Journal of Chromatography, 550 (1991) 741–749
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2154

Isolation of an interleukin 2-binding receptor from activated lymphocytes by high-performance immunoaffinity chromatography

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ABSTRACT

Isolation of a lymphokine-binding receptor, from activated lymphocyte membranes, can be achieved by high-performance immunoaffinity chromatography (HPIAC), using immobilized antibodies against human interleukin 2 (IL-2), as the ligand and natural IL-2 as the receptor probe. Activated lymphocytes were reacted with IL-2, sonically disrupted and their membranes solubilized, prior to passage through the HPIAC column. The IL-2 acted as an efficient receptor probe, which helped to maintain the integrity of the receptor during the isolation procedure and also acted as an attachment antigen for the immunoaffinity ligand. Recovery of the bound receptor was achieved by dissociation of the receptor–antigen–immobilized ligand complex by the action of chaotropic ions and collection of the released receptor from the column effluent during the elution phase of the separation.

INTRODUCTION

The structure of receptors on the membranes of activated lymphocytes is currently of great interest to molecular biologists, membrane biochemists and immunologists. The isolation of these receptors by conventional biochemical techniques is tedious and time consuming, following which the recovered receptor exhibits either decreased activity or no activity at all [1]. However, the isolation and recovery of membrane proteins can be effectively achieved by several different techniques, such as immunoprecipitation [2–5], ion-exchange chromatography [6], size-exclusion chromatography [6], lectin affinity chromatography [7,8], or immunoaffinity chromatography using antibodies, immobilized either directly to activated supports or bound to supports coated with specialized proteins, such as protein A and streptavidin [9–11].

Most cells possess receptors for exogenous growth factors, interaction with which leads to cytoplasmic signaling and cell proliferation. Interleukin 2 (IL-2) is a growth factor that is important in lymphocyte clonal expansion and regulation following antigenic stimulation. Recently, great interest has centered on isolating and

0021-9673/91/$03.50

characterizing IL-2-binding receptors, in an attempt to understand better their involvement in lymphocyte activation.

In this paper, a procedure is described for the isolation of a lymphocyte IL-2-binding receptor, using immobilized anti-human IL-2 antibodies and human IL-2 as the receptor probe.

EXPERIMENTAL

Materials

Solid glass beads (diameter 150–212 μm) and all laboratory chemicals were obtained from Sigma (St. Louis, MO, USA). 3-Aminopropyltriethoxysilane and 1,1'-carbonyldiimidazole were obtained from Aldrich (Milwaukee, WI, USA). Purified streptavidin was purchased as a lyophilized, pure product from Pierce (Rockford, IL, USA) and reconstituted in 50 m*M* sodium carbonate buffer (pH 9.0). Mouse immunoglobulin G_1 (IgG_1) monoclonal antibody (MAb) reactive with human IL-2 was obtained as a purified IgG preparation from benzyme (Cambridge, MA, USA) and purified natural human IL-2 was obtained from Collaborative Research (Bedford, MA, USA). Glass-lined, stainless-steel columns and column fittings were purchased from Alltech (Deerfield, IL, USA). Activated lymphocytes were obtained from consenting patients with systemic lupus erythematosus, seen at the Rheumatology Clinic at the George Washington University Medical Center.

Derivatization of glass beads

The glass beads were prepared as described by Babashak and Phillips [12]. The beads were washed by sedimentation in 0.1 *M* hydrochloric acid, followed by two washes in doubly distilled water. This removed manufactoring impurities from the bead surface, before preparing them for silanization and derivatization by placing 100 g of the washed beads in 500 ml of 0.1 *M* hydrochloric acid and gently sonicating for 25 min, followed by sedimentation in 1000-ml portions of 0.1 *M* hydrochloric acid. This process was repeated, using fresh acid solutions, until the acid supernatant became clear. The beads were then removed and air-dried, before refluxing them for 30 min in 500 ml of 1 *M* nitric acid, with constant agitation. The beads were recovered, air-dried and resuspended in 500 ml of 10% (v/v) 3-aminopropyltrietoxysilane dissolved in toluene. This suspension was gently refluxed for 16 h with constant agitation.

Following silanilization, the beads were recovered and washed twice in 500 ml of 95% methanol before being transferred to fresh 95% methanol and refluxed for 20 min, to remove the excess of silanizing agent. The beads were allowed to settle,washed three times in doubly glass-distilled water and air-dried prior to derivatization of the reactive side-groups.

