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Polymorphism and proteolytic fragments of granulocyte membrane cofactor protein (MCP, CD46) of complement

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Human granulocytes (polymorphonuclear leucocytes, PMN) possess a membrane cofactor protein (MCP, CD46), which is structurally and functionally distinct from the MCPs of other cell types: it shows a single broad band of 56–80 kDa (without the doublet pattern characteristic of MCP) on SDS/PAGE and has less affinity for complement component C3b. We purified PMN MCP using monoclonal antibodies in order to study the molecular differences between it and other MCPs. Several forms of PMN MCP with size heterogeneity were noted on SDS/PAGE and by immunoblotting. *O*-Glycanase treatment decreased this heterogeneity, yielding a fast-migrating component identical in position on SDS/PAGE to the *O*-glycanase-treated MCP of other cells. The cell-specific variation of MCP, therefore, arises from post-translational glycosylation and not from a difference in primary structure. The Factor I cofactor activity of PMN MCP was more efficient in cleaving the methylamine-treated complement components C4/C3 than was MCP from other cells, which shared a similar potency of cofactor activity on a weight basis. Two types of small-form PMN MCP were identified during purification. These were 42 kDa and 30 kDa in size; the former was recognized by M177 (a monoclonal antibody against the active site marker), possessed *N*-linked sugars [located on the short consensus repeats (SCRs)] but not *O*-linked ones (on the Ser/Thr-rich region), and retained cofactor activity for C3b/C4b cleavage, similar in potency to that of other MCPs. The functionally active soluble form of MCP was observed specifically in PMN. Protease inhibitors did not inhibit liberation of the fragments, although the generated fragments became susceptible to serine proteases. The findings show that the SCRs are the functional domain of MCP and that the MCP proteolysis found only in PMN may modulate the properties of PMN MCP. In conclusion, the structural features of PMN MCP largely reflect a variability in the *O*-linked sugars, and the decreased affinity for C3b may be in part attributable to proteolysis.

INTRODUCTION

Activation of the complement cascade is strictly controlled by several complement regulatory proteins. Factor H (Whaley & Ruddy, 1976) and C4b-binding protein (C4bp) (Fujita *et al.*, 1978) are major circulating plasma proteins which prevent spontaneous activation and excess consumption of complement by blocking C3 activation. In addition, C3b/C4b receptor (CR1, CD35) (Fearon, 1979; Iida & Nussenzweig, 1981), decay-accelerating factor (DAF, CD55) (Nicholson-Weller *et al.*, 1982; Medof *et al.*, 1984) and membrane cofactor protein (MCP, CD46) (Seya *et al.*, 1986; Seya & Atkinson, 1989) are membrane regulatory proteins which suppress cell-surface complement activation by inhibiting C3/C5 convertase formation.

MCP is an integral membrane glycoprotein with a molecular mass of 45–70 kDa that was first identified as an iC3/C3b-binding protein present on human blood cells and certain cell lines (Cole *et al.*, 1985). It binds not only to iC3/C3b but also to iC4/C4b, and functions as a cofactor for plasma protease factor I in the inactivation of C3b/C4b by proteolysis (Seya *et al.*, 1986). This function of MCP is closely associated with host cell protection against C3 attack (Seya *et al.*, 1990*b*; Matsumoto *et al.*, 1991). The cDNA for MCP has been cloned and sequenced (Lublin *et al.*, 1988). MCP was found to have repeat units of approx. 60 amino acids each, which match the consensus sequence found in complement regulatory proteins such as CR1, CR2, DAF, C4bp and factor H, approx. 25 amino acids that make up a serine/threonine (Ser/Thr)-rich region containing several *O*-linked glycosylation sites, 17 amino acids of unknown significance, and a 31-amino-acid transmembrane hydrophobic region followed by a 14–23-amino-acid cytoplasmic tail. In addition, MCP has three *N*-linked glycosylation sites in the short consensus repeats (SCRs). The structure of MCP is very similar to that of DAF (Caras *et al.*, 1987; Medof *et al.*, 1987), except that DAF is anchored to the membrane by a phosphatidylinositol tail (Medof *et al.*, 1986).

