

PURIFICATION AND CHARACTERIZATION OF A
MEMBRANE PROTEIN (gp45-70) THAT IS A COFACTOR
FOR CLEAVAGE OF C3b AND C4b

BY TSUKASA SEYA, JERRY R. TURNER, AND JOHN P. ATKINSON

*From the Howard Hughes Medical Institute Laboratories and the Department of Medicine,
Division of Rheumatology, Washington University School of Medicine,
St. Louis, Missouri 63110*

C4b and C3b are components of the membrane-bound and fluid-phase C3 convertases, C4bC2a and C3bBb (1), and these bimolecular complexes cleave C3 to C3a and C3b (2, 3). Complement regulatory proteins limit the activity of C4b and/or C3b, reversibly, by accelerating the decay of the C3 convertases (4-7) or, irreversibly, by acting as a cofactor for I-dependent cleavage (4-6, 8). Two plasma proteins, H and C4b-binding protein (C4bp), and two cell-associated molecules, C3b/C4b receptor (CR1) and decay-accelerating factor (DAF),¹ have been identified with such regulatory activity.

Our laboratory has reported that three distinct proteins are isolated by C3 affinity chromatography (9). One of these has been identified as the C3b/C4b receptor or CR1 (M_r ~200,000), and a second as the C3d receptor or CR2 (M_r 140,000). However, the third one, termed gp45-70 because of its relatively broad bands and cell-specific variations in M_r on SDS-PAGE, has not been purified or functionally characterized. This protein is present on human platelets (10), monocytes, and B and T lymphocytes, but not on erythrocytes (8). Though there is no unequivocal evidence that gp45-70 of each cell population is related, these proteins show similar M_r , with one or two broad bands on SDS-PAGE, binding specificity for C3b but not C3d, and similar alterations in M_r with reduction or enzymatic digestions with deglycosidases (9, 11). C3b binding proteins of similar M_r have been reported on rabbit macrophages (12, 13), and more recently on mouse leukocytes (14, 15). Their relationship to human gp45-70, except for a similar M_r and ligand-binding specificity, is unknown.

Preliminary functional studies (16) of gp45-70 indicated that this protein is a cofactor for I-dependent cleavage of C3b. We have purified a protein from several human cell lines that is a cofactor for I-dependent cleavage of C3b, and we show that it is identical to gp45-70.

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¹ *Abbreviations used in this paper:* DACM, *N*-(dimethylamino-4-methyl coumarinyl) maleimide; DAF, decay-accelerating factor; IAA, iodoacetamide; MA, methylamine; MCP, membrane cofactor protein.

Materials and Methods

Complement Components. Human C1s (17), C4 (18), C2 (19), D (20), and B (21) were purified as described. These components were assessed by gel criteria for purity and by fluid-phase classical and alternative pathway assay systems to be certain that they were free of H, C4bp, and C3 (22). C3 was purified as previously described (8). In the assays of cofactor activity of I-mediated cleavage, C3 was further purified by passage through a column of anti-H antibody conjugated to Sepharose and rechromatography on DEAE-Sephacel to eliminate C4bp (22). C3(H₂O) and methylamine (MA)-treated C3 (C3_{MA}) were prepared as previously noted (22). C3(H₂O) and C3_{MA} are hemolytically inactive C3 produced by repetitive freeze-thaw cycles and MA treatment, respectively. C4_{MA} is hemolytically inactive C4 produced by MA treatment. The conversion of C3 to C3(H₂O) was confirmed by the heat-induced autocleavage reaction (23). Human I (24), C4bp (25), and H (26) were purified as described. H was depleted of C3 by rechromatography on QAE-Sephadex. C4bp contained no detectable C3 or H as assessed by radioimmunoassay (22). All of these components were dialyzed against PBS, pH 7.2, and stored in aliquots at -70°C .

Solubilization and Purification of Cofactor Protein from U937. The human monocytic cell line U937 was cultured at 37°C in a 0.5% CO₂ atmosphere in RPMI 1640 with 2 mg glutamine and 10% FCS and antibacterial/mycotic mixture (Gibco Laboratories, Grand Island, NY). The culture media was doubled every other day, and $1-3 \times 10^{10}$ cells were harvested from 20 liters of the media. The media was removed by centrifugation (1,300 g for 8 min). The packed cells (~20 ml) were washed five times with 3 liters of PBS, and finally once with PBS containing 10 mM EDTA, 10 mM iodoacetamide (IAA), and 2 mM PMSF. The washed cells were solubilized by 100 ml of PBS (pH 8.0) containing 10 mM EDTA, 10 mM IAA, 2 mM PMSF, and 1% NP-40. The mixture was allowed to stir for 1 h at room temperature. The solubilized preparation was then centrifuged (4,800 g for 10 min) at 4°C , and the supernatant was collected, dialyzed against 20 mM acetate buffer with 0.1% NP-40, pH 5.0, overnight at 4°C , and stored at -70°C . Solubilization of a human T-cell line, HSB-2, was performed by the same procedure.

The first section of the Results, Table I, and the two subsequent paragraphs present data on the purification of a cell-associated protein with cofactor activity. Problems that were encountered, alternative chromatographic procedures evaluated, and the purification procedure that was finally used are noted below.

A confounding problem was that the protein was found to stick irreversibly to various surfaces, such as membrane filters for ultra-filtration, and chromatographic media. Concentration of the protein using a Diaflo or Centriflo membrane filters resulted in loss of the cofactor activity. The recovery of the cofactor activity from DEAE-Sephacel (cellulose) or protein conjugated to Sepharose (agarose) was <10 and ~70%, respectively. A similar loss of protein was reported in the purification of C4bp (18). >80% of the cofactor protein also bound to heparin-Sepharose, which has been used to purify other complement regulatory proteins (18, 22, 26). However, the recovery of cofactor protein from heparin-Sepharose was only ~50%, and therefore this column was not used. Satisfactory yields and purity were obtained using the four columns as described in the next paragraph.

The starting material was $\sim 3 \times 10^{10}$ NP-40-solubilized cells. This preparation was dialyzed against 20 mM acetate buffer with 0.1% NP-40, pH 5.0, and the precipitate was removed by centrifugation. The supernatant (~300 ml) was applied to a chromatofocusing column (2.0 \times 50 cm). The equilibration buffer was 20 mM acetate with 0.05% NP-40, pH 4.75. The column was washed with the same buffer and then eluted with a linear (20–250 mM) gradient of acetate buffer (pH 4.3) in 10% (vol/vol) poly buffer 74. The fractions containing cofactor activity (Fig. 1) were pooled after dialysis against 10 mM Tris-HCl plus 0.05% NP-40, pH 7.5. The dialyzed material was loaded on a hydroxylapatite (50 ml, high resolution; Calbiochem-Behring Corp., La Jolla, CA). The equilibration and wash buffer was 15 mM Tris-HCl containing 0.05% NP-40, pH 7.5. This column was eluted with 15 mM phosphate and 0.05% NP-40, pH 7.5, and fractions with cofactor activity were pooled. The pooled fractions were loaded on a C3_{MA}-Sepharose (50 ml) column. The equilibration buffer was 10 mM phosphate with 0.05% NP-40, pH 7.2, and the wash

buffer was 20 mM phosphate with 1 mM PMSF and 0.05% NP-40, pH 7.0. The column was eluted with 0.5 M NaCl and 20 mM phosphate with 0.05% NP-40, pH 7.0. The fractions with cofactor activity were pooled and dialyzed against 20 mM NaCl and 20 mM phosphate with 0.05% NP-40, pH 7.0. The dialyzed material was loaded onto a Mono Q column. The equilibration and wash buffer was 20 mM NaCl and 20 mM phosphate buffer containing 0.05% NP-40, pH 7.0. This column was eluted with a linear gradient of 20–500 mM NaCl in the same buffer as used for equilibration. The flow rate was 1 ml/min and 2-ml fractions were collected. It required ~90 min to complete the gradient (see Fig. 2). Except for the chromatofocusing step, which was performed at 4°C, column work was carried out at room temperature.