The reactive carbonyldiimidazole (CDI) side-groups were attached to the bead surface by suspending the beads in 300 ml of dioxane and slowly adding 6 g of 1,1'-carbonyldiimidazole [13,14]. The mixture was placed in a 500-ml capped conical flask and incubated for 6 h at room temperature in an oscillating shaker. The beads were then recovered and thoroughly washed in dioxane by sedimentation and decantation before being air-dried and immediately coated with streptavidin.

Preparation of streptavidin-coated beads

A 100-g amount of the CDI-derivatized beads was suspended in 200 ml of doubly distilled water prior to the addition of 200 ml of 50 m*M* sodium carbonate buffer (pH 9.0) containing 5 g of streptavidin. The mixture was placed in a 500-ml capped conical flask and incubated for 18 h at 4°C in an oscillating shaker. Following this incubation, the beads were allowed to settle and washed ten times in 0.01 *M* sodium phosphate buffer (pH 7.4) by sedimentation and decantation. Attachment of the streptavidin to the beads was checked by incubating a 500-μl drop of the bead suspension, obtained after the last wash, with fluorescein-labelled biotin and examining the beads under a fluorescence microscope. Following satisfactory coating of the beads, they were sedimented, recovered and resuspended in 500 ml of 0.01 *M* sodium phosphate buffer (pH 7.4).

Biotinylation of monoclonal antibodies

The monoclonal anti-IL-2 antibody was biotinylated by using the hydrazine biotinylation technique described by O'Shannessy and Quarles [15]. This technique requires modification of the carbohydrate portion of the antibody by oxidation with periodate. Briefly, this was achieved by suspending the 100 mg of antibody in 10 ml of 0.1 *M* sodium acetate buffer (pH 5.0) and cooling to 4°C. A 10-ml volume of a 10 m*M* solution of cold sodium metaperiodate was added to the antibody before incubation for 20 min at 4°C in the dark. The reaction was stopped by adding 50 ml of 5% (w/v) ethylene glycol and dialyzing the solution against 0.01 *M* sodium phosphate (pH 7.4) for 18 h at 4°C, with five changes of the dialysate. The antibody was then removed from the dialysis tubing and placed in a capped glass tube. To this were added 10 ml of 0.01 *M* sodium phosphate buffer, (pH 7.4) containing 15 mg/ml of sodium cyanoborohydride and 25 mg/ml of biotin hydrazine, and the mixture was placed in a rotating mixer for 1 h at room temperature. The reaction was stopped by dialysis against 0.1 *M* sodium phosphate buffer (pH 7.4) overnight at 4°C.

Preparation of HPIAC column

The biotinylated MAb preparation was adjusted to 150 μg/ml in 0.1 *M* sodium phosphate buffer (pH 7.4) and 2 ml of this solution were added to 10 g of streptavidin-coated beads. The mixture was placed in a 15-ml capped tube and placed on an end-over-end mixer for 4 h at 4°C. The beads were then washed five times in 0.1 *M* sodium phosphate buffer (pH 7.4). Finally, the MAb-coated beads were slurry-packed into 100 × 4.6 mm I.D. glass-lined stainless-steel high-performance liquid chromatographic (HPLC) columns at 250 p.s.i., using a conventional pump-driven slurry packing apparatus.

Preparation of IL-2–IL-2-binding complex and isolation of lymphocyte membranes

Prior to disruption and solubilization of their membranes, active lymphocytes were isolated from whole blood by centrifugation at 400 *g* for 15 min in a Ficoll gradient [16]. The lymphocyte band was recovered and the cells were washed three times in RPMI 1640 medium and adjusted to $5 \cdot 10^6$ cells/ml. A 100-ng amount of human IL-2 was added to 1 ml of the cell suspension, incubated for 30 min at 37°C and the cells were recovered by centrifugation. The cells were washed twice in RPMI 1640 medium and sedimented by centrifugation at 800 *g* for 10 min. The cell pellet

was recovered, frozen and thawed three times and then sonicated for 2 min at maximum power. The sonicated pellet was resuspended in 2 ml of 0.1 *M* sodium phosphate buffer (pH 7.4) and the membrane fraction was isolated by centrifugation at 10 000 *g* for 30 min. The membrane-enriched supernatant was mixed with an equal volume of 1% (w/v) sodium deoxycholate and incubated for 30 min at room temperature. Finally, the solubilized membrane sample was centrifuged for 1 h at 10 000 *g* and the supernatant was applied to the immunoaffinity columns.

