Isolation of complement-fragment-iC3b-binding proteins by affinity chromatography

The identification of p150,95 as an iC3b-binding protein

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The proteins from labelled human spleen membranes and polymorphonuclear leucocytes which bind to the iC3b fragment of complement component C3 were prepared by iC3b–Sephacryl chromatography in the presence of bivalent cations. Complement receptor type 3 (CR3) was eluted from iC3b–Sephacryl by removal of bivalent cations. Complement receptors type 1 and 2 (present in spleen but not in polymorphonuclear leucocytes) were sequentially eluted by a NaCl gradient. An additional protein of M_r 135000 was eluted from iC3b–Sephacryl under the same conditions as those used to elute CR3. Preabsorption of the starting material on an anti-(CR3 β -subunit) antibody column before iC3b–Sephacryl chromatography removed the α - and β -chains of CR3 and the 135000- M_r protein. Preabsorption with iC3b–Sephacryl before the anti-(CR3 β -subunit) antibody column showed that iC3b binds CR3 and p150,95, the smallest member of the group of three homologous proteins that share the same β -subunit.

INTRODUCTION

Human leucocytes possess receptors for fragments of the third component of complement (C3) (Fearon & Wong, 1983). These receptors are involved in the recognition of opsonins during phagocytosis (Ehlenberger & Nussenzweig, 1971) and may modulate the immune response (Weigle *et al.*, 1983).

After complement activation, C3 binds covalently to the activating surface as the C3b fragment (M_r 178000) (Law & Levine, 1977). The C3b is inactivated by cleavage to iC3b (M_r 175000) (Harrison & Lachmann, 1980). C3b is subsequently degraded to C3c and C3dg (M_r 43000) or C3d (M_r 33000) (Davis *et al.*, 1984). With the exception of C3c, which is released into solution from the activating surface, these fragments all remain bound to the activating surface and are available as ligands for complement receptors.

Complement receptor type 1 (CR1), a 240000- M_r protein (Fearon, 1980), binds C3b and iC3b (Ross *et al.*, 1983) and the activated form of the fourth component of complement (C4). The B-lymphocyte C3d receptor (CR2) is a molecule of 145000 M_r (Iida *et al.*, 1983) that mediates C3d and iC3b binding in B-lymphocytes (Micklem *et al.*, 1984) and can also recognize C3b (Barel *et al.*, 1981). Complement receptor type 3 (CR3) found on phagocytic cells is specific for iC3b and requires bivalent cations for activity (Ross *et al.*, 1983). The protein corresponding to CR3 has been identified as the antigen termed 'Mac1'

(Springer *et al.*, 1979), on the basis of antibody inhibition (Beller *et al.*, 1982) and by formation of a ternary iC3b–receptor–anti-Mac1 complex (Wright *et al.*, 1983). This protein is a non-covalently linked heterodimer with subunits of M_r 165000 and 95000 (Kurzinger *et al.*, 1982) and is one member of the family of three homologous proteins named LFA-1, p150,95 and CR3, which share the same 95000- M_r (β -) subunit (Sanchez-Madrid *et al.*, 1983).

The receptors mediating the C3d-binding activity of cultured monocytes (Inada *et al.*, 1983) and polymorphonuclear leucocytes (Vik & Fearon, 1985) have not been identified, although both anti-CR3 (Vargas *et al.*, 1984) and an antibody directed against the common β -subunit of LFA-1, CR3 and p150,95 (Wright *et al.*, 1984) have been reported to inhibit the monocyte receptor.

Although it is possible to study these receptors by soluble-ligand binding (Vik & Fearon, 1985) or by rosetting of ligand-coated particles (Ross *et al.*, 1983), the application of C3-fragment affinity chromatography has many advantages, especially when many receptors of overlapping specificity are involved (Micklem *et al.*, 1984).

The present paper describes the isolation of the iC3b-fragment-binding proteins from spleen membranes and polymorphonuclear leucocytes. One of these proteins was identified, by using monoclonal antibodies, as p150,95, a homologue of LFA-1 and CR3.

Abbreviations used: PMN, human polymorphonuclear leucocytes; CR1, CR2 and CR3, complement receptor type 1, 2 and 3 respectively; phosphate-buffered saline, 8.2 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 /139 mM- NaCl /3 mM- KCl /1 mM- CaCl_2 /1 mM- MgCl_2 , pH 7.4; DFP, di-isopropyl phosphorofluoridate; PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate; SBTI, soya-bean trypsin inhibitor; NP40, Nonidet P40; lysing buffer, 2% (w/v) Nonidet P40/10 mM- Na_2HPO_4 /5 mM-iodoacetamide/soya-bean trypsin inhibitor (10 $\mu\text{g}/\text{ml}$)/2 mM-phenylmethanesulphonyl fluoride, pH 7.4. The nomenclature of complement components and of C3 and its fragments is as recommended by the World Health Organisation (1968, 1981).