Determination of Cofactor Activity for I-mediated C₃MA and C₄MA Cleavage. MA-treated and *N*-(dimethylamino-4-methyl coumarinyl) maleimide (DACM)-labeled C₃ (DACM-C₃MA) was prepared as a substrate for I as described (27). Briefly, 10 mg of C₃MA was mixed with 10 µl of 2 mg DACM per milliliter acetone. The mixture was allowed to stand for 15 min at room temperature (22, 26). MA-treated and DACM-labeled C₄, DACM-C₄MA, was prepared by the same method. If DACM-C₃MA was to be also labeled by ¹²⁵I, the sample was mixed with Na¹²⁵I in the iodogen-coated glass tube for 30 min at room temperature. The labeled substrate was dialyzed against PBS and stored in aliquots at -70°C.

5–20 µg of substrate (usually 18 µg of DACM-C₃MA in 10 µl) was mixed with 1 µg of I and 100 µl of each column fraction that had been dialyzed against 20 mM phosphate buffer plus 0.05% NP-40, pH 7.2, or PBS plus 0.05% NP-40, pH 7.2. The mixtures were incubated for 3–10 h and subjected to SDS-PAGE (10% rod gels) (28) after the addition of 30 µl of a 30% 2-MER and 1% SDS solution. Modifications of this protocol were used in experiments to compare the cofactor activity of the various regulatory proteins (legends to Figs. 5–7). Cofactor activity was monitored by depletion of the fluorescent labeled α chain of C₃MA and accumulation of a fluorescent labeled α₁ chain (27). Percent cleavage was calculated from the fluorescent intensity at Em/Ex = 375/485 nm, or by gamma counting of the gel slices as previously described (26, 27). In experiments comparing cofactor activity of proteins, 1 U of activity represents the quantity of cofactor required for cleavage of 1 µg of C₃MA per hour under the incubation conditions outlined in the figure legend.

Protein Concentration. In the absence of NP-40, protein concentration was determined by the absorbance at 280 nm, assuming an absorption coefficient value of one for a 1-cm cuvette at 280 nm for a 0.1% solution. Protein concentration of fractions containing NP-40 was determined by the method of Kumar et al. (29) as previously noted (22), or by estimating the difference of the absorbance at 280 nm between sample fractions and the control buffer, which contained the same amount of NP-40.

Labeling of Cofactor Protein. Purified protein and each partially purified fraction were labeled by the iodogen method, according to the manufacturer's booklet. If necessary, free ¹²⁵I was removed by gel filtration using Sephadex G-75. Surface labeling was performed by a modified method using lactoperoxidase (30, 31).

Isolation of Surface-labeled gp45–70. One-step affinity chromatography was performed using C3(H₂O)-Sephacrose. The details of this procedure are given by Cole et al. (9) and Dykman et al. (30). Briefly, after passing the surface-labeled solubilized sample (0.15 M NaCl plus 1% NP-40, pH 7.4) through IgG-Sephacrose, the sample was diluted three times with distilled water. The diluted sample was mixed with C3(H₂O)-Sephacrose, which had been equilibrated with three times-diluted PBS containing 0.3% NP-40, pH 7.4, and agitated for 2 h at room temperature. The mixture was then packed in the column, washed with the equilibration buffer, and eluted with 0.4 M NaCl, 1% NP-40, and 20 mM phosphate buffer, pH 7.4.

Autoradiography. Autoradiography was performed with XAR-5 film (Eastman Kodak, Rochester, NY) and Cronex intensification screens (Dupont, Wilmington, DE) (9, 30). The gels were dried and exposed at -70°C for 2 d in the case of surface-labeled materials, or for 2 h in the case of purified samples.

Determination of Decay-accelerating Activity for Classical and Alternative C₃ Conver-

TABLE I
Purification of a Cofactor Protein of U937

Purification method	Total protein	Cofactor activity*	Recovery	Purification (fold)	Visualiza- tion of band in SDS-PAGE‡
	mg	U	%		
Solubilized preparation	5,823	1,193	100	1	—
Supernatant of acid and low ionic strength precipitation	180	1,039	87	28	—
Chromatofocusing	21	978	82	233	—
Hydroxylapatite	3	955	80	1,646	—
C3 _{MA} -Sephacrose	(0.1) [§]	692	58	33,773	±
Mono Q	(0.02)	668	(56)	163,044	+

These data are derived from a purification in which 3×10^{10} cells were used, and the data are representative of four such purifications.

* Cofactor activity at each step was estimated as described in Materials and Methods. 1U is defined as the cofactor activity (fluorescent method) required for cleavage of 1 μ g of C3_{MA} per hour under the above conditions. Small amounts of CR1 would be present in the solubilized preparation and in the supernatant of the acid and low salt precipitation step. CR1 is eliminated at the chromatofocusing step.

‡ Fractions run on gels were assessed by protein staining or labeling (see Materials and Methods).

§ Numbers in parentheses represent the sum of the protein or cofactor activity (fluorescent method) determined by adding the amount of activity in each fraction.

tases. Fluid-phase assay systems were used (22). The buffer used was PBS with 0.05% NP-40, pH 7.2. For determination of inhibitory activity of the alternative C3 convertase, 20 μ l of a solution containing variable (0.1–0.5 pmol) amounts of C3(H₂O), 20 pmol of B, variable (20–100 pmol) amounts of C3, and 50 nmol of NiCl₂ was mixed with 100 μ l (200 ng) of cofactor protein. After a 2-min incubation at 37°C, the reaction was initiated by adding 0.2 μ g of D. H (200 ng) was used as a positive inhibitory control. For determination of inhibitory activity of the classical C3 convertase, 15 μ l of solution containing 0.1–0.5 pmol of C4, 10 pmol of C2, 40 pmol of C3, and 100 μ mol of MgCl₂ was mixed with 100 μ l (200 ng) of cofactor protein or C4bp. CIs (0.1 μ g) was added to initiate the reaction in the same manner as for the alternative pathway.