MCP is widely distributed and is found on human T and B lymphocytes, natural killer cells, granulocytes, monocytes, platelets, endothelial and epithelial cells, fibroblasts and cultured mononuclear cell lines, but not on erythrocytes (Seya *et al.*, 1988; McNearney *et al.*, 1989). On SDS/PAGE, the MCP of most peripheral blood mononuclear cells (PBMC) and platelets migrates as a doublet (mean molecular masses of ~ 66 kDa and ~ 56 kDa) (Ballard *et al.*, 1988; Purcell *et al.*, 1990*a*). While all individuals express both forms, the quantities of each differ and are inherited in an autosomal co-dominant fashion (Ballard *et al.*, 1987). However, MCP obtained from granulocytes of all individuals migrates as a single broad band (56–80 kDa) regardless of the PBMC MCP phenotype, suggesting that polymorphonuclear leucocyte (PMN) MCP is structurally different from the MCP of PBMC and platelets (Seya *et al.*, 1988).

We have purified MCP from granulocytes and PBMC and studied their structural and functional properties. In addition, we have obtained the functionally active fragment of PMN MCP.

Abbreviations used: $C3_{MA}$, methylamine-treated C3; $C4_{MA}$, methylamine-treated C4; C4bp, C4b-binding protein; CR1, C3b/C4b receptor (CD35); DACM, *N*-(dimethylamino-4-methylcoumarinyl)maleimide; DAF, decay-accelerating factor (CD55); DPBS, Dulbecco's phosphate-buffered saline; HRP, horseradish peroxidase; IAA, iodoacetamide; mAb, monoclonal antibody; MCP, membrane cofactor protein (CD46); NP40, Nonidet P40; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PMN, polymorphonuclear leucocytes; PMSF, phenylmethanesulphonyl fluoride; SCR, short consensus repeat.

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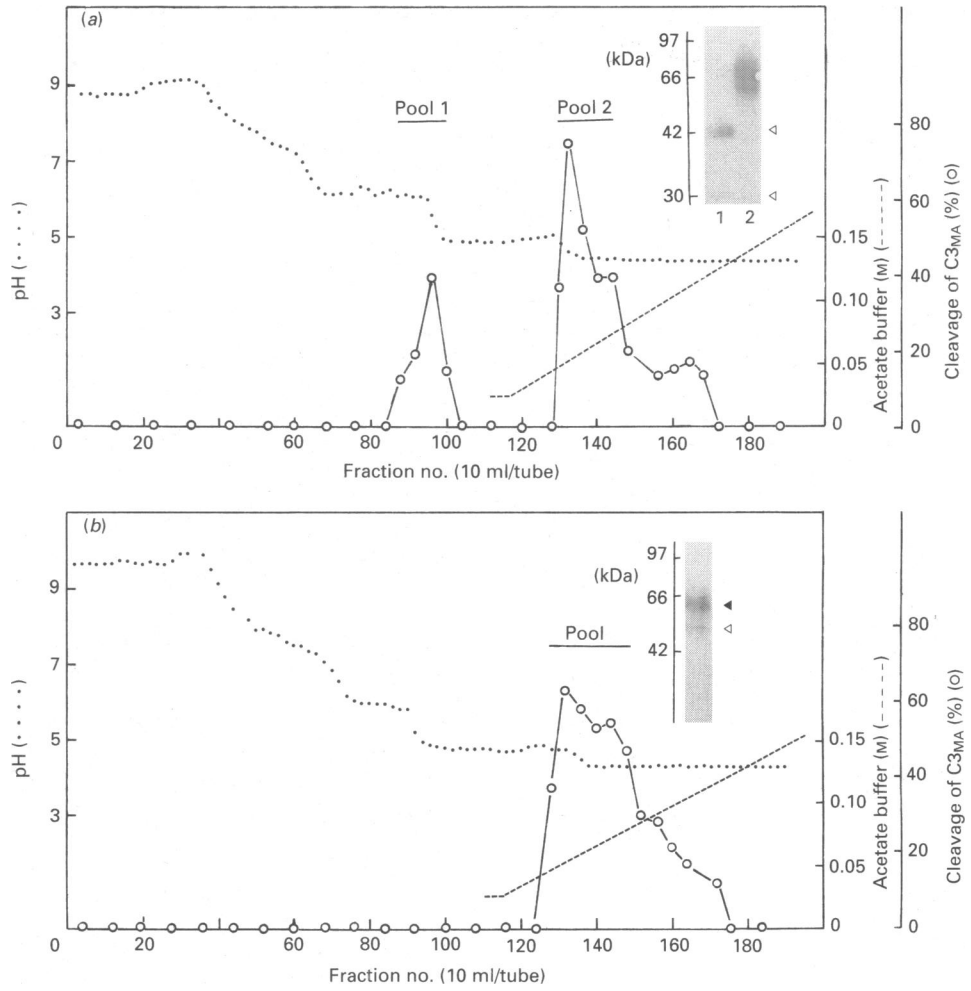

