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MEDIUM-SCALE LIGAND-AFFINITY PURIFICATION OF TWO SOLUBLE FORMS OF HUMAN INTERLEUKIN-2 RECEPTOR

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SUMMARY

Recombinant technology has facilitated the production of two soluble forms of human interleukin-2 receptor (IL-2R) in Chinese hamster ovary cells. We have developed a ligand-affinity method for the medium-scale purification of these two IL-2Rs, based on the biochemical interactions between the matrix-bound ligand (interleukin-2) and its soluble receptor. The affinity-purified IL-2R is further purified by anion-exchange chromatography followed by gel filtration. This method has provided enough highly pure IL-2R for structure and function studies and for use in practical applications such as high-flux drug screening assays and the receptor-affinity purification of human recombinant interleukin-2.

INTRODUCTION

Interleukin-2 (IL-2), previously denoted as T-cell growth factor, is a lymphokine, whose interaction with its high-affinity receptors is essential for the initiation and maintenance of a normal immune response [1]. When resting T-cells are activated with antigen or mitogen in the presence of monocyte-derived interleukin-1, the synthesis of IL-2 is induced as well as the expression of its high-affinity receptors [2]. The interleukin-2 receptor (IL-2R) is characterized as a 55-kDa glycoprotein, consisting of 251 amino acids and containing N- and O-

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linked glycosylation sites [3,4]. Large quantities of the highly pure IL-2R are needed not only for the elucidation of its structure and function but also for use in practical applications such as high-flux drug screening assays [5], receptor-affinity purification of recombinant human interleukin-2 [rIL-2] and possible therapeutic uses [7,8]. Recombinant technology has facilitated the synthesis of two secreted forms of IL-2R in rodent cells denoted as IL-2R-Δ-Nae and IL-2R-Δ-Mst [5,9,10]. The IL-2R-Δ-Nae lacks 28 amino acids at the carboxyl-terminus and contains the naturally occurring N- and O-linked glycosylation sites. It also contains an added proline at the carboxyl-terminus [5]. The Il-2R-Δ-Mst lacks 72 amino acids at the carboxyl-terminus as well as the two naturally occurring N-linked glycosylation sites. An additional carboxyl-terminal threonine is encoded in the IL-2R-Δ-Mst [10]. The amino acid deletions result in the removal of the presumptive transmembrane and the cytoplasmic domains of the IL-2Rs, thus enabling them to be secreted by the transformed rodent cells. An analytical-scale purification method for IL-2R-Δ-Nae has been reported previously [5,6]. Here, we describe the medium-scale purification of these two soluble recombinant IL-2Rs, using ligand-affinity chromatography, which exploits the interaction between the matrix-bound ligand (IL-2) and its soluble receptor.

EXPERIMENTAL

Materials

rIL-2 (Batch No. 9P86) was obtained from Bioprocess Development Department, Hoffmann-La Roche. Silica-based polyaldehyde NuGel-AF and NuGel P-DE 200 were purchased from Separation Industries (Metuchen, NJ, U.S.A.). Sephacryl S-200 and standard molecular mass marker proteins were obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). Sodium cyanoborohydride, sodium azide and ethanolamine were bought from Sigma (St. Louis, MO, U.S.A.). All other chemicals used were of reagent quality.

Production of IL-2Rs

From the information obtained from the DNA sequence of the cDNA clone of human IL-2R [9] expression vectors pIL-2R-Δ-Nae and pIL-2R-Δ-Mst encoding the IL-2R-Δ-Nae and IL-2R-Δ-Mst forms of the IL-2R were constructed [5,10]. These were then mixed with the plasmid pSV2-dhFr and introduced into a dihydrofolate reductase-deficient Chinese hamster ovary (CHO) cell line by the use of gene-linked co-amplification technology [5]. The transfected CHO cells were adapted to medium-scale continuous fermentation in a 3-l airlift bioreactor (Bellco) in the presence of $4 \cdot 10^{-4}$ *M* amethopterin [5]. The CHO conditioned medium containing the secreted IL-2Rs was collected, filtered through a 0.8/0.2 μm filter (Sartorius) and used as the starting material for the purification of IL-2Rs.