At timed intervals, 50 μ l samples were withdrawn and mixed with 50 μ l of the precipitating reagent (Upjohn Diagnostics, Kalamazoo, MI) and 20 μ l of BSA solution containing 100 mg BSA per milliliter PBS. The samples were then centrifuged at 12,000 rpm (Microfuge 12; Beckman Instruments, Inc., Fullerton, CA) for 5 min at 4°C. The aliquots of the supernatants were transferred to the tubes to be used in the RIA. The assay procedure for determination of the released C3a was according to instructions in the kit (812-321-0021; Upjohn).

Results

Purification of Cofactor Protein. A purification procedure was devised based on our recent experience with a multiple column method for the isolation of CR1 (22), the acidic pI of gp45–70 (11), the iC3 and C3b ligand affinity of gp45–70 (9), and preliminary evidence suggesting that solubilized preparations of U937 possessed cofactor activity (16). The purification was followed by assessing cofactor activity of column eluates and/or *M_r* of radiolabeled eluates on autoradiographs of SDS-PAGE. The details of the final procedure are presented in Materials and Methods and Table I, and outlined below.

Supernatant (~300 ml) from solubilized U937 cells ($\sim 3 \times 10^{10}$), containing

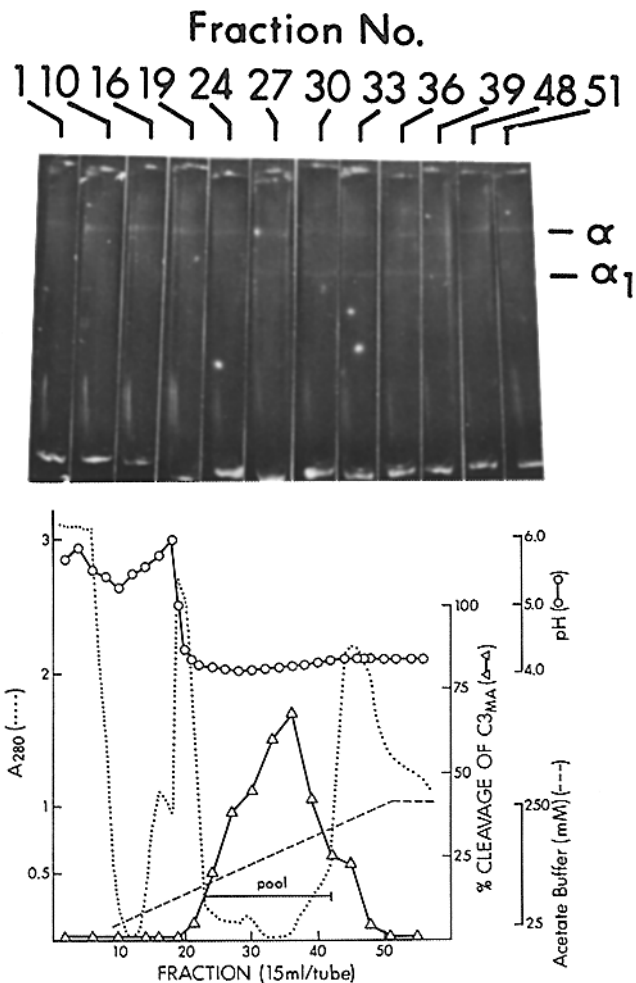
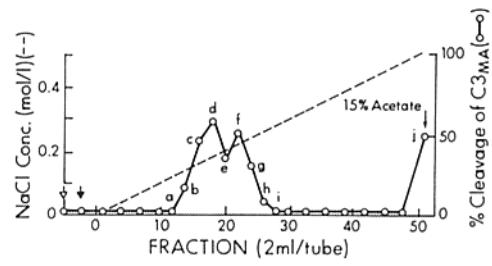
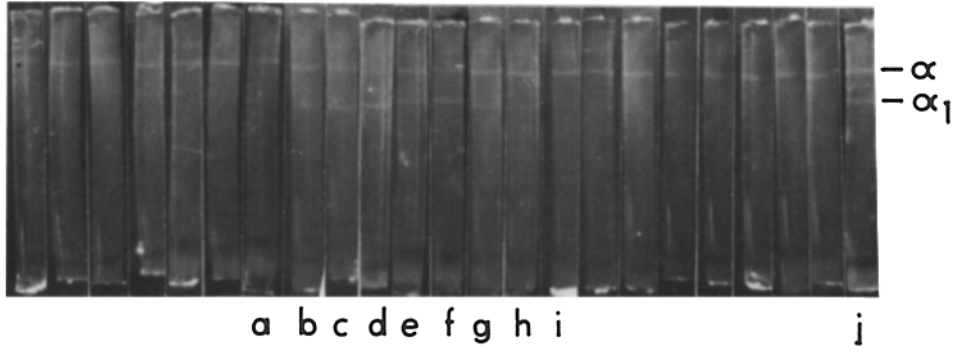


FIGURE 1. Elution profile of the chromatofocusing column. The protein concentration was determined by assessing the absorption of the fractions at 280 nm. For determination of cofactor activity, 5 μ g of DACM-C_{3MA} was incubated for 6 h at 37°C with 1 μ g of I and 100 μ l of each fraction, which had been dialyzed against PBS and 0.05% NP-40. Aliquots were electrophoresed on SDS-PAGE (10% acrylamide) and the degree of cleavage of the α chain into α_1 fragment was monitored by measuring the fluorescent intensity (inset electrophoretogram) (27). Percent cleavage was calculated from the following formula: $F(\alpha_1)/[F(\alpha) + F(\alpha_1)]$ where $F(\alpha)$ and $F(\alpha_1)$ are the fluorescent intensities of α and α_1 band, respectively.

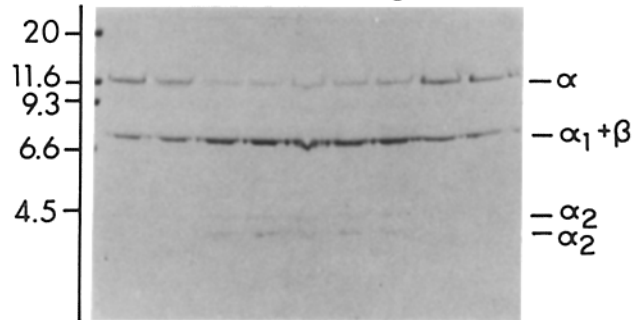
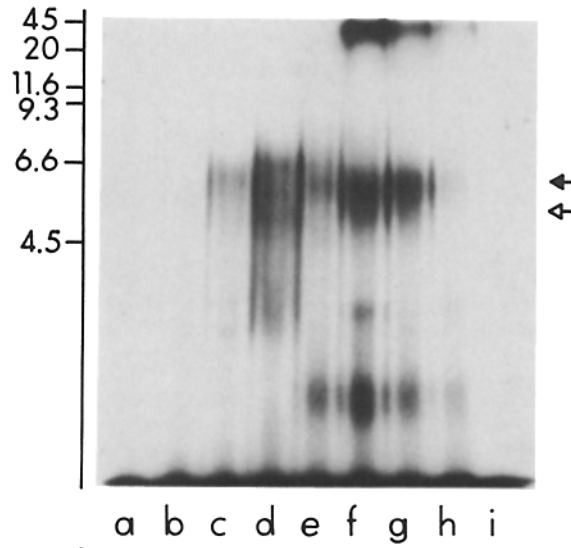
5,823 mg of protein was centrifuged (31,000 g for 20 min) at 4°C, and the supernatant was collected. The supernatant, containing 180 mg of protein, was subjected to chromatofocusing. The column was washed with 300 ml of 0.02 M acetate buffer, and eluted with linear gradient of acetate buffer. The peak of cofactor activity for I-dependent C_{3b} or C_{3MA} cleavage eluted from this column between the two major protein peaks (Fig. 1).

