Biochem. J. (1989) 264, 581–588 (Printed in Great Britain)

Functional properties of membrane cofactor protein of complement

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Membrane cofactor protein (MCP or gp45–70) of the complement system is a cofactor for factor I-mediated cleavage of fluid-phase C3b and C3b-like C3, which opens the thioester bond. In the present study the activity of MCP was further characterized. Unexpectedly, in the absence of factor I, MCP stabilized the alternative- and, to a lesser extent, the classical-pathway cell-bound C3 convertases and thereby enhanced C3b deposition. Soluble MCP, if added exogenously, hardly functioned as cofactor for the cleavage of erythrocyte-bound C3b to iC3b; i.e. its activity, compared with the cofactor activity of factor H, was inefficient, since less than 10% of the bound C3b was MCP-sensitive. Further, exogenously added soluble MCP was also a weak cofactor for the cleavage of C3b bound to zymosan. Likewise, factor I, in the presence of cells bearing MCP, cleaved fluid-phase C3b inefficiently. These results imply that MCP has very little extrinsic cofactor activity for factor I. In contrast, exogenously added MCP and factor I mediated efficient cleavage of erythrocyte-bound C3b if the concentration of Nonidet P40 was sufficient to solubilize the cells. Interestingly, soluble MCP and factor I degraded C3b attached to certain solubilized acceptor membrane molecules more readily than others. The cleavage reaction of fluid-phase and cell-bound C3b by soluble MCP and factor I produced iC3b, but no C3c and C3dg. These and prior data indicate that soluble MCP has potent cofactor activity for fluid-phase C3b or C3b bound to solubilized molecules, but acts inefficiently towards C3b on other cells. This functional profile is unique for a C3b/C4b binding protein and, taken together with its wide tissue distribution, suggests an important role for MCP in the regulation of the complement system.

INTRODUCTION

During activation of the complement system, C4b and C3b attach covalently to targets such as cell membranes. Deposited C4b and C3b serve both as the focus for the assembly of the C3 convertases and as ligands for C3b receptors. Successful formation of bimolecular enzyme complexes between C4b and C2 or between C3b and factor B leads to the cleavage of C3. C3a is released and C3b is deposited on the target. A major goal of this part of the complement-activation process is to amplify the deposition of C3b on foreign material, but inhibit its deposition on 'self' tissues.

Most human peripheral-blood-cell populations are endowed with three C3b/C4b-binding proteins, namely C3b/C4b receptor or complement receptor type one (CR1) (Fearon, 1979), decay-accelerating factor (DAF) (Nicholson-Weller *et al.*, 1982), and membrane cofactor protein (MCP) (Seya *et al.*, 1986). CR1 is a receptor and possesses decay-accelerating and factor I-cofactor activity (Fearon, 1979; Iida & Nussenzweig, 1981). CR1 is involved in controlling the convertases assembled on other targets, that is, extrinsic regulatory activity (Medof *et al.*, 1982). DAF is a potent decay accelerator of the convertases, but has no cofactor activity (Nicholson-Weller *et al.*, 1982; Pangburn *et al.*, 1983). DAF has been shown to be an inhibitor of convertase formation on the same cell surface to which the DAF is anchored, but to have little influence on the formation or stability of the convertases assembled on other cells (Medof *et al.*, 1984). DAF therefore is said to dissociate the convertases in an intrinsic fashion, resulting in protection of host cells. This role of DAF is well illustrated by the excessive sensitivity to complement-mediated lysis of erythrocytes of DAF-deficient patients with paroxysmal nocturnal haemoglobinuria (Pangburn *et al.*, 1983; Nicholson-Weller *et al.*, 1983).

MCP was first identified as an iC3/C3b-binding membrane protein with an M_r of 45000–70000 (initially designated as gp45–70) (Cole *et al.*, 1985). It was subsequently purified and shown to be a potent cofactor for factor I-mediated cleavage of fluid-phase C3b (Seya *et al.*, 1986). This protein did not accelerate the decay of the C3 convertases. Therefore its functional profile was complementary to that of DAF (Seya *et al.*, 1986). Like DAF, MCP is widely distributed, being found on all

Abbreviations used: complement components are named according to the World Health Organisation (1981) recommendations, except for C3ma and iC3ma, which are methylamine-treated C3, a C3b-like product, and an iC3b-like product that was made from C3ma by the treatment with factor I and its cofactor respectively; sheep erythrocytes sensitized with haemolysin (EA) and the intermediate cells, and buffers for haemolytic assay, were prepared as described by Mayer (1961); E*, erythrocyte ghosts; C4bp, C4b-binding protein; CR1, C3b/C4b receptor; DACM, the fluorescent thiol reagent *N*-(dimethylamino-4-methylcoumarinyl)maleimide; DAF, decay-accelerating factor; IAA, iodoacetamide; MCP, membrane cofactor protein (gp45–70); NP-40, Nonidet P40; NS, normal saline; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride; SCR, short consensus repeat (characteristic of C3b/C4b binding proteins); the definitions/compositions of VBS, GVB^{++}, EDTA-GVB, $GGVB^{++}$., EDTA-GGVB, NS and PBS are given in the text.

peripheral-blood cells except for erythrocytes (Seya *et al.*, 1988) and on epithelial and endothelial cells and fibroblasts (McNearney *et al.*, 1989). Moreover, except for the glycolipid anchor of DAF, the structure of MCP and DAF are similar, consisting of four short consensus repeats (SCRs) (Lublin *et al.*, 1988) and their structural genes are less than 300 kb apart on the long arm of chromosome one at the regulator-of-complement-activation gene cluster (Bora *et al.*, 1989). In the present work we have further characterized the cofactor activity of MCP towards C3b.