Fig. 1. Elution profile of PMN MCP and PBMC MCP from a chromatofocusing column

The column was equilibrated with 10 mM-Tris/HCl/0.05% NP40 (pH 9.0). After the solubilized samples were loaded, the column was washed with 0.02 M-acetate buffer/0.05% NP40 (pH 4.75) in 10% (v/v) Polybuffer 74, and then eluted with a linear (0.02–0.25 M) gradient of acetate buffer (pH 4.3) in 10% Polybuffer 74. The cofactor activity of MCP was determined as described in the Materials and methods section. pH (· · · ·), the molar concentrations of the acetate buffer (----) and cofactor activity (○) are shown. (*a*) Elution profile of PMN MCP. Two peaks with cofactor activity can be seen. The fractions with cofactor activity were pooled separately (indicated as pool 1 and pool 2) and applied to an mAb M177–Sepharose column. The final samples purified from the two pools are shown on the immunoblot in the inset. (*b*) Elution profile of PBMC MCP. A single cofactor peak was eluted from the column. Purified proteins were analysed under non-reducing conditions on SDS/PAGE (7.5% gel) followed by immunoblotting (inset).

The properties of the soluble fragments of MCP derived from granulocytes by proteolysis are also discussed.

MATERIALS AND METHODS

Proteins

Human complement proteins C3 (Nagasawa & Stroud, 1977), C4 (Nagasawa & Stroud, 1980) and factor I (Nagasawa *et al.*, 1980) were purified as previously described. Methylamine (MA)-treated C3 ($C3_{MA}$) and C4 ($C4_{MA}$) were prepared by incubation of C3 or C4 with 100 mM-methylamine for 2 h at 37°C in phosphate-buffered saline (PBS), pH 7.5. $C3_{MA}$ and $C4_{MA}$ were then labelled with *N*-(dimethylamino-4-methylcoumarinyl)-maleimide (DACM) as described (Seya & Nagasawa, 1982).

Mouse monoclonal antibodies (mAbs) against human MCP, M177 and M160, were prepared (Seya *et al.*, 1990*a*) and purified on Protein A–Sepharose (Pharmacia, Uppsala, Sweden).

Cathepsin G and human leucocyte elastase were purified as described by Matsuda *et al.* (1985). Cathepsin D was purchased from Sigma (St. Louis, MO, U.S.A.).

Cells

K562 and HSB2 cells were gifts from Dr. J. P. Atkinson (Washington University, St. Louis, MO, U.S.A.). These cells were cultured at 37 °C in a 5% CO_2/95% air atmosphere in RPMI 1640 medium supplemented with fetal calf serum (Cell Culture Laboratories, Cleveland, OH, U.S.A.) and antibiotics. Cells were harvested from 5 litres of medium and centrifuged.