IL-2R assay

IL-2R activity was determined by the bimolecular solid-phase receptor binding assay method of Hakimi et al. [5], which utilizes the binding of IL-2 to the re-

ceptor. The activity was expressed as U/ml, in reference to a standard which is arbitrarily chosen as 1000 U/ml [11]. A serially diluted receptor standard was used to construct a reference curve, from which the receptor concentrations of unknown samples were determined.

Preparation of IL-2 affinity adsorbent

rIL-2 was immobilized to polyaldehyde NuGel-AF support (500 Å, 40–50 μm) by reductive amination in the presence of sodium cyanoborohydride by a modified method of Roy et al. [12]. A 100-g amount of activated support equivalent to 140 ml per gel was washed with cold water in a coarse sintered glass funnel. The gel was transferred into a 500-ml stoppered Erlenmeyer flask containing 140 ml of rIL-2 at a protein concentration of 5 mg/ml. The coupling buffer was 0.1 *M* potassium phosphate containing 0.1 *M* sodium chloride, pH 5.0. The mixture was mixed on a Labline Junior Orbital shaker in the presence of 0.05% (w/v) sodium cyanoborohydride (added as a dry powder) for 16 h at 4°C. The uncoupled rIL-2 was collected by filtration and the gel was washed once with an equal volume of phosphate-buffered saline (PBS). The filtrate and washing were combined and the final volume was noted. The protein content of the uncoupled rIL-2 was determined by the method of Lowry et al. [13]. The rIL-2-coupled gel was resuspended in an equal volume of 0.5 *M* ethanolamine hydrochloride, pH 7.0, and mixed for 4–6 h in the presence of 0.05% sodium cyanoborohydride. The gel was then washed thrice with an equal volume of PBS and stored in the presence of 0.02% sodium azide at 4°C.

Affinity purification of IL-2R-Δ-Nae

IL-2 affinity sorbent (100 ml) was packed into an Amicon 6.6 cm×4.4 cm column fitted with two adapters and equilibrated with PBS. An 18-l volume of filtered CHO-conditioned medium containing the IL-2R-Δ-Nae was applied to the affinity column at a flow-rate of 20 ml/min using an FMI Lab. pump (Fluid Metering, Oyster Bay, NY, U.S.A.). The column effluents were monitored by a Gilson 111B UV detector and recorded by a Kipp & Zonen recorder (Gilson Medical Electronics, Middletown, NJ, U.S.A.). The column was washed with PBS until the effluent absorbance returned to baseline. The adsorbed IL-2R activity was eluted with 1–2 bed volumes of 0.2 *M* acetic acid containing 0.2 *M* sodium chloride. The affinity eluate was dialyzed against PBS.

Anion-exchange chromatography. The dialyzed affinity eluate was applied to a 20 cm×4.4 cm DEAE-silica (NuGel P-DE 200) column equilibrated with PBS at a flow-rate of 8 ml/min. The column was washed with PBS until all the unadsorbed materials were removed from the column. The adsorbed IL-2R-Δ-Nae was eluted with 0.2 *M* sodium chloride in PBS and a strongly bound contaminant was removed from the column with 1 *M* sodium chloride wash.

Size-exclusion liquid chromatography. IL-2R-Δ-Nae from the previous step was concentrated to 15–20 ml in a stirred-cell Amicon thin channel concentrator fitted with a YM5 membrane. It was then subjected to gel permeation in a 90 cm×4.4 cm Sephacryl S-200 column, at a flow-rate of 1 ml/min using PBS as the mobile phase. The column effluents were monitored as described earlier. Fractions of 10

min were collected in an LKB RAC-7000 fraction collector (LKB-Produkter, Bromma, Sweden). The peaks corresponding to the dimeric and monomeric IL-2R-Δ-Nae were pooled separately, concentrated to 2–5 mg/ml and stored frozen at −20°C in the presence of 0.02% sodium azide.

Affinity purification of IL-2R-Δ-Mst

IL-2R-Δ-Mst from 17 l of CHO-conditioned medium was purified according to the same protocol as for IL-2R-Δ-Nae, but without the DEAE-step.