MATERIALS AND METHODS

Preparation of buffer, cells and cellular intermediates

The following buffers were prepared: veronal-buffered saline (VBS), which contains 0.145 M-NaCl/0.05 M-sodium barbiturate, pH 7.3; VBS/0.1% gelatin/0.15 mM-$CaCl_2$/1.0 mM-$MgCl_2$ (GVB^{++}); VBS/0.1% gelatin/100 mM-EDTA (EDTA-GVB); iso-osmotic glucose VBS containing 1.0 mM-$MgCl_2$ and 0.15 mM-$CaCl_2$ ($GGVB^{++}$); glucose VBS, containing 10 mM-EDTA (EDTA-GGVB); normal saline (NS) (pH 7.4), containing 0.15 M-NaCl; and phosphate-buffered saline (PBS) (pH 7.4) containing 0.15 M-NaCl, 1.92 mM NaH_2PO_4 and 8.7 mM-K_2HPO_4.

Sheep erythrocytes (E) and rabbit IgM antibody (A) (haemolysin) (Cordis, Miami, FL, U.S.A.) were used to prepare sensitized sheep erythrocytes (EA). The EA were treated sequentially with guinea-pig C1 and human C4 at concentrations sufficient to give 2000 C4b sites/cell. EAC142 were prepared by the addition of sufficient C2 to EAC14 to yield more than 1.0 haemolytic site/cell (Mayer, 1961).

In some experiments, ghosts (E*) of the intermediates were prepared by the addition of water containing the proteinase inhibitors [10 mM-EDTA, 25 mM-iodoacetamide (IAA) and 1 mM-phenylmethanesulphonyl fluoride (PMSF)] and then washed in PBS.

Complement components and complement regulatory proteins of plasma origin

Guinea-pig C1 was purchased from Cordis. Human complement components, including C4 (Nagasawa & Stroud, 1980), C2 (Nagasawa & Stroud, 1977*b*), D (Volanakis *et al.*, 1977), B (Seya *et al.*, 1985*b*), and C3 (Nagasawa & Stroud, 1977*a*) and human complement regulatory proteins factor H (Seya & Nagasawa, 1985), C4bp (Nagasawa *et al.*, 1982) and factor I (Nagasawa *et al.*, 1980) were purified as described in the references cited. These proteins were stored in aliquots at −70 °C, except for C3 and C4, which were stored at 4 °C and were dialysed against the appropriate buffer before use.

Cell-associated complement regulatory proteins and their antibodies

Human MCP was purified from HSB-2 cells as described by Seya *et al.* (1986). Human DAF was purified from a butanol extract of human erythrocytes by a four-step sequential column-chromatographic procedure (Seya *et al.*, 1987). Octyl glycoside rather than NP-40 was employed as a solubilizer in order to facilitate determination of the protein concentration at 280 nm. Human CR1 was purified from ghosts of human erythrocytes of donors homozygous for the most common phenotype, CR1-A (Seya *et al.*, 1985*a*). Rabbit polyclonal antibodies to MCP, DAF and CR1 were prepared as described (Seya *et al.*, 1985*a*, 1987, 1988), and their monospecificities were further characterized (T. Seya, S. Nagasawa & J. P. Atkinson, unpublished work).

Radiolabelling of proteins, surface-labelling of cells and SDS/PAGE followed by autoradiography

Proteins were labelled with ^{125}I by the Iodogen method (Pierce Chemical Co., Rockford, IL, U.S.A.). Before labelling MCP, DAF or CR1, the samples were concentrated and the NP-40 concentration was reduced to less than 0.005% by MonoQ f.p.l.c. chromatography. After labelling, free iodine was removed by dialysis.

SDS/PAGE was performed by the method of Laemmli (1970). Gels were stained with Coomassie Blue R-250, destained, and fixed with methanol/acetic acid and dried. Autoradiography was performed with XAR-5 film (Kodak, Rochester, NY, U.S.A.) and Cronex intensification screens (du Pont, Wilmington, DE, U.S.A.).

Assay for factor I-mediated C3b cleavage (cofactor activity)

The assay employing a fluid-phase system was performed as described by Seya *et al.* (1986).

The assay for a solid-phase system employed EAC3b or ghosts of EAC3b (E*AC3b). EAC3b were prepared by the addition of ^{125}I-labelled C3 to EAC14b plus C2 or to EAC4b3b plus B and D. EAC3b prepared via the classical pathway had ~2000 C3b sites and 6000–8400 c.p.m./10^7 cells. EAC3b activated via the alternative pathway had 6000 C3b sites. To prepare the ghosts, these cells were lysed in water containing 1 mM-PMSF and washed once with EDTA-GVB and twice with NS. ^{125}I-C3b bearing cells (3×10^7 cells if prepared by the classical pathway or 1×10^7 cells if prepared via the alternative pathway) were used as a substrate for factor I.

EAC3b or E*AC3b were mixed with 1 μg of factor I and 0.5–4.0 μg of factor H or 15–200 ng of MCP, in a total volume of 160 μl of PBS containing various amounts of NP-40. The reaction was stopped and cells were solubilized by the addition of 10 μl of 3% NP-40, 10 μl of 10% SDS, 45 μl of the dye solution for SDS/PAGE and 5 μl of 2-mercaptoethanol. Analysis was by SDS/PAGE followed by autoradiography.

When HSB-2 or K562 cells were used as a source of cofactor, 5×10^7 cells were mixed with E*AC3b (~6,000 C3b sites/cell; 1×10^7 cells) and factor I (1 μg) in RPMI 1640 medium without NP-40. After 60 min at 37 °C, the mixture was centrifuged (800 ***g*** for 5 min) and the supernatants containing the ghosts were removed. The ghosts were recovered by centrifugation (10000 ***g*** for 10 min), solubilized and analysed. Solubilized preparations of these cells were also used as a source of cofactor. In this case, the cells (5×10^7 cells) were solubilized with 1% NP-40, and the supernatants were recovered after acidic treatment (Seya *et al.*, 1986). The cofactor assay was performed in the presence of ~0.4% NP-40 (which is sufficient to solubilize the co-existent E*AC3b) as described above. In other experiments, HSB-2 or K562 ($1 \times 10^{7\sim8}$ cells) were incubated with factor I (1 μg) and fluorescent-labelled fluid-phase C3ma (5 μg) in RPMI/0.2% bovine serum albumin for up to 180 min at 37 °C. The supernatants were recovered by centrifugation and the C3ma cleavage was analysed by SDS/PAGE and spectrofluorimetry (Seya & Nagasawa, 1985).

