

[1] About 95% of the peptide chain in IL-2 was found to contain a N-terminal methionine which was not removed during the post-ribosomal processing. Since this methionine is not detected in the natural IL-2, it was designated by Met^{O}.

by trypsin, suggesting that they were either involved in IL-2 binding or near the site of interaction. The result is consistent with the finding by site-directed mutagenesis of IL-2R in which amino acid substitutions in the segment covering 35-43 severely affect the binding of IL-2 (10). However, it should be noted that Arg^{35} and Arg^{36} were found to be sensitive to tryptic digestion in solution but had no apparent effect on IL-2 binding (11; and Pan et al., unpublished results) and thus ruled out the involvement of Arg^{36} in IL-2 binding. The role of Lys^{38} in interleukin-2/receptor interaction is currently under further investigation.

In conclusion, this investigation has demonstrated that protein covalently immobilized on the N-hydroxysuccinimide derivative of poly-hydroxy silica gel is suitable for N-terminal sequence analysis and may be applicable for studying other protein-ligand interactions as the one described here.

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REFERENCES

1. Morgan, D. A., Ruscetti, F. W., and Gallo, R. C. (1976) Science 193, 1007-1008.
2. Robb, R. J., Munck, A., and Smith, K. A. (1981) J. Exp. Med. 154, 1455-1474.
3. Weber, D. V., Keeney, R. F., Familletti, P. C., and Bailon, P. (1988) J. Chrom. (Biomedical Applications), 431, 55-63.
4. Bailon, P., Weber, D. V., Keeney, R. F., Fredericks, J. E., Smith, C., Familletti, P. C. and Smart, J. E. (1987) Bio/Technology 5, 1195-1198.
5. Hewick, R. M., Hunkapiller, M. W., Hood, L. and Dreyer, W. J. (1981) J. Biol. Chem. 256, 7990-7997.
6. Aebersold, R. H., Teplow, D. B., Hood, L. E. and Kent, S. B. (1985) J. Biol. Chem. 261, 4229-4238.
7. George, D. G., Barker, W. C., and Hunt, L. T. (1986) Nucleic Acid Res. 14, 11-15.
8. Mihalyi, E. (1978) in Application of Proteolytic Enzymes to Protein Structure Studies (CRC Press, Inc.) pp. 153-191.
9. Ju, G., Collins, L., Kaffka, K. L., Tsien, W-H., Chizzonite, R., Crowl, R., Bhatt, R., and Kilian, P. L. (1987) J. Biol. Chem. 262, 5723-5731.
10. Robb, R., Rusk, C. M., and Neeper, M. P. (1988) Proc. Natl. Acad. Sci. USA 85, 5654-5658.
11. Miedel, M. C., Hulmes, J. D., and Pan, Y.-C. E. (1989) J. Biol. Chem. in press.