The fractions with cofactor activity were pooled and dialyzed against 10 mM Tris-HCl overnight at 4°C. The dialyzed sample was then applied to the hydroxylapatite column. The column was washed with the equilibration buffer and

A



B
Mr ($\times 10^{-4}$)



eluted with 15 mM phosphate buffer. ~300 ml of eluate was pooled from fractions containing >80% of the initial cofactor activity and 20 mg of protein. This material was applied to a column of C3_{MA} conjugated to Sepharose-6 B, (50 ml, 4 mg of C3_{MA} per milliliter of Sepharose). The column had been equilibrated with 10 mM phosphate buffer. Under these conditions, the cofactor activity was completely and reproducibly bound to this column. After being washed with 20 mM phosphate buffer, the column was eluted with 0.5 M NaCl. ~60% of the initial cofactor activity was present, and 100 µg of protein were obtained. The fractions having the cofactor activity were pooled and dialyzed against 20 mM NaCl plus 20 mM phosphate buffer. The sample was applied to a Mono Q HPLC column (Pharmacia Fine Chemicals, Piscataway, NJ) (Fig. 2*a*) using an HPLC system (Millipore, Bedford, MA). The column was eluted with a linear (20–500 mM) NaCl gradient. Cofactor activity eluted between 140 and 220 mM. These fractions were radiolabeled and analyzed by SDS-PAGE (Fig. 2*b*). The cofactor activity is composed of several species, some of which can be separated in this step. The four to six fractions with cofactor activity contained 20 µg of protein and 60% of the cofactor activity of the initially solubilized material, representing a 163,000-fold purification.

The fractions eluted from the Mono Q column (Fig. 2*b*) with cofactor activity were of similar M_r (45,000–70,000) to that previously described for gp45–70 of U937 (arrows in Fig. 2*b*; see also Fig. 4 and Cole et al. [9]). Therefore, although it is most likely that the protein of M_r 45,000–70,000 is responsible for the cofactor activity, labeled bands at the top of the gel and at an M_r of ~20,000 are present in some fractions. The material at the top of the gel is usually observed in the late-eluting fractions, but its presence does not correlate with cofactor activity. Likewise, the band at M_r ~20,000 is only observed in some of the preparations (for example, it is not present in the column eluates shown in Fig. 3*a*), and again, even when it is present there is no correlation with cofactor activity. The apparent extension of the major band to below M_r 45,000 in fraction d was not observed in other purifications (see below and Fig. 3).

To further evaluate this protein, it was isolated by the same procedure from two additional sources of U937. Interestingly, the cofactor protein from each source of U937 showed similar but not identical M_r and the relative quantity of

FIGURE 2. Elution profile of the Mono Q HPLC column. The eluate of C3_{MA}-Sepharose was dialyzed (Table I) and applied to the Mono Q column (*A*, bottom). An open arrow indicates the pass through fractions and a solid arrow represents the wash fraction. Percent cleavage of DACM-C3_{MA} was determined (*A*, top) by SDS-PAGE (10% acrylamide) as described in Fig. 1. Each fraction (30 µl) obtained from the Mono Q column was assessed for cofactor activity. The fractions possessing such activity are labeled *a–j*. 50 µl of the fractions *a–i* were labeled by ¹²⁵I using the Iodogen method. The samples were subjected to SDS-PAGE (10% acrylamide) (*B*, top), and after drying the gel was exposed at –70°C for 2 h. Solid arrow, upper band of surface-labeled gp45–70; open arrow, lower band of surface-labeled gp45–70. Bio-Rad M_r markers were used as standards. The identity of the labeled material at <30,000 M_r in lanes *f* and *g* is unknown (See Fig. 3 and Results). Also, a variable amount of counts were observed at the top of the gel in lanes *f* and *g*. 50 µl of the column fractions (*a–i*) were incubated for 5 h at 37°C with 1 µg of I and 10 µg of DACM-C3_{MA} (*B*, bottom). Aliquots were reduced and analyzed by SDS-PAGE (10% acrylamide). α2 bands with M_r of 43,000 and 46,000 were present and are similar in M_r to what others (4–6, 8) have reported for the first cleavage of C3b by I and other cofactors.