PMN and PBMC were isolated from venous blood (supplemented with 10 mM-EDTA) of normal volunteers by methylcellulose sedimentation and the Polyprep method (Seya *et al.*, 1990*a*). The purity of each cell population was > 95%.

Purification of MCP from various cells

Large-scale isolation of PMN was performed as follows. Platelet-free concentrated blood (900 ml, from three donors) was diluted with an equal volume of 0.9% NaCl, mixed with 200 ml of 2% methylcellulose and allowed to stand for 60 min at room temperature. The leucocyte-rich fraction was collected and centrifuged at 800 *g* for 15 min. The pellet was washed once and

resuspended in 50 ml of 0.9% NaCl. The PMN and PBMC were then separated with Polyprep (Nycomed, Oslo, Norway). The contaminating red blood cells were removed by hypo-osmotic lysis. The degree of purity of the PMN and PBMC preparations was > 90%. The cells were washed twice in saline and solubilized with 20 ml of Dulbecco's PBS (DPBS) containing 10 mM-EDTA, 10 mM-iodoacetamide (IAA), 1 mM-phenylmethanesulphonyl fluoride (PMSF) and 1% Nonidet P40 (NP40). The material was stirred for 30 min at room temperature and centrifuged at 2400 *g* for 10 min, and the supernatant was collected and frozen at −70 °C until use. Finally, 100 ml of the extract was obtained by five solubilization cycles and used as a starting material for purification of PMN MCP. PBMC were also isolated from 2 litres of fresh heparinized blood by another method in which methylcellulose sedimentation and Ficoll/Hypaque gradient centrifugation were employed. After three washes, the PBMC were solubilized as described above.

The supernatants of the solubilized PMN and PBMC were dialysed against 20 mM-acetate buffer containing 0.05% NP40, pH 5.0, and the precipitate was removed by centrifugation. The supernatant was applied to a chromatofocusing column (2 cm × 70 cm) equilibrated with 10 mM-Tris/HCl/0.05% NP40, pH 9.0. The column was washed with 20 mM-acetate buffer/0.05% NP40, pH 4.75, in 10% (v/v) Polybuffer 74 (Pharmacia), and eluted with a linear (20–250 mM) gradient of acetate buffer (pH 4.3) in 10% Polybuffer 74 (Seya *et al.*, 1986). The fractions containing cofactor activity were pooled and the sample (~ 300 ml) was dialysed against 10 mM-PBS/0.05% NP40, pH 7.4. The dialysed material was applied to an M177-conjugated Sepharose column (30 ml). The column was sequentially washed with PBS/0.05% NP40, pH 7.4, and PBS/0.5 M-NaCl/0.05% NP40, pH 7.4. MCP was eluted with 0.17 M-glycine/HCl/0.05% NP40, pH 2.5, and immediately neutralized with 1 M-Tris, and then dialysed overnight against 20 mM-PBS/0.05% NP40, pH 6.0. MCP was concentrated with water-absorbing gel (Ms. BTAURY-KN) (ATTO Co., Tokyo, Japan) after dialysis.

The methods described above were applied to purification of MCP from other cell types.

The concentrations of purified MCP were determined by three methods: (1) a densitometric assay by scanning the silver-stained gels at 632.8 nm, (2) a dye-binding assay using an ISS protein-gold kit (Integrated Separation Systems, Hyde Park, MA, U.S.A.) and (3) an e.l.i.s.a. (M. Matsumoto, T. Seya & S. Nagasawa, unpublished work). These three methods all gave similar MCP concentrations.

Deglycosylation of MCP

Purified MCP (~ 20 ng) was dialysed against 20 mM-Tris/maleate/10 mM-D-galactono-γ-lactone/1 mM-calcium acetate/0.1% NP40, pH 6.0, for 15 h and then incubated with 50 munits of neuraminidase (Sigma) for 1 h at 37 °C. The desialylated samples were incubated with 3 munits of *O*-glycanase (Genzyme Co., Boston, MA, U.S.A.) for 16 h at 37 °C (Matsumoto *et al.*, 1989). *N*-Glycanase (Genzyme) digestion was performed in the presence of PBS/0.05% NP40 for 16 h at 37 °C (Matsumoto *et al.*, 1989).