Another substrate, C3b-zymosan, was prepared by

using purified B, D and ^{125}I-labelled C3 (Fearon & Austen, 1977). In some experiments the C3b-zymosan was subsequently treated with hydroxylamine (Law *et al.*, 1979) and then with 25 mM-Tris/HCl/0.5% SDS, pH 7.5. The recovered C3 fragments were analysed by SDS/PAGE and autoradiography.

Determination of C3 convertase activity

E*AC14b (10^7 cells; 600 C4b sites/cell) were mixed with 0–200 ng of DAF or MCP and then incubated with 2 μg of C2 and 4 μg of ^{125}I-C3 in a total volume of 200 μl of GVB** at 37 °C. After 30 min, EDTA-GVB was added to the samples. The ghosts were recovered by centrifugation (2000 ***g***, 10 min), washed once with 0.5 M-NaCl, twice with NS, and solubilized as described above. The deposition of C3b was evaluated from the radioactivity in the solubilized preparations and by SDS/PAGE followed by autoradiography.

To determine the activity of the alternative-pathway C3 convertase, the E*AC4b3b (10^7 cells with 2800 C3b sites/cell) were mixed with 2 μg of B, 0.5 μg of D, and 4 μg of ^{125}I-C3 in 200 μl of GVB^{++} in the presence of DAF or MCP.

RESULTS

Modulation of the C3 convertase by MCP

The first question we asked was 'does exogenously added MCP, in the absence of factor I, modulate the cell-bound C3 convertases (Fig. 1)?' MCP enhanced C3b deposition, especially by the alternative-pathway convertase, a result compatible with its higher avidity for C3b than for C4b (Cole *et al.*, 1985; Seya & Atkinson, 1986; Seya *et al.*, 1986). As expected, DAF inhibited C3b deposition by both convertases. The M_r and pattern of the deposited C3b were not altered by MCP or DAF. The gel analysis also confirmed that C3b deposition was inhibited by DAF and enhanced by MCP.

Cofactor activity of purified MCP for cell-bound C3b

E*AC3b bearing ~ 6000 C3b sites/cell were incubated with soluble MCP or factor H and factor I. Under conditions in which the concentration of NP-40 was < 0.005% and thus the cells were intact, > 90% of the radioactivity remained bound to the erythrocytes (results not shown). Therefore these two cofactors do not release C3c. Some of the cell-bound C3b, though, was converted

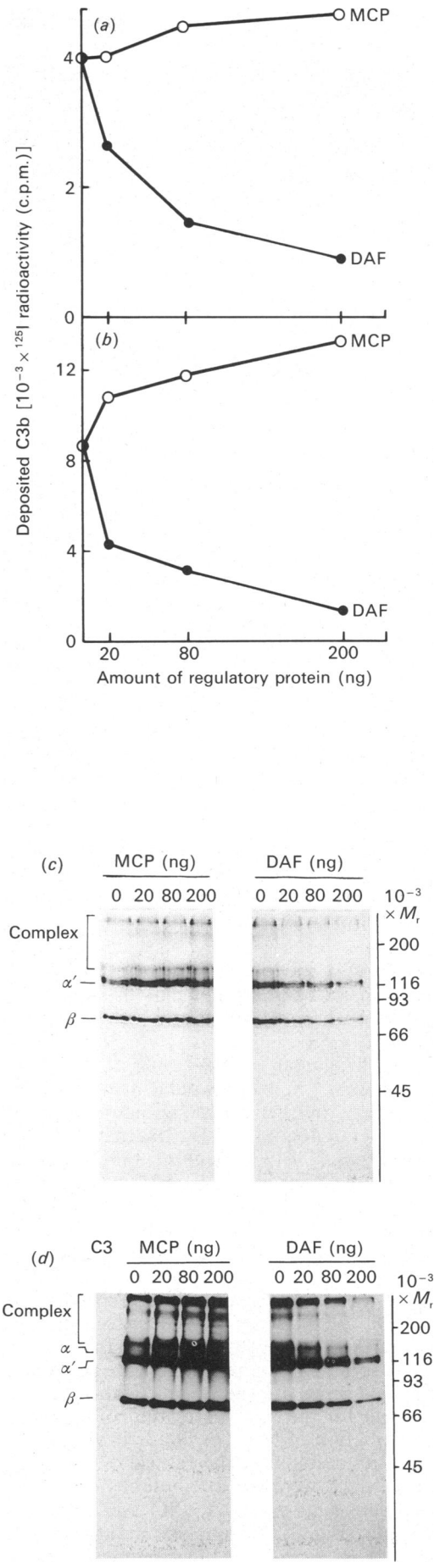

Fig. 1. Effect of MCP and DAF on cell-bound C3 convertases

(*a*) E*AC14b were mixed with various amounts of purified MCP or DAF and then incubated with C2 and ^{125}I-C3 for 30 min at 37 °C (see the Materials and methods section). The quantity of deposited C3b was calculated on the basis of radioactivity bound to the ghosts. (*b*) E*AC4b3b were mixed with various amounts of MCP or DAF and then incubated with D, B, and ^{125}I-C3 for 30 min at 37 °C. (*c*) After incubation, cells of (*a*) were solubilized. The solubilized material was reduced and analysed by SDS/PAGE (10% gel). (*d*) After incubation, erythrocyte ghosts of (*b*) were solubilized. The solubilized material was reduced and analysed by SDS/PAGE (10%). α, β, and α′ in (*c*) and (*d*) refer to the chains and chain fragment of C3b. The brackets denote C3b complexed to acceptor molecules. Fig. 1 is representative of three experiments. Bio-Rad M_r markers were used as standards.