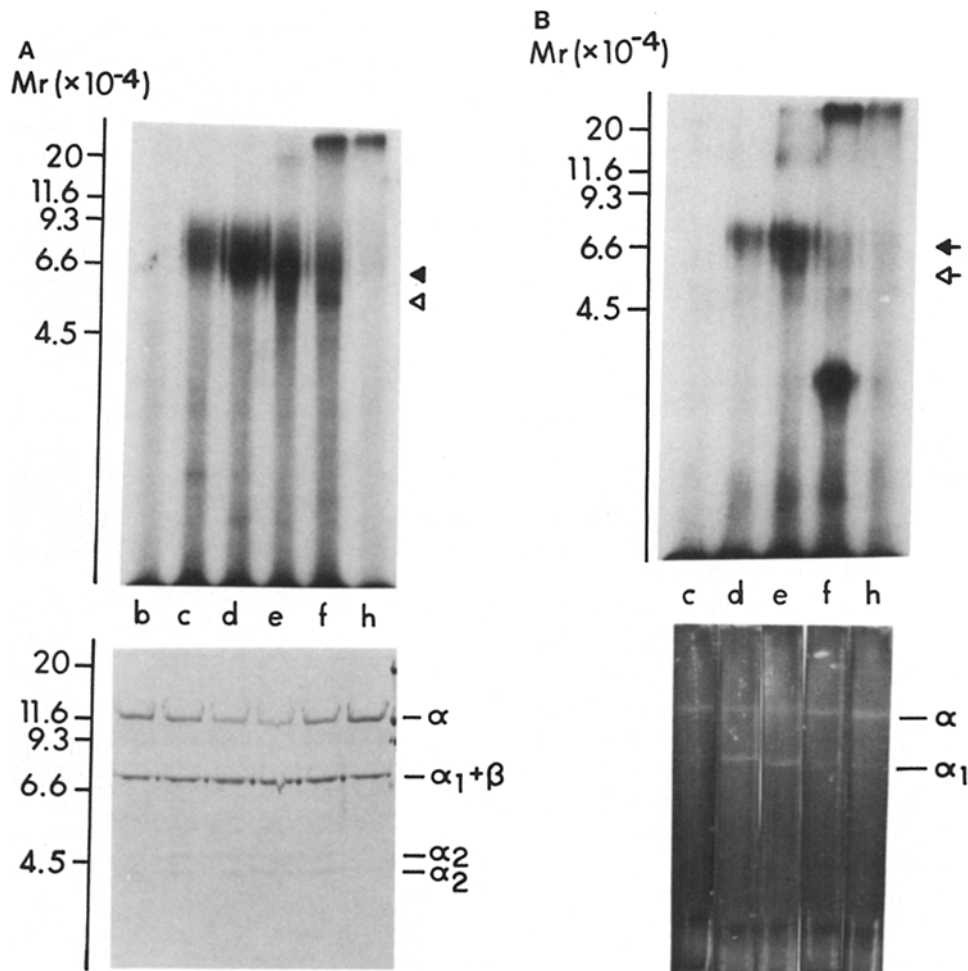


FIGURE 3. Mono Q column elution profile of cofactor protein from two additional strains of U937. 50 μ l of the fractions encompassing the area of cofactor activity were labeled and subjected to SDS-PAGE as per Fig. 2b (A and B, top). The position of surface-labeled gp45-70 is marked by solid arrow (upper band) and open arrow (lower band). Fraction number is indicated alphabetically, and these fractions are the same as those in Fig. 2. 50 μ l (A, bottom) or 100 μ l (B, bottom) of the column fractions were incubated for 5 h at 37°C with 1 μ g of I n 10 μ g of DACM-C3_{MA}. The aliquots were analyzed by slab (A), as described in Fig. 2b, or rod (B) SDS-PAGE (10% acrylamide). In B, cofactor activity was estimated by fluorescent intensity of α_1 -fragment.

the major M_r components varied (Fig. 3, a and b). The M_r of the cofactor protein in Fig. 3a was higher (50,000–85,000) than that of the usual gp45–70 of U937. However, the labeled proteins seen in Figs. 2b and 3a and b were eluted in the same fractions of the Mono Q column and showed a cofactor activity that correlated with the quantity of labeled protein with an M_r of 50,000–85,000. In the experiment shown in Fig. 3a, cofactor activity is present in fractions that do not contain the contaminating proteins. In the experiment of Fig. 3b, a correlation is seen between protein at $\sim 70,000 M_r$ and cofactor activity (i.e. prominent cofactor activity in tracks d and e but not in f, which has the labeled band at

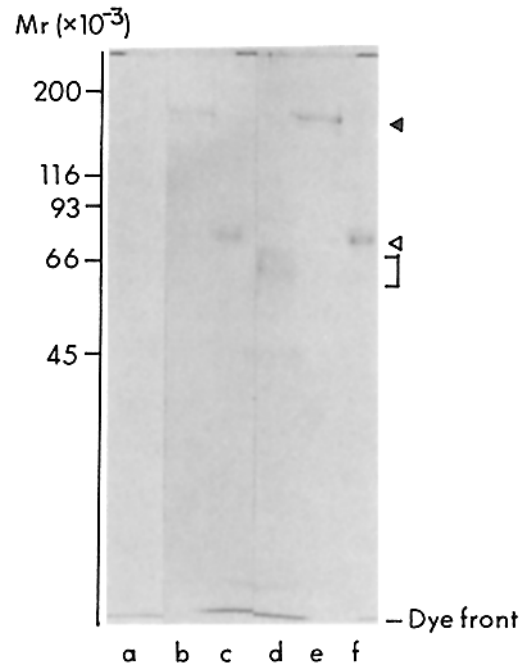
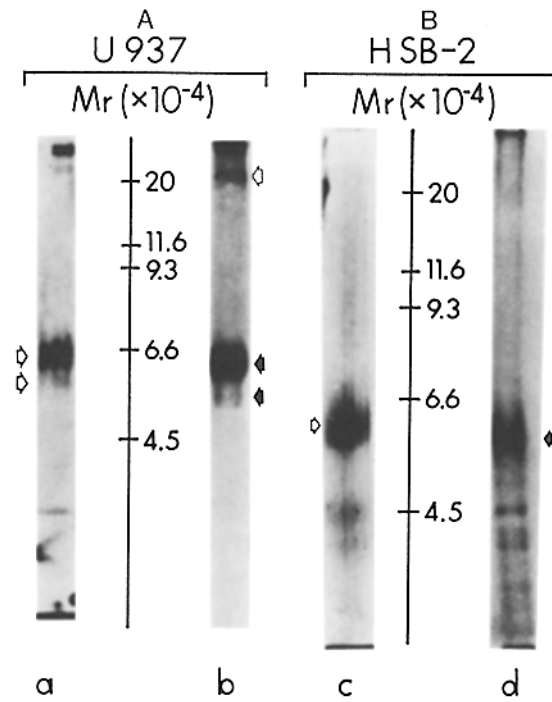
~20,000). These elution profiles of the cofactor protein from the Mono Q column always showed a tendency for the cofactor protein with the slower M_r to elute earlier than the faster-migrating proteins. The main peak of the cofactor protein is reproducibly observed between fractions c and g (160 mM to 200 mM NaCl concentration). As noted, fraction f usually contains larger (M_r ~450,000) and smaller (M_r ~20,000) proteins.

Comparative Analysis of Cofactor Protein Purified from U937 and gp45-70 Isolated from Surface-labeled U937. The above purification procedure allowed the isolation of a protein with cofactor activity with an M_r similar to gp45-70, a membrane protein of U937 that binds C3b and C3(H₂O) (9, 11). To further compare this protein to gp45-70, U937 cells were surface-labeled and solubilized (9). gp45-70 was isolated by C3(H₂O) affinity chromatography and the eluate was evaluated on SDS-PAGE. Purified cofactor protein from the same strain was electrophoresed on the same gel (Fig. 4a). The stained cofactor protein shows a similar mobility and a broad two band pattern with a predominant upper band in SDS-PAGE as the labeled gp45-70. Also, note that this protein-stained material is derived from fraction g of Fig. 2b, and that the radiolabeled bands (Fig. 2b) and protein bands (track a, Fig. 4a) are the same. Multiple such comparisons have indicated that the bands defined by radiolabeling the column fractions identified the same proteins in approximately the same concentrations as those visualized by protein staining.

Under reducing conditions, each subcomponent of the cofactor protein, like gp45-70 (9), shifts (~8,000 increase in M_r) to an upper position (not shown), suggesting that the cofactor protein is composed of a single polypeptide chain containing intrachain disulfide bridges.

Isolation of Cofactor Protein from Another Cell Line and Comparison to gp45-70 of Its Surface-labeled Material. A second cell line was evaluated to determine whether the purification procedure would lead to the isolation of a protein with cofactor activity which aligned with gp45-70 (purified from surface-labeled material by affinity chromatography). On the T-cell line, HSB-2, gp45-70 is a single broad band with a mean M_r of 62,000 (9). The purification (exactly as that for U937) of a protein with cofactor activity was performed on two occasions, and a cofactor protein with the same binding and elution characteristics as the cofactor protein of U937 was isolated. The purified cofactor protein of HSB-2 was compared to gp45-70 of surface-labeled HSB-2 that was isolated by affinity chromatography (Fig. 4b). The cofactor protein aligned with gp45-70 under nonreducing and reducing (not shown) conditions, and the protein-stained band was predominantly a single band with a mean M_r of 62,000. These results, taken together with those for U937, indicate that for these two cell lines, the purified cofactor protein and gp45-70 have the same M_r .