Protein determination

Protein content of the IL-2R samples was determined from their absorbances at 280 nm and confirmed by quantitative amino acid analysis. The predetermined absorbance of standard solutions (1 mg/ml) of IL-2R-Δ-Nae and IL-2R-Δ-Mst were 1.65 and 1.3, respectively.

Sodium dodecyl (lauryl) sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Purified IL-2Rs were analyzed by SDS-PAGE with 12% polyacrylamide under reducing and non-reducing conditions according to the method of Laemmli [14].

Amino acid analysis

Amino acid analyses were performed in a post-column fluorescamine amino acid analyzer [15]. Samples were hydrolyzed in 6 *M* hydrochloric acid containing 4% thioglycolic acid at 110°C for 20–24 h in vacuo. Proline and cysteine were determined after performic acid oxidation.

Molecular mass determination

Molecular mass of the IL-2Rs was determined from electrophoretic mobility data and also from gel permeation results. Both forms of IL-2R were subjected to gel permeation using PBS, as well as PBS containing 6 *M* guanidine hydrochloride as the mobile phases. Molecular masses were determined from the respective log M_r versus K_{av} curves where $K_{av} = (V_e - V_{void})/(V_{total} - V_{void})$ (V_e = elution volume; V_{void} = void volume; V_{total} = total volume of the packed bed; K_{av} = partition coefficient).

These standard curves were constructed using data (not shown) obtained from standard molecular mass markers: blue dextran = 2 000 000; catalase = 232 000; aldolase = 158 000; bovine serum albumin = 67 000; chymotrypsinogen A = 25 000; ribonuclease A = 13 700.

RESULTS AND DISCUSSION

IL-2 affinity adsorbent

Calculations based on the difference between the starting (700 mg) and uncoupled (46 mg) amounts of rIL-2 and the gel volume (140 ml) gave the coupling density as 4.7 mg/ml of gel. Normally, proteins are coupled at pH 7.0. However, this pH was not suitable for IL-2 coupling due to solubility problems. Since rIL-2 (p*I* 7.75) was soluble at pH 5.0, we chose that pH for immobilization. The coupling efficiency at this pH was 93%. The high-flow, low-pressure silica-based

IL-2 affinity sorbent allowed the processing of large volumes of CHO-conditioned medium in a very short time.

Purification of IL-2Rs

IL-2R purification results are summarized in Table I. Recoveries after the affinity step were 81 and 77% for IL-2R-Δ-Nae and IL-2R-Δ-Mst, respectively. Their specific activity at this stage was $315 \cdot 10^6$ U/mg for both proteins. The IL-2R-Δ-Nae was further purified by anion-exchange chromatography in DEAE-silica. A strongly anionic contaminant present in the affinity-purified material was removed by this step. At this step, 62% of the activity was recovered with a specific activity of $334 \cdot 10^6$ U/mg. The IL-2R-Δ-Mst was less charged possibly due to the absence of N-linked glycosylation sites and thus did not bind to the DEAE column. Consequently, it was purified without the DEAE step.

The gel permeation profiles are shown in Fig. 1A and B. The presence of two molecular forms of IL-2R in the IL-2R-Δ-Nae preparation is indicated in Fig. 1A. They were identified as a reducible dimer (first peak) and monomer (second peak) from their electrophoretic mobilities (Fig. 2, lanes 4–7) and by Western blotting (data not shown). Their respective specific activities were 343 and $346 \cdot 10^6$ U/mg. The results (Table I) also indicate that the monomer-to-dimer ratio was 3:1. The IL-2R-Δ-Mst did not contain the dimeric form (Fig. 1B). After the gel permeation step, it had a specific activity of $444 \cdot 10^6$ U/mg.

Characterization of IL-2Rs

SDS-PAGE analysis (Fig. 2) not only confirmed the existence of monomeric and dimeric forms of the IL-2R-Δ-Nae but also showed that the dimer was redu-

TABLE I

IL-2R PURIFICATION RESULTS

Volumes of 18 and 17 l of CHO-conditioned medium were used as the starting materials for IL-2R-Δ-Nae and IL-2R-Δ-Mst, respectively. Protein contents of the IL-2Rs were determined from their absorbances at 280 nm of a 1 mg/ml solution of the respective protein. IL-2R activity was measured by the bimolecular solid-phase assay of Hakimi et al. [5]. See text for details. N.A. = not applicable.