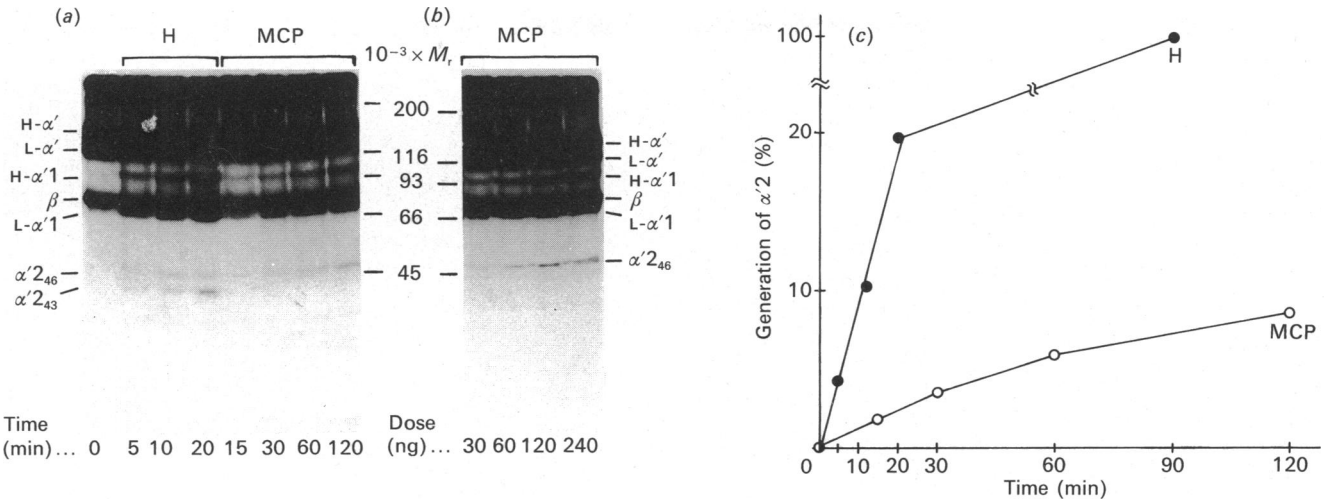

Fig. 2. Analysis of the extrinsic cofactor activity of MCP

(*a*) E*AC3b were incubated with factor I (1 μg) and MCP (60 ng) or factor H (3 μg), in a total volume of 160 μl, for the indicated times at 37 °C. These amounts of MCP and factor H are functionally equivalent for cleavage of fluid-phase C3b. The buffer was PBS/0.005% NP-40, pH 7.4. The reaction was terminated by the addition of 10 μl of 10% (w/v) SDS and 2 μl of 2-mercaptoethanol. The samples were subjected to SDS/PAGE (10% gel). These autoradiographs were exposed in order to illustrate the formation of the α′2 46 and 43 kDa (α′2$_{46}$ and α′2$_{43}$) fragments. Shorter exposures were used to analyse the higher-M_r fragments (see also Figs. 5 and 6). (*b*) E*AC3b were incubated with 1 μg of factor I and variable amounts of MCP in a total volume 160 μl for 60 min at 37 °C. α′, β, α′1, α′2$_{46}$, α′2$_{43}$, were identified according to standard nomenclature (see Ross *et al.* 1982): H-α′ (135 kDa), α′-chain of C3b that formed a complex with a membrane protein of ~ 25 kDa; L-α′ (110 kDa), α′-chain of C3b that probably formed a complex with a small molecule (see Law & Levine, 1977); H-α′1 (95 kDa) and L-α′1 (68 kDa) represent fragments of H-α′ and L-α′ respectively (see Figs. 5 and 6). (*c*) Densitometric analysis for determination of the quantity of the α′2 fragment generated. E*AC3b were treated with factor I and MCP or factor H as in (*a*). The resultant autoradiogram was analysed by densitometric scanning. The percentage generation of α′2 in each time point was calculated by assuming that all C3b was converted into iC3b by incubation with factor H (3 μg) and factor I (1 μg) for 90 min at 37 °C. (Under these conditions the 135 kDa and 110 kDa bands were not detectable.)

into iC3b by the action of factor H or MCP and factor I (Fig. 2). Factor H and factor I converted all of the C3b into iC3b within 90 min (Fig. 2*c*). iC3b generation was increased a little, but not in a dose-dependent manner, by the addition of excess MCP (Figs. 2*b* and 2*c*) or with more prolonged incubations (up to 120 min). Even in these experiments, though, the maximal quantity of C3b converted into iC3b by MCP and factor I was less than 10% (Fig. 2*c*).

The α′ 2 fragment of cell-bound iC3b produced by MCP and factor I had a molecular mass of 46 kDa (Fig. 2*a*,*b*), whereas that generated by factor H and factor I was a doublet of 46 and 43 kDa (Harrison & Lachmann, 1980; Sim *et al.*, 1981; Ross *et al.*, 1982). During longer incubations, cleavage by MCP and factor I of the 46 kDa fragment did not occur on the cell-bound C3b. Under fluid-phase or solubilized conditions, on the other hand, factor H, C4bp, CR1 and MCP all produced both the 46 and 43 kDa fragments, together with factor I (results not shown). In these experiments and, as reported previously (Seya *et al.*, 1986), soluble MCP was ~ 50-fold more efficient for the first cleavage of C3b than is factor H only when the 'solubilized' conditions were provided.