High-performance immunoaffinity chromatography

The MAb/bead-packed HPIAC column was installed in a Beckman (Palo Alto, CA, USA) Model 340 isocratic HPLC system equipped with a Model 112 pump, a Model 160 UV detector (set at 280 nm) and a Shimadzu (Columbia, MD, USA) C-R1B recording peak integrator. The elution profile was automatically controlled by a Model III OPG/S gradient controller (Autochrom, Milford, MA, USA). Samples (2.5 μg of protein) were introduced into the system by injection through an Altex 210 injection port, equipped with a 100-μl sample loop.

The column was isocratically developed in either 0.1 *M* sodium phosphate buffer (pH 7.4) with no additives, 0.1 *M* sodium phosphate buffer (pH 7.4) plus 0.5% of (w/v) sodium deoxycholate (a detergent which causes minimal effect on antibody–antigen interactions) or with 0.1 *M* sodium phosphate buffer (pH 7.4) to which 0.5% (w/v) of sodium deoxycholate and 0.1% (w/v) of polyvinylpolypyrrolidone (PVP) had been added. In all instances, the initial isocratic phase was maintained for 15 min at a flow-rate of 0.5 ml/min. Throughout the entire run, the column temperature was maintained at 4°C by a glass column jacket, attached to a recycling ice-bath. Following the initial 15-min run, during which the IL-2 of the II-2–IL-2-receptor complex bound to the immobilized antibody, an elution recovery phase was started. An elution gradient was developed by adding 0–2.5 *M* sodium thiocyanate to the running buffer over a further 15 min and maintaining the upper limit of the gradient for a further 5 min, before recycling the column. This was achieved by reversing the gradient until the column was returned to the initial running conditions. Fractions of 200 μl of the eluted material were collected in 500-μl Beckman microfuge tubes in a modified ISCO Cygnet fraction collector (ISCO, Lincoln, NB, USA) and dialyzed overnight at 4°C against 0.01 *M* sodium phosphate buffer (pH 7.4). Analysis of the isolated materials was performed by polyacrylamide gel electrophoresis (PAGE) on a 10–30% linear gradient gel containing 0.1% (w/v) sodium dodecyl sulfate (SDS). Following separation, the gels were fixed in methanol–acetic acid (4:1) and silver stained [17]. To establish the identity of the SDS-PAGE-isolated bands, the gel profiles were blotted to nitrocellulose membranes by the technique described by Towbin *et al.* [18]. The blotted membranes were stained with I^{125}-labelled human IL-2 by incubating them for 2 h in a solution of 0.1 *M* sodium phosphate, (pH 7.4) to which 1 ng/ml of radiolabelled IL-2 had previously been added. The membranes were washed five times in 0.1 *M* sodium phosphate buffer (pH 7.4) and the bound IL-2 probe was rendered visible by autoradiography.

Receptor binding studies

The functional ability of the immunoaffinity-isolated IL-2 receptor to bind its substrate was tested by incubating the HPIAC-isolated receptor isolated in the three

different buffers with I^{125}-labelled human IL-2 and plotting the binding efficiency against time [19]. Briefly, 5 ng of membrane protein from the immunoaffinity peak B, separated by the three buffer systems, were incubated at 37°C for 30 min with 500 fg of labelled IL-2. The IL-2 binding capacities of the three immunoaffinity-isolated membrane preparations were compared with the binding capacity of $1 \cdot 10^6$ intact lymphocytes, incubated with an identical amount of labelled IL-2.

RESULTS AND DISCUSSIONS

Analysis of over 200 batches of streptavidin-coated glass beads has shown that a 1-g batch of beads can be coated with between 0.76 and 1 mg of streptavidin. Once coated, the beads were able to bind between 120 and 185 μg of biotinylated antibody, which represents *ca.* 200 μg of bound antibody per column. Stability analysis showed that the columns could effectively be recycled between 20 and 30 times before the immobilized antibody became either detached or lost its activity. All columns remained stable for up to 6 months when stored at 4°C.

Fig. 1 shows a typical immunoaffinity chromatogram produced by passing the solubilized lymphocyte membrane preparation over the immobilized antibody. Three major peaks are produced, at 8 min (peak A), 23 min (peak B) and 25 min (peak C). SDS-PAGE of the starting materials and the contents of the three peaks revealed fifteen visible bands in the starting material and fourteen bands in peak A. Analysis of peak B demonstrated the presence of only one band at *ca.* 55 kilodalton, which is similar to that described for the IL-2 receptor isolated by other immunochemical techniques [20–22] and corresponded to the band missing from the profile of peak A (Fig. 2). Peak C contained a single band of *ca.* 15 kilodalton, which is similar to the molecular weight, estimated by the manufacturer, of the human IL-2 probe. Ligand-