Another example of a protein stain of the purified cofactor protein is shown in Fig. 4c. In this figure, 2 or 4 μ g of cofactor protein (derived from HSB-2), H, and C4bp are compared on one gel. While at 4 μ g, all three complement regulatory proteins are easily visualized, at 2 μ g, cofactor protein is not seen, but C4bp and H are clearly visible. The result is in part secondary to the fact that gp45-70 is a broader band than C4bp or H. However, in multiple such compar-



isons it appears that this protein is also not as efficiently stained by Coomassie R as C4bp, H, or marker proteins (32).

Further Evaluation of Cofactor Activity. The purpose of these experiments was to evaluate the cofactor activity of the purified protein for I-mediated C3_{MA} and C4_{MA} cleavage (Figs. 5–7), and to compare the activity of the various fractions eluting from the Mono Q column. C3_{MA} and C4_{MA} were incubated with fractions of the purified cofactor protein and I. H and C4bp were used as positive controls in the C3_{MA} and the C4_{MA} cleavage studies, respectively. The C3_{MA} cleavage products produced by cofactor protein plus I were identical in M_r to those of H plus I; that is, α chain of C3_{MA} was cleaved into $\alpha 1$ (M_r 75,000) and $\alpha 2$ (M_r 46,000 and 43,000) (4). In the absence of I, cofactor protein had no proteolytic activity toward C3_{MA} or C4_{MA}. Also, the two (early- vs. late-eluting) fractions had similar specific activities.

The protein of U937 also serves as factor for the I-mediated cleavage of the α chain of C4_{MA} (Fig. 6, *left*) but required a higher concentration. The cleavage product of the α chain was located between the trace amount of the α' chain and the β chain. The fluorescent electrophoretogram shows that this cleavage product was derived from the α chain (Fig. 6, *right*). Based on these data, and judging from M_r , the product yielded by I plus cofactor protein is iC4_{MA}, first described by Nagasawa et al. (24). Higher concentrations of C4bp yielded C4c and C4d, while cofactor protein produced only trace amounts, even with a six-fold increase in cofactor protein and excess I (Fig. 6, *middle*).

Fig. 5 suggests that the cofactor protein is a more efficient cofactor than H. Further experiments in which cofactor protein and H (Fig. 7) or cofactor protein and C4bp (not shown) were compared indicate that on a weight basis, cofactor protein is ~50 times more effective than H in the case of the first cleavage of C3_{MA}, while it is slightly weaker than C4bp in the case of the first cleavage of C4_{MA}. Cofactor protein is not able to efficiently mediate the second cleavage of fluid-phase iC3_{MA} (Fig. 5) or iC4_{MA} (Fig. 6, *middle*).

In other experiments (designed as per Figs. 5 and 7), the cofactor activity of various fractions eluting from the Mono Q column was compared (not shown). The activity correlated with the quantity of protein eluting between 50,000 and

FIGURE 4. Comparison by SDS-PAGE of cofactor protein (MCP) and gp45–70. Using U937 (A), 500 ng of fraction g (Fig. 2b) was electrophoresed (track a) together with the eluate of an iC3 affinity column purification of ¹²⁵I surface labeled U937 (track b). The gel was first stained by Coomassie R followed by silver staining. The lane containing gp45–70 was cut out and the autoradiogram (b) obtained as described in Materials and Methods. Open arrow in autoradiogram (b), CR1; closed arrows, gp45–70; open arrow in silver-stained gel (a), cofactor protein. The identity of the stained material at the top of gel in track a is unknown but does correspond to labeled material in Fig. 2b, fraction g. In multiple similar comparisons, the same bands were identified by radiolabeling or protein staining of column fractions. Using HSB-2 (B), 500 ng of purified cofactor protein was added to lane c (open arrow). An autoradiograph of the eluate of an iC3 affinity column purification of surface-labeled HSB-2 is in track d (gp45–70 marked by filled arrow). The experimental procedure was identical to that of A. The lighter bands with M_r of 45,000 or less are usually not present (9) and their identity is unknown. Comparison of the cofactor protein of HSB-2 with C4bp and H (C). 2 μ g of purified cofactor protein (a), H (b), and C4bp (c), and 4 μ g of cofactor protein (d), H (e), and C4bp (f), were mixed with 30% 2-ME and subjected to SDS-PAGE (10% acrylamide). The gels were stained by Coomassie R. Filled arrow, H; open arrow, C4bp; brackets, cofactor protein.

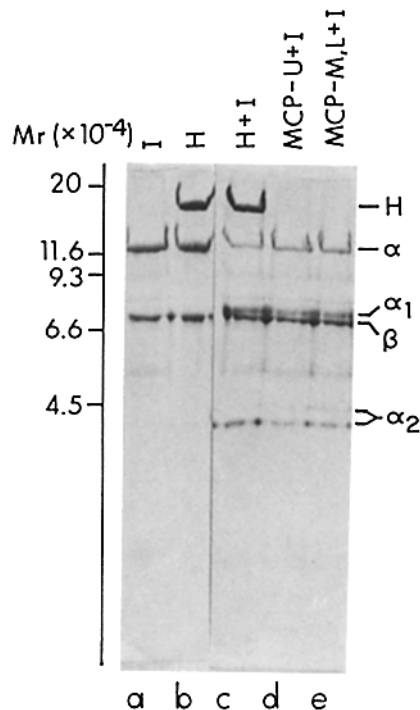


FIGURE 5. Cofactor activity for I-mediated cleavage of $C3_{MA}$. Samples containing cofactor protein were dialyzed against PBS, pH 7.2, with 0.05% NP-40. $C3_{MA}$ (10 μ g) was mixed with (a) 1 μ g of I, (b) 10 μ g of H, (c) 1 μ g of I and 10 μ g of H, (d) 1 μ g of I and 200 ng of upper part of cofactor protein (MCP-U) (fraction d of Fig. 3A), and (e) 1 μ g of I and 200 ng of middle and lower parts of cofactor protein (MCP-M+L) (fraction g of Fig. 2). The mixtures were incubated for 3 h at 37°C. 40 μ l of a solution containing 10% SDS and 30% 2-ME was added, and the samples subjected to SDS-PAGE (10% acrylamide) and stained by Coomassie R. H, α chain of C3, β chain of C3, and the cleavage products derived from the first cleavage of the α chain are marked.

80,000 M_r , and thus, despite variability in M_r and charge, no differences in specific activity were detected among the fractions. These results are in accord with the data shown in Figs. 2, 3, and 5, in which the specific activity of each fraction was similar. Also, the specific activity of the cofactor protein of U937 and HSB-2 was similar (not shown).