In the next series of experiments, C3b-zymosan served as the target for the analysis of the cofactor activity of MCP. Incubation of MCP or factor H with factor I and C3b-zymosan resulted in the release of a small number of counts, which were not attributable to C3c, on SDS/PAGE, whereas, as expected, CR1 and factor I released many counts (Fig. 3). Gel analysis indicated that only in the case of CR1 was a 140 kDa fragment, characteristic of C3c, released (results not shown). Moreover, a 27 kDa protein was observed under reducing conditions and this is one fragment of C3c (Medof *et al.*, 1982). Thus MCP or factor H and factor I did not cleave C3b to C3c + C3dg. However, whereas factor H converted ~ 80% of the releasable C3b into iC3b, MCP converted less than 20%, regardless of the concentration of MCP (results not shown).

Cofactor activity of endogenous MCP for cell-bound C3b or fluid-phase C3ma

HSB-2 cells, which possess MCP but no CR1 (Cole *et al.*, 1985), were added to E*AC3b or fluid-phase C3ma and the results analysed as for Fig. 2. iC3b or iC3ma was barely detectable (Table 1). Similar results were obtained with K562 cells (Table 1). By contrast, both cell-bound C3b and fluid-phase C3ma were degraded efficiently under the conditions with NP-40 sufficient to solubilize the cells (Table 1). Polyclonal anti-MCP antibody blocked these C3b and C3ma cleavages by factor I and MCP partially, although polyclonal anti-DAF and −CR1 did not (Table 1).

Effect of detergent on the cofactor activity of MCP

Preliminary results had indicated that purified MCP was a potent cofactor for factor I-dependent cleavage of fluid-phase C3b, and the present studies demonstrated that MCP is a weak cofactor for C3b bound to E or zymosan. Consequently erythrocytes bearing C3b were solubilized, and the ability of MCP and factor I to cleave C3b bound to acceptor molecules was examined. MCP

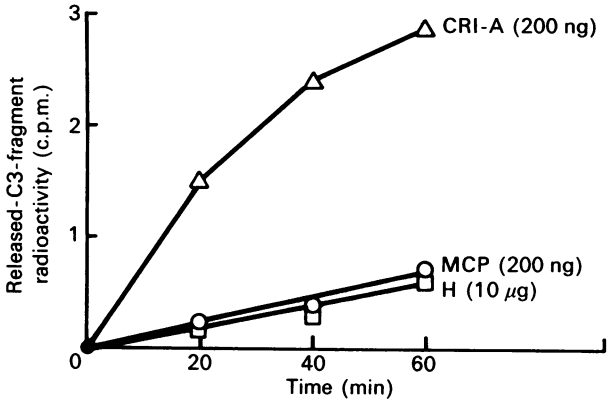

Fig. 3. Liberation of C3 fragments from zymosan by treatment with factor I and cofactors

^{125}I-C3b zymosan (50 μl; 2500 c.p.m./μl) was incubated with factor I (2 μg) and CR1-A (the predominant phenotype of CR1), MCP or factor H in a total volume of 200 μl of GVB^{++} at 37 °C. To assess the background, factor I and the C3b-zymosan without cofactor were incubated under the same conditions (results not shown). The reaction was terminated with 2.5 μl of 20% acetic acid. The supernatants were recovered by centrifugation and counted for radioactivity. The specific release of C3 derivatives was obtained by subtracting each value of the supernatant from that of the corresponding backgrounds. On analysis by SDS/PAGE and autoradiography the sample with CR1 and factor I exhibited a 140 kDa band corresponding to C3c (results not shown). No C3c band was observed in the samples with factor H and MCP, although a little radioactivity was detected in the supernatant at 60 min (results consistent with those of Venkatech *et al.*, 1984).

exhibited potent cofactor activity if the concentration of NP-40 was 0.05% or greater, i.e. sufficient to solubilize the cells (Fig. 4). In contrast, MCP cleaved < 5% of the C3b bound to the cells if the concentration of NP-40 was < 0.01%, a concentration that did not disrupt the integrity of the membranes. In multiple experiments the concentration of NP-40 that caused dissolution of the cells coincided with that which produced cleavage of C3b. (It is noteworthy that it is not a direct effect of NP-40 on MCP, as cleavage of fluid-phase C3b by MCP and factor I was not affected by the NP-40 concentration.)

This processing of C3b deposited on E was further examined in the presence or absence of 0.1% NP-40 (Fig. 5). Only if the buffer contained NP-40 did α-chain degradation fragments accumulate in the presence of MCP and factor I. These same fragments were also observed in the factor H + factor I samples, but were less prominent. In contrast with MCP and factor I, the presence or absence of NP-40 did not modulate the cleavage of E*AC3b by factor H and factor I. C3b deposited by the alternative or classical pathway was similarly degraded.

Next, the dose-dependency and kinetics of the degradation of the two most prominent forms of C3b–acceptor complexes on E (C3b–135 kDa designated 'H-α'' and C3b–110 kDa designated 'L-α'') were analysed (see Figs. 2, 5 and 6). Factor H or MCP and factor I cleaved C3b–110 kDa (L-α' of Figs. 2, 5 and 6) to a 68 kDa fragment, consistent with the attachment of C3b to a low-M_r acceptor molecule of the erythrocyte membrane. The cleavage of C3b–135 kDa or (H-α' of Figs. 2, 5 and

Table 1. Generation of iC3b or iC3ma using MCP-bearing cells as a source of cofactor activity

Factor I (1 μg) and DACM-C3ma (5 μg) or E*AC3b (18420 c.p.m., ~ 6000 sites/cell) were incubated with the indicated cells (5×10^7) or the solubilized materials as sources of MCP (see the Materials and methods section). The final NP-40 concentration was ~ 0.4% in the samples containing the solubilized cell preparations, so that the co-incubated substrates were also solubilized. In the fluid-phase system, the percentage conversion into iC3ma was determined as described by Seya & Nagasawa (1985). In the case of E*AC3b, the percentage conversion of 135 kDa into 95 kDa was determined by densitometric scanning. iC3b (iC3ma)-generating activity was blocked partially by the anti-MCP. (The reason of this incomplete inhibition by the antibody is unknown.) The other antibodies did not affect iC3b (iC3ma) generation.