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METHODS AND MATERIALS

Spleen and PMN preparations

Spleen membranes, which were a gift from Dr. S.-K. Law (Department of Biochemistry, University of Oxford, Oxford, U.K.), were prepared by homogenizing chopped human spleen suspended in 2.5% (v/v) Tween 40 (Sigma)/10 mM-Tris/HCl, pH 8, containing 10 mM-iodoacetamide, 2.5 mM-DFP and 2 mM-PMSF, with a Potter homogenizer (six strokes, 100 rev./min) on ice. This material was centrifuged at 3000 *g* for 20 min and the supernatant centrifuged at 40000 *g* for 20 min. The second pellet was washed twice in 10 mM-Tris/HCl, pH 8, and stored at -70°C .

PMN were prepared from the blood of healthy donors by centrifugation over Histopaque (Sigma, Poole, Dorset, U.K.). The pellet was subjected to three washings with iso-osmotic NH_4Cl to lyse the erythrocytes. The resulting cells were washed twice in phosphate-buffered saline to remove erythrocyte ghosts.

Radioiodination

PMN (10^7 cells) in 1 ml of phosphate-buffered saline containing 0.5 mM- CaCl_2 and 0.5 mM- MgCl_2 were iodinated by using 1 mCi of Na^{125}I (Amersham International, Amersham, Bucks., U.K.) in an Iodogen (Pierce and Warriner, Chester, U.K.)-coated polyethylene tube for 10 min at room temperature. The cells were washed twice with phosphate-buffered saline and lysed by resuspension in lysing buffer for 20 min on ice.

The extract was centrifuged (10000 *g* for 1 min) and the supernatant applied to a Sepharose G-25 column (PD10; Pharmacia, Milton Keynes, Bucks., U.K.) equilibrated with 1% NP40/10 mM-sodium phosphate (pH 7.4)/0.5 mM- CaCl_2 /0.5 mM- MgCl_2 . The excluded material containing labelled membrane proteins was stored at -70°C before use. This material contained 0.15% of the initial ^{125}I . Spleen membranes (10 mg of protein) in 0.5 ml of 10 mM-sodium phosphate, pH 7.4, were iodinated in a similar way to cell suspensions, except that the iodination was terminated by the addition of 0.5 ml of lysing buffer without washing. The membrane preparation was solubilized and gel-filtered exactly as described for the cells. This material contained 2.5% of the initial ^{125}I .

Affinity chromatography

iC3b-Sepharose was prepared by coupling purified human iC3b to CNBr-activated Sepharose 4B (Pharmacia) as described previously (Micklem *et al.*, 1984). The ligand concentration was 2.2 mg of iC3b/ml of Sepharose.

The solubilized extracts were applied to an iC3b-Sepharose column (2 ml) equilibrated with 1% NP40 in 10 mM-sodium phosphate/1 mM- CaCl_2 /1 mM MgCl_2 at 6 ml/h. The column was washed with this buffer until the eluted radioactivity was at a minimum. The buffer was changed to 10 mM-sodium phosphate, pH 7.4, containing 2 mM-EDTA, and continued until the eluted radioactivity returned to the baseline value. The remainder of the iC3b-binding proteins were then eluted with an NaCl gradient (total volume 40 ml) up to 500 mM in the same buffer.

Immunoaffinity chromatography was performed by using H52 monoclonal antibody bound to CNBr-activated Sepharose (a gift from Dr. S.-K. Law). Extracts were applied (6 ml/h) to the H52 column (1 ml) equilibrated with 1% NP40/10 mM-sodium phosphate, pH 7.4. The column was washed with this buffer and then with 200 mM-NaCl in the same buffer. The bound proteins were eluted with 1% NP40/0.1 M-glycine/HCl, pH 2.

Immunoprecipitation

Mouse monoclonal antibodies were obtained as follows: OKM1, which recognizes CR3 α -chain (Wright *et al.*, 1983), Ortho Diagnostics, High Wycombe, Bucks., U.K.; H52, an antibody directed against the common β -subunit of LFA-1, p150,90 and CR3 (Hildreth & August, 1985), a gift from Dr. J. Hildreth, Johns Hopkins Medical School, Baltimore, MD 21205, U.S.A.; MHM24, an anti-(L-FA1 α -chain) antibody (Hildreth *et al.*, 1983), a gift from Professor A. McMichael, Nuffield Department of Surgery, John Radcliffe Hospital, Oxford, U.K.