Effect of Cofactor Protein on Decay of Alternative and Classical C3 Convertases. Dialyzed samples of cofactor protein, pretreated with 2 mM PMSF, 5 mM EDTA, and 5 mM IAA and adjusted to 200 ng per 100 μ l, were mixed with the alternative pathway complement components and $NiCl_2$. Cofactor protein was not a decay accelerator of the alternative pathway, but rather behaved as a modest C3 convertase potentiator (Fig. 8a). Although under these conditions this effect was reproducible, higher concentrations of cofactor protein did not produce increasing stability of the convertase. Cofactor protein or an equivalent amount of C4bp was mixed with the classical pathway components and $MgCl_2$. Cofactor protein did not accelerate the decay of the classical C3 convertase while C4bp was an efficient decay accelerator (Fig. 8b).

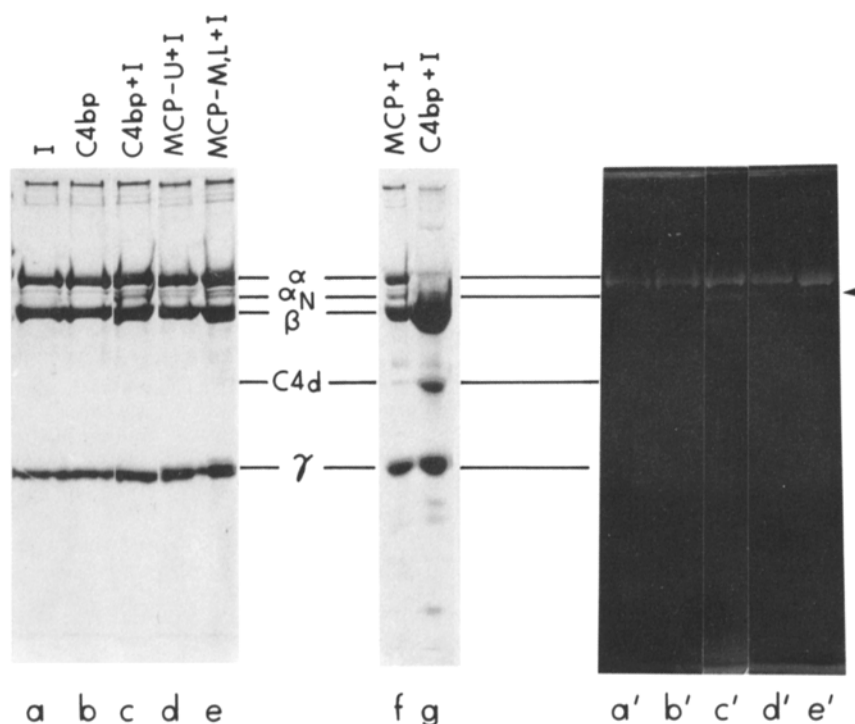


FIGURE 6. Cofactor activity for I-mediated $C4_{MA}$ cleavage. Samples were dialyzed as for Fig. 5. (Left) DACM- $C4_{MA}$ (20 μ g) was mixed with (a) 1 μ g of I, (b) 400 ng of C4bp, (c) 1 μ g of I and 400 ng of C4bp, (d) 1 μ g of I and 400 ng of cofactor protein (MCP) upper part (fraction d of Fig. 3A), and (e) 1 μ g of I and 400 ng of middle and lower parts of cofactor protein (fraction g of Fig. 2). The mixtures were incubated for 6 h at 37°C and subjected to SDS-PAGE (10% acrylamide) under reducing conditions. Lanes a-e were stained by Coomassie R. Before staining, the gel was observed (right) under UV illumination (365 nm). The fluorescent electrophoretogram (lanes a'-e' correspond to a-e of the left panel) is shown. The solid arrow points to α -N, the amino-terminal fragment of the α chain of $C4_{MA}$. DACM- $C4_{MA}$ (20 μ g) were mixed with (f) 3 μ g of I and 2.4 μ g of cofactor protein (fraction f in Fig. 2) or (g) 3 μ g of I and 5 μ g of C4bp. The mixtures were incubated for 6 h at 37°C and subjected to SDS-PAGE (10% acrylamide). The gel was stained with Coomassie R.

Discussion

The goal of this study was to purify gp45-70 in quantities sufficient for functional analysis. Because of the lack of an antibody to this protein, its biologic characteristics, including an acidic pI, C3b-ligand specificity, and pattern and M_r on SDS-PAGE were used in devising a purification procedure. However, the most important biologic characteristic, which was central to the success of this endeavor, were our preliminary experiments suggesting that gp45-70 of U937 cells possessed cofactor activity (16, 33).

The initial step in the procedure, low ionic strength precipitation under acidic conditions, was based on the knowledge that gp45-70 had an acidic pI and therefore would probably not be denatured by such treatment. The first column (chromatofocusing) also took advantage of the acidic pI of the molecule. In this step, the protein was also separated from CR1, since this membrane glycoprotein has a pI of ~ 7.0 (22). The C3(H₂O) affinity step was based on the ligand

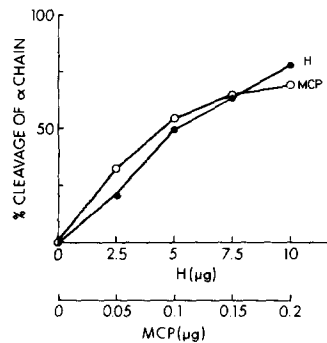


FIGURE 7. Comparison of the cofactor activity of MCP to that of H. Variable amounts of H and MCP were mixed with radiolabeled DACM-C3_{MA} (18 μg) and I (1.2 μg) at a final volume of 200 μl and incubated for 3 h at 37°C under the same conditions as noted in Materials and Methods. Reaction was stopped by adding 10 μl of 10% SDS and 30% 2-ME, and the mixture was then subjected to SDS-PAGE. Percent cleavage was determined by counting the gel fragments corresponding to the α2 band. The counts in the gel band containing the α2 fragment generated in the presence of excess I and H was used as the 100% value (22).

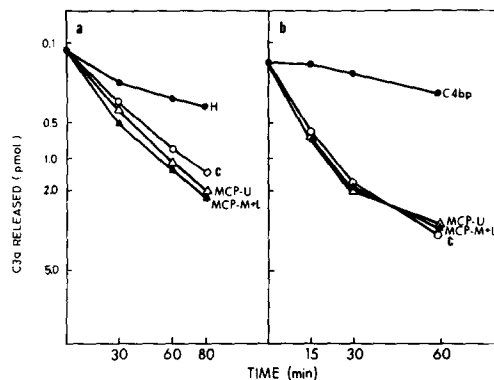


FIGURE 8. Effect of cofactor protein on convertase activity. Alternative pathway C3 convertase (a). The samples, containing constant amounts of C3(H₂O), B, C3 and NiCl₂, were mixed with 200 ng of H, 200 ng of the upper part of cofactor protein (MCP-U) (fraction d of Fig. 3a), 200 ng of middle and lower parts of cofactor protein (MCP-M+L) (fraction g of Fig. 2) or PBS, pH 7.2, with 0.05% NP-40 (C, control). After adding D, generation of C3a was determined at timed intervals (22). Classical pathway C3 convertase (b). The samples, containing constant accounts of C4, C2, C3, and MgCl₂, were mixed with 200 ng of C4bp, 200 μg of upper part of cofactor protein (MCP) (fraction d of Fig. 3a), 200 ng of middle and lower parts of cofactor protein (MCP) (fraction g of Fig. 2) and PBS, pH 7.2, with 0.05% NP-40 (C, control). After adding CIs, C3a generated was determined as for Fig. 7.

specificity of gp45-70 of U937 (9). The Mono Q column provided an efficient means for concentrating the protein, separation of the major species comprising the protein, and removal of a variable quantity of minor contaminants. We routinely obtained ~30 μg of cofactor protein from 3×10^{10} U937 or HSB-2 cells and, based on the percent recovery, this calculates to a mean of ~10,000 molecules/cell.