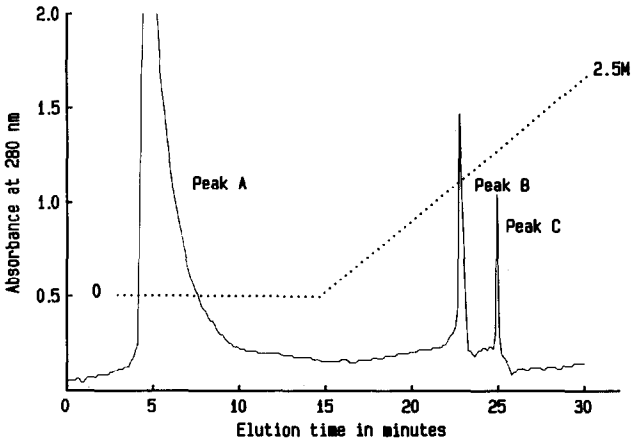

Fig. 1. HPIAC isolation of an IL-2 receptor from activated human lymphocytes using a 100 × 4.6 mm I.D. column, packed with anti-human IL-2 antibody, immobilized on streptavidin-coated glass beads. Peak A represents the unbound material, peak B contains the IL-2 receptor and peak C contains the IL-2 probe. The chromatogram was developed at 0.5 ml/min in 0.1 *M* sodium phosphate buffer–0.5% (w/v) sodium deoxycholate–0.1% (w/v) PVP buffer (pH 7.4). The elution profile was monitored at 280 nm with the detector set at 0.008 a.u.f.s. The dotted line represents the elution gradient.

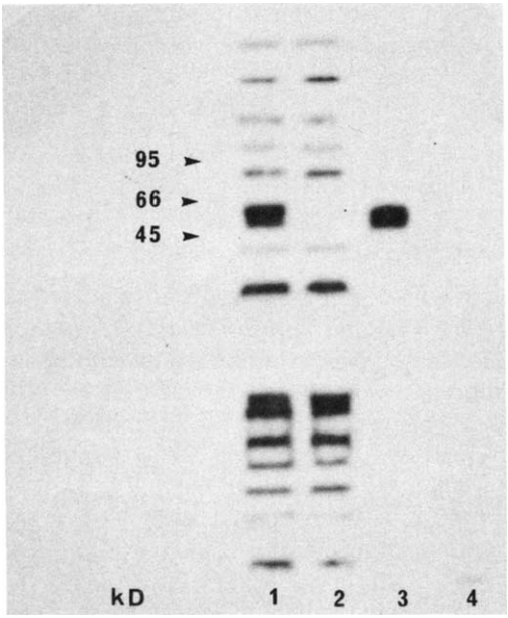

Fig. 2. SDS-PAGE of the immunoaffinity chromatographic peaks. Lane 1, 2 μg of starting cell membrane material; lane 2, unreactive membrane material from peak A of the immunoaffinity chromatogram; lane 3, material retained by the immobilized antibody and eluted in peak B of the chromatogram; lane 4, the material eluted in peak C of the chromatogram. Numbers above kD indicate reference molecular weights in kilodaltons, calculated from molecular weight standards.

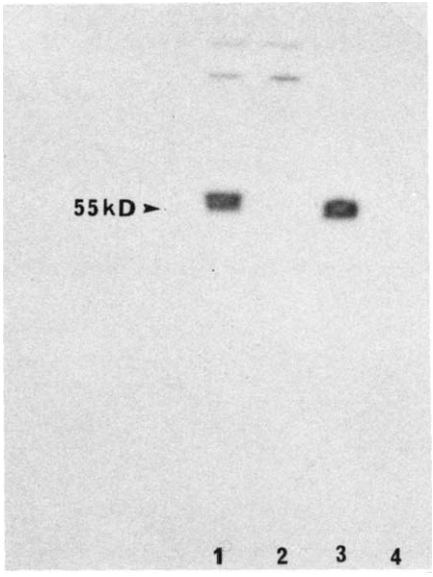

Fig. 3. IL-2 ligand blotting of the SDS-PAGE profiles shown in Fig. 2. Lane 1, starting membrane material; lane 2, peak A from the immunoaffinity chromatogram; lane 3, peak B from the immunoaffinity chromatogram; lane 4, peak C from the immunoaffinity chromatogram.