Purification step	Protein (mg)	Activity ($\times 10^6$ U)	Specific activity ($\times 10^6$ U/mg)	Recovery (%)
IL-2R-Δ-Nae				
CHO-conditioned medium	–	31240	–	100
Affinity	80	25200	315	81
DEAE	58	19400	334	62
Monomer	42	14550	346	47
Dimer	14	4800	343	15
IL-2R-Δ-Mst				
CHO-conditioned medium	–	20760	–	100
Affinity	51	16060	315	77
DEAE	N.A.	N.A.	N.A.	N.A.
Monomer	36	16000	444	77
Dimer	N.A.	N.A.	N.A.	N.A.

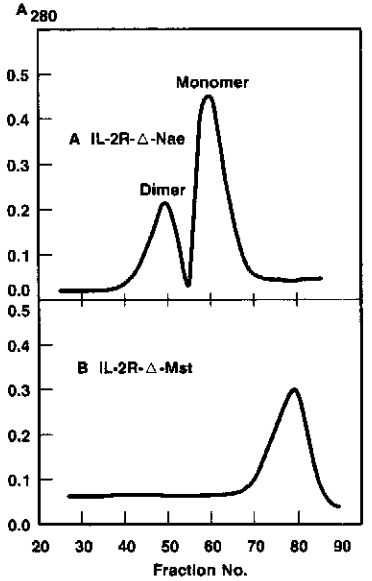

Fig. 1. Gel permeation profiles of IL-2R-Δ-Nae and IL-2R-Δ-Mst. The column, 90 cm×4.4 cm, was packed with Sephacryl S-200. The mobile phase used was PBS; 58 mg of IL-2R-Δ-Nae and 51 mg of IL-2R-Δ-Mst were applied to the column in 20 ml buffer, at a flow-rate of 1.0 ml/min, and 10-min fractions were collected. Fractions corresponding to each peak were pooled separately and concentrated to 2–5 mg/ml and stored frozen at −20 °C, in the presence of 0.02% sodium azide.

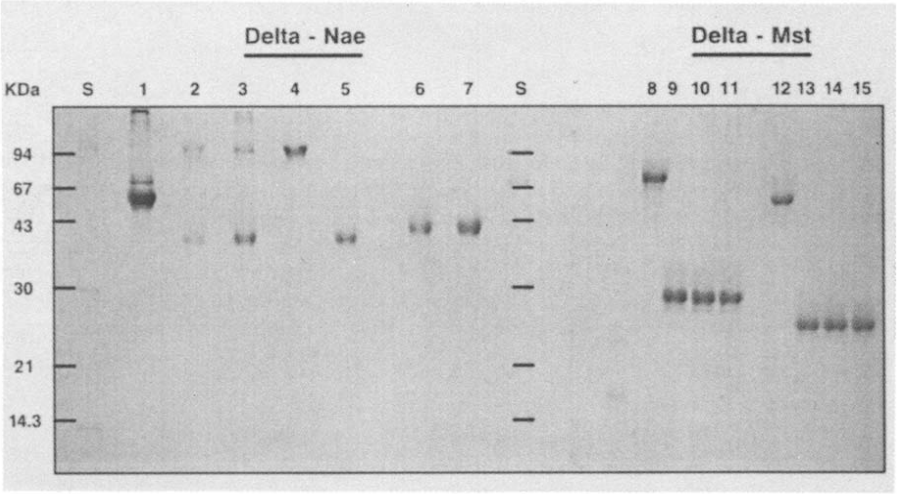

Fig. 2. SDS-PAGE analysis of IL-2R-Δ-Nae and IL-2R-Δ-Mst. Lanes 1–5 and 12–15 were done under non-reducing conditions and lanes 6–11 under reducing conditions. Lane S: standard molecular mass markers; lanes 1, 8 and 12: CHO-conditioned media; lanes 2, 9 and 13: affinity-purified IL-2Rs; lanes 3, 10 and 14: DEAE-purified IL-2Rs; lanes 4 and 6: IL-2R-Δ-Nae dimer; lanes 5 and 7: IL-2R-Δ-Nae monomer; lanes 11 and 15: IL-2R-Δ-Mst after gel permeation. Note that the initial purification scheme for IL-2R-Δ-Mst contained a DEAE-step, which was later eliminated.