Assay of MCP cofactor activity for factor I-mediated $C3_{MA}/C4_{MA}$ cleavage

MA-treated and DACM-labelled C3 and C4 (DACM-$C3_{MA}$ and DACM-$C4_{MA}$) were used as substrates. DACM-$C3_{MA}$ or DACM-$C4_{MA}$ (20 μl, 10 μg) was incubated for 5 h at 37 °C with 50 μl of factor I (0.5 μg) and 80 μl of each column fraction (dialysed against 20 mM-PBS/0.05% NP40, pH 6.0) or purified MCP. The reaction was stopped by the addition of 60 μl of 1% SDS and 10 μl of 2-mercaptoethanol and the samples were subjected to SDS/PAGE (7.5% gel). Cofactor activity was assessed by measuring the fluorescence intensity of the α and α_1 fragments of DACM-$C3_{MA}$ or the α, α_1 and α_2 fragments of DACM-$C4_{MA}$ (Seya *et al.*, 1986).

Proteolytic cleavage of PMN MCP by granulocyte-derived proteases

PMN or PBMC [(1–2) × 10^7] were solubilized with 200 μl of 1% NP40/DPBS with or without 10 mM-EDTA, 10 mM-IAA and 1 mM-PMSF. The mixtures were allowed to stand for 30 min at room temperature and centrifuged at 2400 *g* for 10 min. The samples were subjected to SDS/PAGE under non-reducing conditions, and transferred to a nitrocellulose sheet. The sheet was then blocked with 3% BSA/PBS for 1 h at 37 °C, subjected to overnight blocking at 4 °C and finally incubated sequentially with M177, horseradish peroxidase (HRP)-conjugated goat anti-(mouse IgG) (Bio-Rad, Richmond, CA, U.S.A.) and substrate for HRP (Konica Co., Tokyo, Japan).

In another experiment, PMN MCP was treated with proteases purified from PMN. PMN MCP (10 ng) was incubated with various concentrations of cathepsin G (0.1–1 μg) or neutrophil elastase (0.1–1.2 μg) in 80 μl of PBS/0.02% NP40, pH 7.4, for 1 h at 37 °C. The reaction was terminated by the addition of 40 μl of 1% SDS. These preparations were analysed by SDS/PAGE followed by immunoblotting. To examine the effect of acid protease on the cleavage of MCP, we incubated PMN MCP with various concentrations of cathepsin D in 20 mM-acetate buffer containing 50 mM-NaCl and 0.05% NP40, pH 4.0.

SDS/PAGE and Western blotting

SDS/PAGE was performed by the method of Laemmli (1970). Electrophoretic transfer of proteins to a nitrocellulose sheet was performed according to the method of Towbin *et al.* (1979).

RESULTS

Purification of MCP of granulocytes and mononuclear cells

PMN MCP and PBMC MCP were purified by chromatofocusing and with M177 conjugated to Sepharose. Chromato-