Source of cofactor	iC3b or iC3ma generation (%)	
	Fluid-phase C3ma	E*AC3b
HSB-2, intact	3.0	4.1
K562, intact	2.6	4.8
HSB-2, solubilized	62.2	> 98.0
K562, solubilized	61.3	> 98.0
HSB-2, solubilized		
+Anti-MCP (20 μg)	24.6	48.8
+Anti-DAF (20 μg)	66.0	> 98.0
+Anti-CR1 (20 μg)	60.1	> 98.0

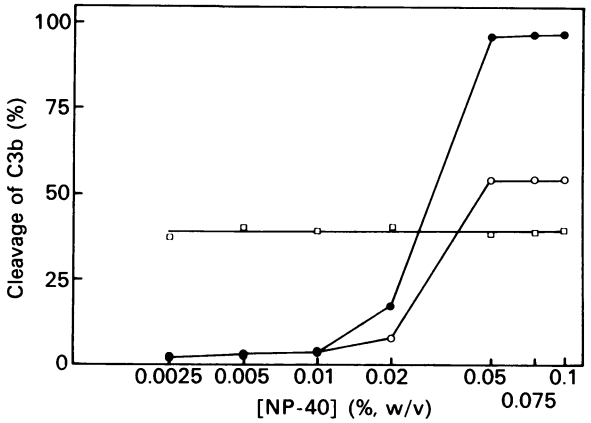

Fig. 4. Effect of NP-40 concentration on cofactor activity of MCP for cell-bound and fluid-phase C3b

E*AC3b of fluid-phase C3b (almost the same amounts as in E*AC3b) were incubated for 45 min at 37 °C with factor I (1 μg) and MCP (40 ng) in 120 μl of PBS containing various concentrations of NP-40. The samples were reduced and analysed by SDS/PAGE, followed by autoradiography. The autoradiogram was scanned and percentage cleavage of C3b was calculated by using the following formula:

Percentage cleavage = sample $A(\alpha'2)$/control $A(\alpha'2)$

where A stands for the absorbance of the fragment. The control ($\alpha'2$) was prepared by treatment of C3b with excess factor I and factor H. ○, ●, Cleavage of E*AC3b (○) and H-α' (●). High-M_r α' (including H-α') consists of an α'-chain of C3b and a membrane protein. □, Fluid-phase cleavage of C3b.

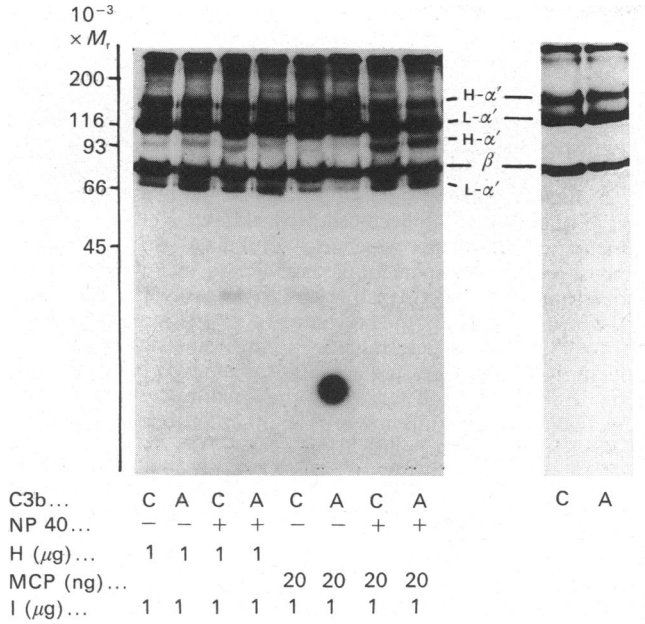

Fig. 5. Cleavage of cell-bound C3b by MCP + factor I or factor H + factor I in the presence or absence of NP-40

E*AC3b was prepared via the classical- or the alternative-pathway C3 convertases (see the Materials and methods section). About 20000 c.p.m. of the substrate was incubated with constant amounts of factor I and factor H, or factor I and MCP for 30 min at 37 °C. The buffer used in this assay was PBS (indicated as NP-40 ' − ' or PBS/0.2% NP-40 (indicated as NP-40 ' + '). MCP used in this assay was extensively dialysed against PBS containing 0.002% NP-40. The samples were solubilized with SDS and run on SDS/PAGE. Abbreviations: C, E*AC3b prepared by the classical pathway; A, E*AC3b prepared by the alternative pathway.

6) by MCP generated a 95 kDa fragment, consistent with the α-chain of C3b being bound to an acceptor molecule with an M_r of ~25 kDa. This interpretation of the degradation of C3b–135 kDa and C3b–110 kDa is supported by the concomitant increase in 95 kDa and 68 kDa fragments as the 135 kDa and C3b–110 kDa bands decrease. This degradation profile of the α′ chain of C3b–135 kDa and C3b–110 kDa is shown schematically in Fig. 6(*d*).

Whereas factor H + factor I cleaved C3b–135 kDa and C3b–110 kDa to a similar extent, there was preferential cleavage of C3b–135 kDa by MCP + factor I (Figs. 6*a*, 6*b* and 6*c*). Most of the C3b–135 kDa was degraded by 60 ng of MCP, whereas most of the C3b–110 kDa remained uncleaved. Likewise, in samples containing 2 μg of factor H or 75 ng of MCP and a constant amount of factor I (Figs. 6*a* and 6*b*), MCP was a more efficient cofactor for C3b–135 kDa than for C3b–110 kDa. This point was further confirmed by densitometric scanning of the autoradiograph (Fig. 6*c*).