Solubilized antigen preparations were incubated with antibodies at 5°C for 1 h, and then rabbit anti-(mouse immunoglobulin) antibody bound to CNBr-activated Sepharose (0.1 ml of a suspension containing 2.5 mg of antibody bound to 50 μl of gel) was added for a further 1 h. The Sepharose was then washed twice with 1% NP40 in 10 mM-sodium phosphate, pH 7.4, twice with 200 mM-NaCl in the same buffer, and finally twice in 0.05% SDS. The absorbed proteins were eluted with 1% NP40 in 0.2 M-glycine/HCl, pH 2. This material was analysed by SDS/polyacrylamide-gel electrophoresis.

SDS/polyacrylamide-gel electrophoresis

Samples were prepared by precipitation with 10% (w/v) trichloroacetic acid at 5°C by using 50 μg of SBTI as carrier. The precipitate was washed with 5% trichloroacetic acid and twice with acetone before solubilization in 1% SDS/4 M-dithiothreitol/200 mM-Tris/HCl, pH 8, at 80°C for 10 min. Polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970) on a 6.5% gel. Marker proteins were detected with Coomassie Blue staining, and the gels dried for autoradiography with an intensifying screen (Du Pont, Stevenage, Herts., U.K.) exposed at -70°C .

RESULTS AND DISCUSSION

iC3b-binding proteins from spleen

As a source of complement receptors a membrane preparation from human spleen was labelled with ^{125}I , solubilized in non-ionic detergent and applied to an iC3b-Sepharose column in a low-ionic-strength buffer containing Ca^{2+} and Mg^{2+} as described in the Methods and materials section. In order to elute CR3 specifically, after washing the unbound material from the column, EDTA was substituted for the bivalent cations without altering the ionic strength. This was followed by an NaCl gradient as described in the Methods and materials section to remove sequentially CR1 and CR2, together with any other iC3b-binding proteins.

Fig. 1 shows an autoradiograph of a polyacrylamide gel of fractions of the material eluted from the column. At least six distinct radioactive peptides are visible. Four of these can be identified by size and elution conditions. The 240000- M_r protein eluted at 10–30 mM-NaCl (tracks 6–9) is CR1. The 145000- M_r protein eluted at 20–50 mM-NaCl (tracks 8–11) is CR2. The bands at M_r 150000 and 98000 eluted with EDTA (track 1) are the α - and β -subunits of CR3.

The most prominent unidentified band in the EDTA eluate, of M_r 135000, is in the expected position of the α -chain of the third, relatively uncharacterized, member of the LFA1, CR3 family of antigens, which share a common β -subunit. This protein has been termed 'p150,95' (Sanchez-Madrid *et al.*, 1983). M_r estimates,

Fig. 1. Analysis of iC3b-binding proteins from labelled spleen membranes

Solubilized spleen membranes labelled with ^{125}I were applied to an iC3b-Sepharose column as described in the Methods and materials section. The column was washed until the eluted radioactivity was decreased to a constant level. The elution buffer was changed to 2 mM-EDTA and 4 ml fractions were collected. After the peak of radioactivity had been eluted with EDTA, elution with a 40 ml gradient of 0–500 mM-NaCl in the same buffer was started. Samples (2 ml) of each fraction were precipitated with trichloroacetic acid and analysed by SDS/polyacrylamide-gel electrophoresis. The Figure shows an autoradiograph of the fractions. Tracks 1–4 are the EDTA elution. The NaCl gradient begins at track 5. The M_r standards were: fibronectin, M_r 220000; α_2 -macroglobulin, 167000; phosphorylase, 92000. M_r values for the sample proteins were calculated by interpolation on a semilogarithmic plot of the migration of standard proteins against M_r .

from SDS/polyacrylamide-gel electrophoresis, for these proteins vary. Although in the present paper the M_r estimates are different from those reported by Sanchez-Madrid *et al.* (1983), the nomenclature proposed by those workers is used here. The apparent M_r values we observe for these proteins are very similar to those reported by Anderson *et al.* (1984).

A suggestion, based on indirect evidence, that p150,95 might be responsible for C3d receptor activity in cultured monocytes (Wright *et al.*, 1984) prompted a closer examination of the protein eluted with EDTA. The identification of this protein with a monoclonal antibody directed against the common β -subunit of LFA-1, CR3 and p150,95 is described below.

The other unidentified bands in Fig. 1 include a protein migrating at 90000 M_r , eluted with 15–40 mM-NaCl (tracks 7–10), and a faint band at 160000 M_r , eluted at 10–20 mM-NaCl (tracks 6 and 7). These may be fragments of CR1 or CR2, although the 83000- M_r active fragment of CR2 (Micklem *et al.*, 1985) has the same ionic-strength-dependence of elution as intact CR2, suggesting that the unknown 90000- M_r band is not a fragment of CR2.

iC3b-binding proteins from PMN

Preliminary results indicated that the 135000- M_r iC3b-binding protein eluted by EDTA was present in human peripheral-blood PMN extracts. As PMN are relatively easy to obtain, these cells were used in subsequent experiments as a more defined cell population.