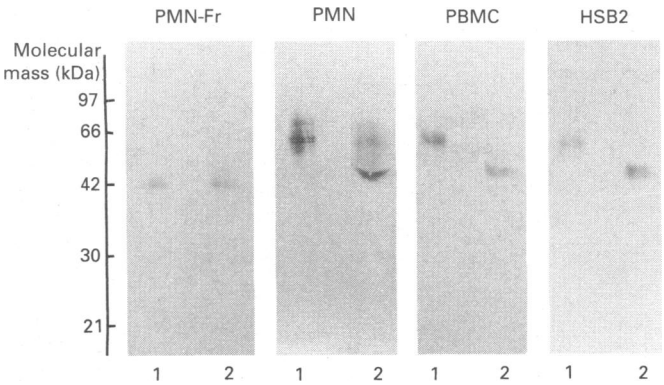

Fig. 2. *O*-Glycanase treatment of purified MCP

The 42 kDa and 30 kDa PMN MCP fragments (PMN-Fr) and PMN, PBMC and HSB-2 MCPs were digested with neuraminidase followed by *O*-glycanase. The samples were subjected to SDS/PAGE (10% gel) under non-reducing conditions and transferred to nitrocellulose sheets. The sheets were then blocked with 3% BSA/PBS and developed with M177, HRP-conjugated anti-(mouse IgG) and substrate. Lane 1, untreated MCP; lane 2, *O*-glycanase-treated MCP. *O*-Glycanase-treated K562 MCP also aligned with the deglycosylated MCP shown in the Figure (results not shown). Although the 30 kDa fragment is not clearly visible in the Figure, in other experiments it was not altered by *O*-glycanase digestion.

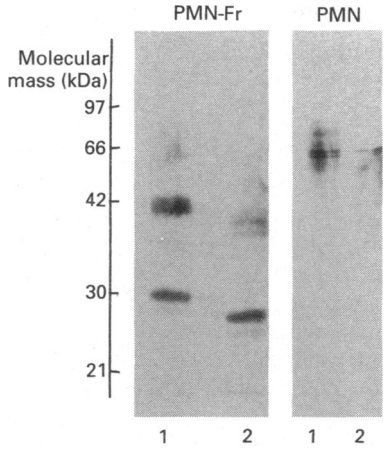

Fig. 3. *N*-Glycanase treatment of PMN MCP and its fragments

The 56–80 kDa PMN MCP, and the 42 kDa and 30 kDa fragments (PMN-Fr) were digested with *N*-glycanase and analysed as described in Fig. 2. The molecular sizes of the 42 kDa and 30 kDa fragments were reproducibly decreased by *N*-glycanase treatment, while PMN MCP was partially digested. Lane 1, untreated MCP; lane 2, *N*-glycanase-treated MCP.

focusing revealed a distinctive characteristic of PMN MCP (Fig. 1*a*). Two cofactor peaks appeared separately and, after subsequent purification using M177-coupled Sepharose, each was found to contain proteins of various sizes which reacted with anti-MCP (Fig. 1*a*, inset). The second peak contained a 56–80 kDa protein which exhibited nearly the same electrophoretic migration and profile as that reported for PMN MCP (Seya *et al.*, 1988). The first peak, however, consisted of 42 kDa and 30 kDa components which have not been identified by the surface-labelling/immunoprecipitation method. Based on the elution profile obtained with chromatofocusing, these two components were found to be more basic than the conventional MCP.

PBMC MCP was purified using the same procedure (Fig. 1*b*). Its elution profile from the chromatofocusing column, with a single cofactor peak, was similar to those of U937 MCP and HSB2 MCP (Seya *et al.*, 1986).

Immunoblotting analysis (Fig. 2) of these purified MCPs (non-reducing conditions) indicated that PBMC MCP and the three types of PMN MCP were recognized by mAb M177, a marker of the active site of the MCP (Seya *et al.*, 1990*b*), as well as by M160, a non-active-site marker (result not shown). Using purified materials, one of the PMN MCPs displayed a broad band of 56–80 kDa (Fig. 1*a*, lane 2 of the inset), and the PBMC MCP appears on SDS/PAGE as a doublet of 63 kDa and 56 kDa (Fig. 1*b*, inset). These findings were in agreement with those obtained with the surface-labelled materials. The 56–80 kDa PMN MCP appeared to be heterogeneous, since several distinct bands appeared on the immunoblots (Figs. 1 and 2).

Endoglycosidase treatment of MCP

MCP possesses *O*-linked sugars in the *C*-terminal Ser/Thr-rich region and *N*-linked sugars in the *N*-terminal SCRs. Purified MCP was digested with neuraminidase followed by *O*-glycanase. The 56–80 kDa PMN MCP, PBMC MCP and HSB2 MCP all decreased in size, while the 42 kDa and 30 kDa PMN MCPs were unaffected (Fig. 2). By this treatment, the broad band of heterogeneous PMN MCP was converted into a single entity, which migrated identically to the *O*-glycanase-treated PBMC and HSB2 MCPs.