Having isolated a protein possessing cofactor activity, we then addressed several questions: (a) Utility of this approach in isolating a protein with cofactor activity from other cell lines. (b) The relationship of the purified cofactor protein to gp45-70. (c) The function of this protein, especially in comparison to that of

the previously identified complement proteins with regulatory activity for the C3 convertases.

In regard to the first question, the same procedure (elution profiles from each column were nearly identical) led to the purification of a protein with cofactor activity from two additional sources of U937 and from HSB-2. The relationship of the cofactor protein to gp45-70 was initially addressed by comparing their M_r . For U937, this comparison was complicated by the variability in M_r of gp45-70 on this cell line. Depending on the source of the U937, the cofactor protein purified from U937 had a slightly different M_r . However, from a given strain the surface labeled gp45-70 and the cofactor protein aligned by SDS-PAGE. To further address this second question, cofactor protein and gp45-70 of HSB-2 were compared. This cell line has a single band pattern for gp45-70 (9). The cofactor protein was also a single band and aligned with gp45-70.

The preceding M_r analysis combined with the very acidic pI of both molecules and their ligand binding specificity indicate that gp45-70 and cofactor protein are identical molecules. Further evidence in support of this conclusion has recently been obtained in our laboratory by demonstrating that the carbohydrate composition of gp45-70 (11) and cofactor protein (our unpublished data) are similar. Also, for reasons that are yet to be determined, gp45-70 is variably glycosylated, and differences in sugar composition largely account for the cell-specific as well as the strain-specific (U937) variation in M_r (9, 11).

The protein isolated from U937 and HSB-2 was a cofactor for the first but not the second cleavage of C3b and C4b. It is a remarkable cofactor for C3b cleavage, being ~50 times more efficient than H, which had been reported (22) to be the most potent cofactor for C3b cleavage. In the case of C4b, the activity for the first cleavage was less than that for equal quantities of C4bp. These results are in accord with previous studies of gp45-70 indicating that it bound iC4 or C4b but with a lower affinity than C3(H₂O) (9, 16). C4bp is also a cofactor for the I-mediated first but not second cleavage of C3b (8, 26), and has a lower efficiency than H (4, 8). Based on these data, the natural ligand of cofactor protein is probably C3b.

To allow a functional comparison of the species with different M_r (50,000-85,000), we purposely did not pool the fractions eluting from the Mono Q column. The cofactor protein isolated from U937 cells consisted of several species within this M_r range. In addition to their charge differences, the earlier eluting bands had a higher M_r than the later eluting species. No differences in specific activity were detected among the fractions or between cofactor protein of U937 and HSB-2. Also, none of the fractions possessed decay-accelerating activity. With these comparative data in hand, the various fractions can now be pooled and the protein further purified and characterized.

The activity profile of this cofactor protein is summarized and compared to the other regulatory proteins in Table II. Interestingly, the activity of cofactor protein is complementary to those of DAF (7, 34) (Table II). DAF lacks cofactor activity but decays both C3 convertases (7, 35), while the protein characterized in this report has cofactor activity but no decay-accelerating activity. We suspect that the reason these two proteins are widely distributed on human peripheral blood cells relates to their synergistic activity profile such that complement

TABLE II
Comparison of Activity Profile of Membrane Cofactor Protein to that of Other Complement Regulatory Proteins

Activity	Membrane associated			Fluid phase	
	CR1	DAF	MCP	H	C4bp
C3b binding	+	—*	+	+	+
Cofactor for first cleavage of C3b	+	—	+	+	+
Cofactor for second cleavage of C3b	+	—	—	± [‡]	—
Alternative complement pathway decay	+	+	—	+	—
C4b binding	+	—*	+	—	+
Cofactor for first cleavage of C4b	+	—	+	± [‡]	+
Cofactor for second cleavage of C4b	+	—	—	± [‡]	+
Classical complement pathway decay	+	+	—	—	+
Rosette formation	+	—	—*		

* Unpublished data.

[‡] Relatively inefficient at promoting this cleavage.

activation on autologous tissue is inhibited. An important question, the relative intrinsic (cofactor activity for C3b on the same cell) vs. extrinsic (cofactor activity for C3b bound to other cells or to soluble and particulate immune complexes) activity of this regulatory protein remains to be determined.

A purification procedure has been devised that permits isolation in reasonable yields and purity of an additional cell-associated complement regulatory protein. The structure of this protein indicates that it is identical to a previously identified C3b-binding membrane protein of human PBL, platelets, and cell lines, termed gp45–70 (9, 10). The functional studies reported herein indicate that it has strong cofactor activity for the I-mediated first cleavage of C3b, but no decay-accelerating activity. Based on these results, we suggest that gp45–70 be renamed membrane cofactor protein (MCP). The activity profile of MCP is unique for the regulatory proteins, whose activity is directed at the C3 convertases and suggests an important role for MCP in controlling complement activation on autologous tissue.

Summary

Based on preliminary evidence indicating that a cell-associated protein of U937 (a human monocyte-like cell line) possessed cofactor activity and was not the C3b/C4b receptor, we sought to further characterize this protein. A sequential four-column purification procedure was devised that includes C3(H₂O) affinity chromatography to isolate in reasonable yields and purity a cell-associated protein of U937 and several other human cell lines. Based on its pattern and *M_r* on SDS-PAGE, acidic pI, and ligand specificity, it is identical to a recently described C3(H₂O) or C3b-binding membrane glycoprotein of human PBL and cell lines; having no presently identified function, it was termed gp45–70. After purifying this protein, we determined its functional capabilities and compared them to those of the other complement proteins with regulatory activity directed at components comprising the C3 convertases. This protein was the most efficient (50 times that of H) yet-described cofactor for the I-mediated first cleavage of C3b. It also was

a cofactor for the first cleavage of C4b, but was not as efficient as C4bp. The second cleavage of C3b and C4b was not efficiently mediated. It had no ability to accelerate decay in the classical or alternative pathway C3 convertases. Based on this unique activity profile and ability to be surface labeled, we have renamed this molecule membrane cofactor protein (MCP). We suggest that this protein plays a major role in preventing autologous complement activation.

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