DISCUSSION

The purpose of the present study was to analyse the cofactor activity of MCP towards C3b. In a fluid-phase system, MCP had been previously shown to be a 50-fold more efficient cofactor for factor I-mediated cleavage of fluid-phase C3b than H, but not a decay accelerator of the C3 convertases (Seya *et al.*, 1986). The present experiments further our knowledge about the functional activity of MCP in three respects.

1. Stabilization of the cell-bound C3 convertases

An unanticipated observation was that MCP possessed C3 convertase-potentiating activity, especially for the alternative-pathway enzyme. In preliminary experiments with fluid-phase convertases (Seya *et al.*, 1986) there were indications of this positive modulatory activity, and the present analysis establishes this point. Three kinds of C3bBb potentiators have been described, namely Ni^{2+} (Fishelson *et al.*, 1983), properdin (Pensky *et al.*, 1968) and C3 nephritic factor (Daha *et al.*, 1976). These factors bind to the convertase complex, stabilize it, and thereby prolong its half-life. Presumably, by binding to C3b, MCP converts the complex into a less labile form. The physiological meaning of this activity of MCP is unclear, since factor I would be present in a physiological system. However, this result establishes that MCP can bind to the C3b or C4b in the convertase and thereby modulate its activity.

2. Cofactor activity of MCP

Purified MCP or solubilized cell extracts bearing MCP have previously been shown to possess potent cofactor activity for C3b (Seya & Atkinson, 1986; Seya *et al.*, 1986; Yu *et al.*, 1986). As had been reported (Seya & Atkinson, 1986), the polyclonal anti-MCP we prepared blocked ~60% of cofactor activity of MCP in the fluid-phase assay system. The reason for this incomplete inhibition of the antibody, even in the purified system, is unsettled.

The new results relative to functional activity of MCP are as follows. First, purified MCP added to erythrocytes or zymosan-bearing C3b is a poor cofactor. Secondly, MCP-bearing cells were inefficient cofactors for fluid-phase C3ma or erythrocytes bearing C3b. The last two points strongly suggest that MCP is not primarily an extrinsic cofactor. Thirdly, purified MCP has a potent cofactor activity for C3b bound to acceptor molecules on erythrocytes, but only if the cells are solubilized by detergent. The role for NP-40 in the above experiment is to solubilize the cells, thereby permitting MCP access to C3b acceptors. The presence of detergent did not affect the activity of CR1 and factor H. This finding is related to the current concept that both act on cell-bound C3b in an extrinsic manner. In kinetic and dose-dependent analyses of this finding, MCP was a more efficient cofactor for some C3b acceptors than others; i.e. MCP more specifically degrades protein-bound C3b than small-acceptor-molecule (presumably oligosaccharide)-bound C3b. Factor H again lacks this tendency. Therefore, MCP would be distinct from factor H and CR1 in these functional properties.

This functional profile of MCP is unique for a complement regulatory protein and contrasts with that of the known extrinsic cofactor proteins. On the basis of findings suggesting that MCP hardly acts as an extrinsic cofactor, and its functional properties are distinguishable from those of factor H and CR1, we hypothesize that MCP is an intrinsic complement regulatory protein. This idea may be further supported by the fact that MCP is a widely distributed membrane protein (Cole