N-Glycanase treatment was also performed with purified MCP. The 42 kDa and 30 kDa PMN MCPs decreased by 2–4 kDa and 2 kDa respectively (Fig. 3). Intact PMN MCP (Fig. 3) and HSB2 MCP (results not shown) were only partially decreased in size by *N*-glycanase treatment, presumably because digestion was incomplete, which supports the previous report stating that native MCP is relatively resistant to *N*-glycanase (Ballard *et al.*, 1988). The fact that both the 42 kDa and 30 kDa MCPs possess *N*-linked sugars but not *O*-linked ones suggests that these components are fragments of PMN MCP which consist of *N*-terminal SCRs.

Cofactor activity of PMN MCP

Equal amounts of MCP purified from various cells (Fig. 4*a*) were incubated with factor I and DACM-labelled $C3_{MA}$, and

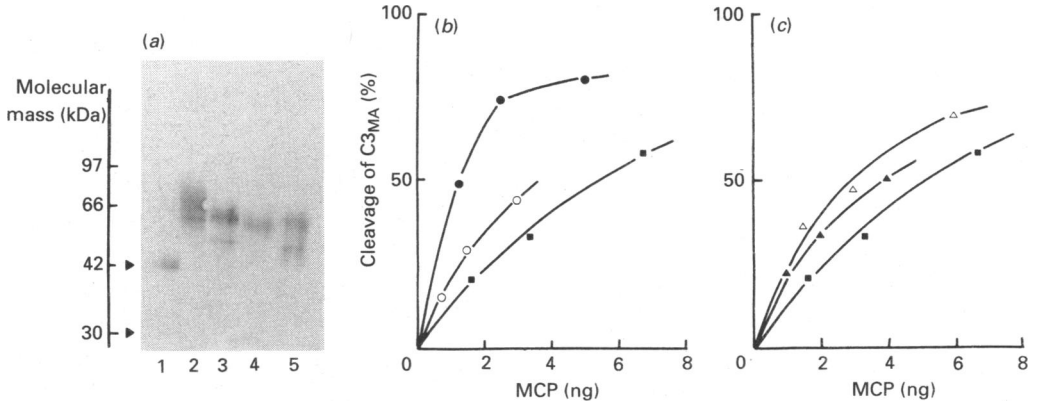

Fig. 4. Cofactor activity of MCPs from various cells

(*a*) Immunoblotting of the MCPs purified from PMN (lane 2), PBMC (lane 3), HSB-2 cells (lane 4) and K562 cells (lane 5) and of purified PMN MCP fragments (lane 1). Equal amounts of MCP (~ 20 ng) were subjected to SDS/PAGE (7.5% gel) under non-reducing conditions and transferred to nitrocellulose sheets. The MCP was detected with mAb M177, HRP-conjugated goat anti-(mouse IgG) and substrate. Arrows indicate the 42 kDa and 30 kDa fragments derived from PMN MCP. (*b*) Comparison of the cofactor activity of PMN MCP with that of PBMC MCP. Various amounts of MCPs were incubated with DACM-treated $C3_{MA}$ (10 μg) and factor I (0.5 μg) in a total volume of 150 μl for 5 h at 37 °C. The reaction was stopped by adding 20 μl of 10% SDS/30% 2-mercaptoethanol, and the mixture was subjected to SDS/PAGE. The percentage cleavage of $C3_{MA}$ was determined by measuring the fluorescence intensity of the α and α_1 fragments of DACM-$C3_{MA}$. ●, PMN MCP; ○, the 42 kDa/30 kDa fragments of PMN MCP; ■, PBMC MCP. (*c*) Comparison of the cofactor activity of PBMC MCP with that of K562 and HSB-2 MCPs. The experiment was performed as in (*b*). ■, PBMC MCP; △, K562 MCP; ▲, HSB-2 MCP.