TABLE I

EFFECT OF HPIAC BUFFERS ON PROTEIN RECOVERY

Buffer	Starting material[a] (ng)	Peak A (ng)	Peak B (ng)	Peak C (ng)	Total recovery (%)
0.1 *M* NaH_2PO_4	2500	2219	61	22	92.1%
0.1 *M* NaH_2PO_4–0.5% deoxycholate	2500	2240	74	29	93.7%
0.1 *M* NaH_2PO_4–0.5% deoxycholate–0.1% PVP	2500	2259	87	31	95.1%

[a] Total protein content of the starting material, including the IL-2 probe, as measured by the BCA method (Pierce, Rockford, IL, USA).

staining of nitrocellulose blots of the SDS-PAGE profiles with radiolabelled IL-2 demonstrated strong binding to the 55 kilodalton band of the starting material and peak B. In addition, the ligand bound to an 85- and a 100-kilodalton band of both the starting material and peak A. No staining was observed in the 55-kilodalton range in peaks A or C (Fig. 3).

IL-2 binding studies demonstrated that the composition of the buffer used in the primary phase of the HPIAC isolation had an effect on the activity of the eluted receptor, even though little difference could be detected in the total protein recovered in each peak (Table I). The maximum IL-2 binding by intact cells took place at 60 min and all comparison measurements were taken at that time point. Taking the intact cell binding as a reference, it was found that the receptor isolated with the three different chromatographic buffers exhibited different binding capacities (Fig. 4). The receptor

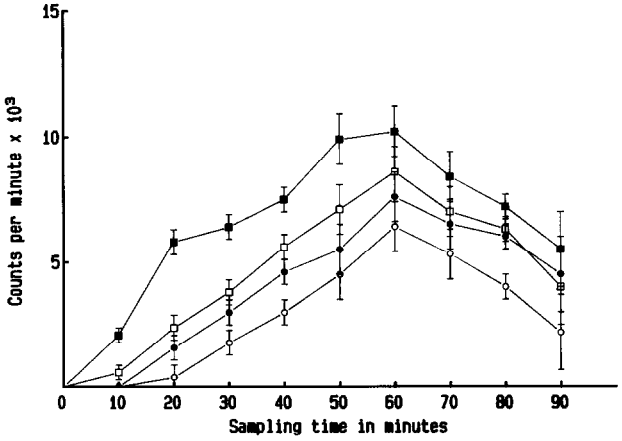

Fig. 4. Binding of radiolabelled IL-2 to HPIAC IL-2-isolated membrane receptor and intact cells. (○) Receptor isolated in 0.1 *M* sodium phosphate buffer (pH 7.4); (●) receptor isolated in 0.1 *M* sodium phosphate–0.5% (w/v) sodium deoxycholate buffer (pH 7.4); (□) receptor isolated in 0.1 *M* sodium phosphate–0.5% (w/v) sodium deoxycholate–0.1% (w/v) PVP buffer (pH 7.4); (■) intact lymphocytes. Points are the means of ten experiments ± the standard error of the mean.

isolated in 0.1 *M* sodium phosphate–0.5% (w/v) sodium deoxycholate–0.1% (w/v) PVP buffer (pH 7.4) retained the highest degree of binding activity, which equalled 85% of that shown by intact cells. The receptor isolated with 0.1 *M* sodium phosphate–0.5% (w/v) sodium deoxycholate buffer (pH 7.4) demonstrated a binding efficiency that was equal to 74% of the intact lymphocytes, while the receptor isolated in 0.1 *M* sodium phosphate buffer (pH 7.4) gave the lowest reactivity (63%).

Although HPIAC can isolate a receptor in less than 30 min, a significant amount of activity is lost during the process. It is unknown whether the loss of receptor function occurs during the initial binding of the receptor–substrate complex to the immobilized antibody or during the elution phase. Experiments with both acid and chaotropic elution have shown no difference in the activity of the recovered receptor and new elution agents are under investigation. It has been demonstrated that both acid and choatropic ions interfere with the tertiary structure of proteins [23], and perhaps these agents cause structural changes which a affect the binding capacity of the isolated receptor. However, sodium thiocyanate has been shown to be an effective elution agent for immunoaffinity procedures, especially membrane receptor [24,25], and has the added advantage that it does not appreciably damage the immunoaffinity support. Improved elution agents are required before HPIAC can achieve its full potential as the separation technique of choice for membrane receptor isolation.

CONCLUSIONS

Lymphocyte membrane receptors can be isolated by HPIAC using immobilized antibodies directed against the receptor substrate, which is used as a receptor probe. The technique is rapid and reasonably efficient, isolating receptors which retain 70–85% of their original binding efficiency in a form suitable for further biochemical or immunochemical analysis.

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