Fig. 2. Analysis of iC3b-binding proteins from labelled PMN

Solubilized PMN labelled with ^{125}I were applied to an iC3b-Sepharose column as described in the Methods and materials section. The column was washed and eluted as described in the legend to Fig. 1. Tracks 1–4 represent the elution with EDTA. The NaCl gradient begins at track 5. The M_r standards were: fibronectin, 220000; α_2 -macroglobulin, 167000; C3b, 105000 and 70000; phosphorylase, 92000.

Human PMN were prepared, labelled with ^{125}I and solubilized as described in the Methods and materials section. The extract was applied to an iC3b-Sepharose column. After washing the column the iC3b-binding proteins were eluted with EDTA and NaCl as described above. Fig. 2 shows an autoradiograph of the gel of material eluted with EDTA (tracks 1–4) and NaCl (tracks 5–14). The radioactive bands are similar to those given by the spleen preparation, with the exception of the absence of CR2, which is only found on B-lymphocytes (Nadler *et al.*, 1981). The relative intensities of the bands are different from those given by the spleen material, the α - and β -subunits of CR3 being especially prominent. The 90000- M_r band is also more intense. The 135000- M_r band eluted with EDTA is present at lower intensity, but is still readily detectable.

To identify positively the CR3 subunits and to investigate whether the 135000- M_r band is the α -subunit of p150,95, the PMN extract was passed down an anti-(β -subunit) antibody (H52) column to remove all β -subunit-containing molecules before chromatography on iC3b-Sepharose. In a reciprocal experiment the material that had passed through the iC3b column was applied to the anti-(β -subunit) antibody column.

PMN extract was applied to an H52-Sepharose column as described in the Methods and materials section. The material that did not bind was applied to an iC3b-Sepharose column as described above. Fig. 3 shows the EDTA eluates (tracks 1 and 2) from iC3b columns both before (track 1) and after (track 2) passing the starting material through an H52-Sepharose column. All three bands eluted by EDTA are removed by the H52-Sepharose column (compare track 1 with track 2), indicating that the two larger bands (135000 and 155000 M_r) are associated with the 98000- M_r β -subunit removed by the H52-Sepharose column.

The material from the original extract that bound to the H52-Sepharose is shown in Fig. 3, track 3. The

Fig. 3. Identification of iC3b-binding proteins from PMN

Extracts (1 ml) of ^{125}I -labelled PMN were applied to an iC3b-Sepharose column as described in the Methods and materials section. Material eluted with EDTA (track 1) is shown. Identical PMN extracts were run on an H52-Sepharose column (1 ml) at 6 ml/h and the material passing through the column was applied to the iC3b-Sepharose column. The material eluted from iC3b-Sepharose with EDTA (track 2) is shown. The material binding to the H52-Sepharose column was eluted with pH 2 buffer and analysed (track 3). Similarly the material bound to H52 from a sample which had been pre-adsorbed with iC3b-Sepharose was analysed (track 4). The standards were as described in the legend to Fig. 2.

material that binds to H52 after iC3b-Sepharose chromatography, that is, material which ran through the iC3b-Sepharose column, is shown in track 4. The H52 column binds all three β -subunit-containing proteins (track 3) in the original extract (the 165000- M_r LFA1 α - and the 150000- M_r CR3 α -subunits are poorly resolved on this gel). After passing the PMN extract down an iC3b-Sepharose column, only LFA1 remains to be bound to the H52 column (track 4).

These results show that the three bands eluted from the iC3b column by EDTA are the subunits of CR3 and the protein termed p150,95.

The significance of the binding of p150,95 to iC3b is not at present known. It is not known if it acts as a receptor for iC3b-coated particles, or perhaps for soluble C3 fragments. Cole *et al.* (1985) have reported a C3b-binding protein that does not mediate rosetting with C3b-coated particles, whereas Vik & Fearon (1985) have described a receptor activity on PMN which appears to bind soluble C3 fragments but does not mediate rosetting (PMN do not rosette with C3d-coated erythrocytes). p150,95 may be associated with the cultured monocyte C3d receptor (Inada *et al.*, 1983), which can apparently be inhibited by an anti-(β -chain) monoclonal antibody (Wright *et al.*, 1984).

The 90000- M_r iC3b-binding protein may be associated with the receptor for soluble C3d on PMN reported by Vik & Fearon (1985), which is not dependent on the presence of bivalent cations for activity.

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