TABLE II

AMINO ACID COMPOSITION OF IL-2Rs

Amino acid analyses were performed in a post-column fluorescamine amino acid analyzer. Samples were hydrolyzed in 6 *M* hydrochloric acid containing 4% thioglycolic acid at 110°C for 20–24 h in vacuo. Proline and cysteine were determined after performic acid oxidation.

Amino acid	Residues			
	IL-2R-*Δ*-Nae		IL-2R-*Δ*-Mst	
	Observed	Expected	Observed	Expected
Aspartic acid	11.5	11	9.7	10
Threonine	22.3	25	17.2	17
Serine	13.9	16	11.8	12
Glutamic acid	42.5	41	33.3	32
Proline	17.8	18	15.2	15
Glycine	11.8	12	12.2	11
Alanine	14.9	13	7.9	8
Cysteine	10.4	11	8.1	10
Valine	10.3	10	6.3	7
Methionine	10.1	10	7.9	8
Isoleucine	5.9	6	3.1	4
Leucine	8.6	8	6.8	7
Tyrosine	7.1	7	5.8	6
Phenylalanine	6.2	6	4.0	4
Histidine	6.3	6	5.8	6
Lysine	9.9	10	9.8	10
Arginine	11.9	11	10.3	10
Tryptophan	1.6	3	4.1	3

TABLE III

MOLECULAR MASS DETERMINATIONS OF IL-2Rs

Molecular masses were calculated from gel permeation data (using PBS or PBS containing 6 *M* guanidine hydrochloride as mobile phase) and also from electrophoretic mobilities. See text for details.

Compound	Molecular mass (kDa)		
	Gel permeation		Electrophoretic mobility
	PBS	PBC + guanidine hydrochloride	
IL-2R-*Δ*-Nae monomer	115	33	40
IL-2R-*Δ*-Nae dimer	330	67	100
IL-2R-*Δ*-Mst	38	21	25

cible (lanes 4–7). The latter was indicated by the conversion of the dimer to the monomer under reducing conditions (lane 6). It also indicated that the dimer contained intermolecular disulfide bond. The reduced bands (lanes 6 and 7) moved slightly slower than the non-reduced ones, suggesting the presence of intradomain disulfide bonds. The IL-2R-*Δ*-Mst preparation contained a major and a

minor band (lanes 9–11 and 13–15). Both bands were shown to be IL-2Rs by Western blotting (data not shown). No dimer was observed in the IL-2R-Δ-Mst preparations.

Amino acid analyses of both forms of IL-2R agreed well with that of the expected values, thus confirming the identity of the molecules (Table II).

Molecular masses of both forms of IL-2R, determined from electrophoretic mobility and gel permeation data, are listed in Table III. The coded M_r of IL-2R-Δ-Nae is 25 000. However, in SDS gels the monomer showed an apparent M_r of 40 000 and the dimer an M_r of 100 000. Thus, both molecular forms of the IL-2R-Δ-Nae appeared to be heavily glycosylated. The apparent M_r of the IL-2R-Δ-Mst, determined from electrophoretic mobility data, was 25 000. Molecular masses of both forms of IL-2R, calculated from gel permeation data using PBS as the mobile phase, were much higher than expected (see Table III). When the mobile phase contained 6 M guanidine hydrochloride, the monomeric and dimeric forms of IL-2R-Δ-Nae behaved like true monomer and dimer with M_r values of 33 000 and 67 000, respectively. Under the same conditions, the IL-2R-Δ-Mst had an apparent M_r of 21 000. The anomalous behavior of these IL-2Rs during gel permeation at the physiological pH cannot be fully explained at this time, other than to say that the IL-2R-Δ-Mst appeared to migrate like a globular protein, whereas both forms of IL-2R-Δ-Nae migrate as rod-like proteins.

In summary, ligand-affinity chromatography involving the use of an IL-2 affinity sorbent has been shown to be an efficient method for the preparation of highly pure IL-2Rs. The IL-2Rs purified by IL-2 affinity chromatography are expected to be fully capable of recognizing the biologically important epitopes of the IL-2 molecule.

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