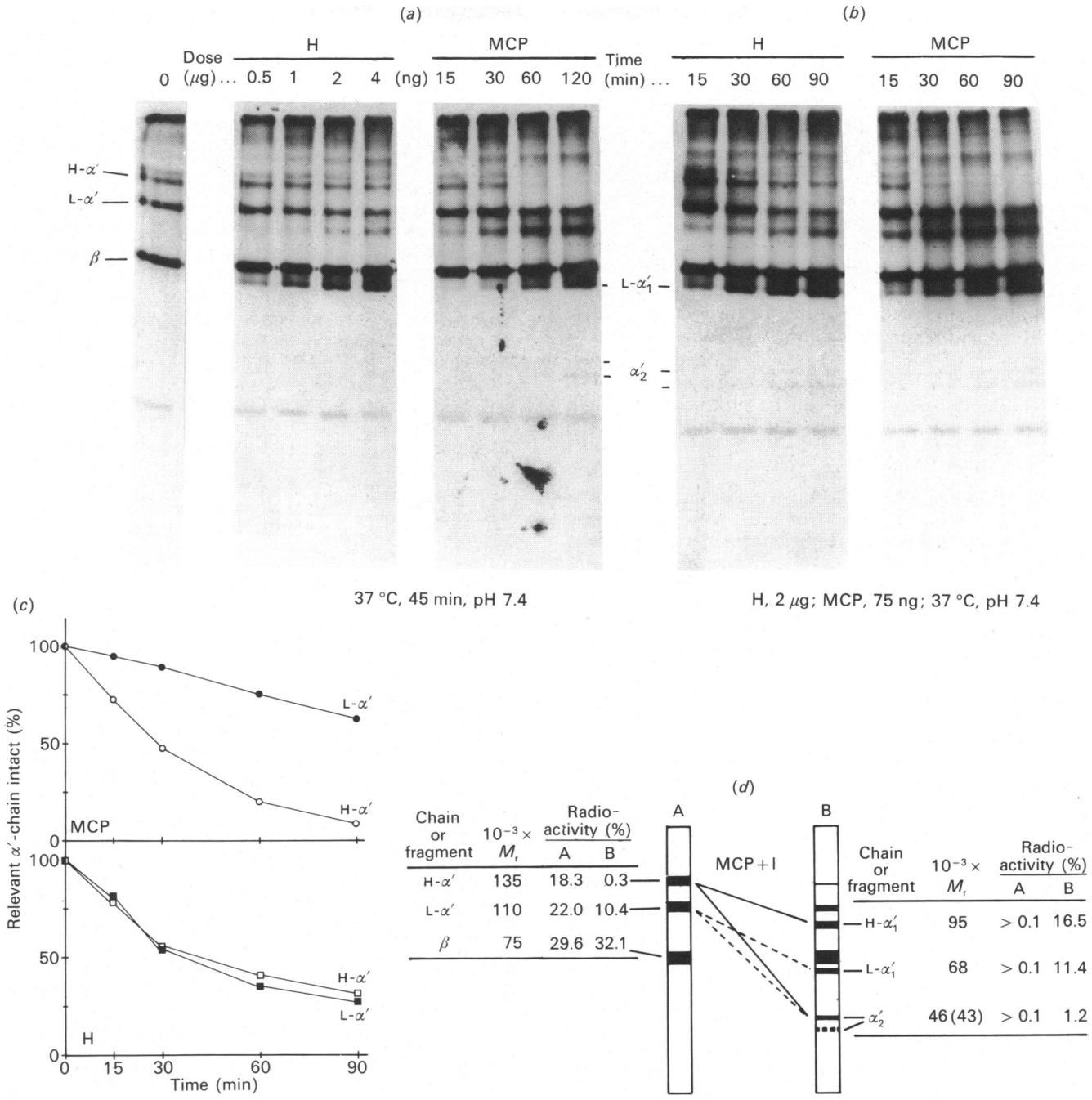

Fig. 6. Cleavage of cell-bound C3b by MCP and factor I in the presence of NP-40

The buffer used in the assay was PBS/0.1% NP-40, pH 7.4. The far left panel shows an untreated sample. (*a*) E*AC3b (1×10^7 cells prepared by activation of the alternative pathway) were incubated with factor I (1 μg) and variable amounts of factor H or MCP in a total volume of 160 ml for 45 min at 37 °C. (*b*) Kinetic analysis. Experimental conditions were similar to those of (*a*). In this assay 1 μg of factor I, 2 μg of factor H and 75 ng of MCP were used. (*c*) Kinetic analysis. The resultant autoradiography was scanned with a densitometer. Factor I (1 μg) and factor H (1.5 μg) or factor I (1 μg) and MCP (60 ng) were incubated with E*AC3b (2×10^7 cells) in a total volume of 125 μl at 37 °C. Peaks of H-α′ and L-α′ in each scanning profile were measured and the percentage of remaining H-α′ and L-α′ was calculated by the following equation:

$$\text{Percentage remaining} = P\,(\text{time})/P(0) \times 100$$

where P(time) is the height of the H-α′ (or L-α′) peak at the indicated times and $P(0)$ is the height of H-α′ (or L-α′) at zero time. (*d*) A scheme showing the cleavage of bound C3b by MCP and factor I. A and B represent SDS/PAGE profiles of reduced E-bound C3b that was incubated with MCP and factor I for 0 min (A) and 60 min (B) under the conditions described in (*b*). The nomenclature of the C3 chains and fragments used are shown in the two Tables. The M_r and the percentage radioactivity at zero time (A) and 60 min (B) are indicated. Percentage radioactivity was obtained from densitometric analysis in which the sensitivity of the densitoscanner was adjusted to the height of the β-chain. As shown here and in Fig. 5, there are several high-M_r bands, presumably from the α′-chain–membrane acceptor complex seen on autoradiography. These bands and their products are omitted from the scheme. Products are shown in the right-hand table. The 43000 M_r α′2 fragment is shown as a broken line, since it appeared only in the presence of sufficient NP-40.

et al., 1985; Seya *et al.*, 1988) which binds weakly (compared with CR1) to C3b-Sepharose (Cole *et al.*, 1985), MCP-bearing cells such as platelets and most T-cells do not rosette with C3b-coated erythrocytes (Cole *et al.*, 1985; Yu *et al.*, 1986) and its functional profile is complementary to that of DAF. Its structural similarities to DAF (having four SCRs), but not to CR1 and H (more than 20 and 30 SCRs respectively), also suggest that its main function will not be as an extrinsic cofactor for target-bound or fluid-phase C3b.

These experiments have not, though, specifically addressed the question of MCP's ability to serve as a cofactor for breakdown of C3b bound to the same membrane as the MCP. To investigate this possibility, monoclonal antibodies that completely block the cofactor activity of MCP need first to be developed. Secondly, now that cDNAs are available (Lublin *et al.*, 1988), transfectants bearing MCP can be produced and then tested for their sensitivity to complement-mediated lysis.

3. Fragments of C3b produced

Like CR1 and factor H, MCP acted as factor I-dependent cofactor for generation of iC3b from fluid-phase C3b, but this did not lead to efficient further degradation to C3c and C3dg. Also, MCP and factor H did not mediate factor I-dependent production of C3c and C3dg from target-bound C3b, whereas CR1 and factor I did accomplish this additional proteolytic cleavage. Concerning the production of 46 and 43 kDa fragments, in the absence of NP-40, factor H, C4bp and CR1 produced both fragments, together with factor I, but MCP and factor I only produced the 46 kDa fragment. These differences are as yet unexplained.

In summary, soluble MCP is a potent cofactor for fluid-phase C3b and C3b bound to solubilized acceptor molecules. It has cofactor activity for C3b covalently bound to intact erythrocytes but, compared with H, MCP is much less potent. On the basis of its having very little extrinsic cofactor activity, we hypothesize that MCP is a regulatory protein with cofactor activity whose primary function is to inhibit C3b in an intrinsic fashion on autologous tissue.

This work was supported in part by National Institutes of Health Grant 5RO1-AI19642 (U.S.A.), the Uehara Memorial Foundation, the Mochida Memorial Foundation, the Ichiro Kanehara Foundation, the Naito Memorial Foundation and the Taiyo Life Insurance Fund (Japan). We are grateful to Dr. E. J. Brown (Washington University) for his valuable discussions. We are also grateful to Dr. K. Inoue, Dr. T. Kinoshita, Dr. H. Kitamura, Dr. K. Nagaki and Dr. H. Akedo for their encouragement to complete this work in Japan.

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Received 19 September 1988/12 April 1989; accepted 9 August 1989

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