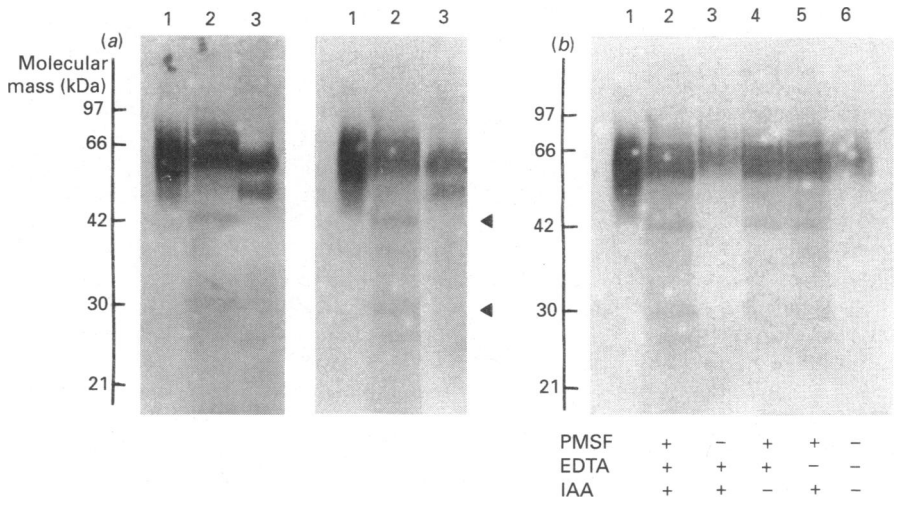

Fig. 5. Proteolytic liberation of PMN MCP fragments

(*a*) Detection of MCP fragments in solubilized extract of PMN by immunoblotting. PMN and PBMC from a donor having approximately equal quantities of large and small molecular MCP components (left panel) and those from a donor having predominantly large MCP components (right panel) were solubilized in 100 μl of DPBS containing 1 % NP40, 1 mM-PMSF, 10 mM-IAA and 10 mM-EDTA. Supernatants from 3×10^6 cells were subjected to SDS/PAGE (10 % gel) under non-reducing conditions and electroblotted to nitrocellulose sheets. MCP was detected with mAb M177. Lane 1, control PMN MCP purified from pool 2 shown in Fig. 1(*a*); lane 2, MCP detected in the PMN extract; lane 3, MCP detected in the PBMC extract. (*b*) Effect of protease inhibitors on fragmentation of PMN MCP. PMN from one donor (predominantly expressing large MCP on PBMC) were solubilized under the indicated conditions, electrophoresed and immunoblotted. The solubilization buffer was DPBS/1 % NP40 containing 1 mM-PMSF, 10 mM-IAA, and/or 10 mM-EDTA as indicated. Lane 1, control 56–80 kDa PMN MCP. Arrowheads indicate the 42 kDa and 30 kDa MCP fragments.

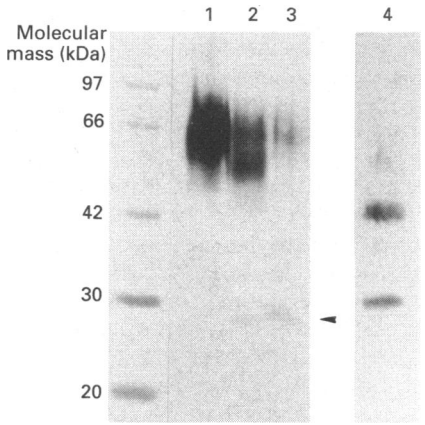

Fig. 6. Treatment of PMN MCP with human leucocyte elastase or cathepsin G

PMN MCP (60 ng) was incubated with granulocyte elastase or cathepsin G (1 μg) [enzyme/substrate = 15 (w/w)] for 120 min at 37 °C in a total volume of 85 μl. The reaction was stopped by adding 2 μl of 100 mM-PMSF. The mixture was subjected to SDS/PAGE (10 % gel) under non-reducing conditions and then transferred to nitrocellulose sheets. MCP was detected with mAb M177, HRP-conjugated goat anti-(mouse IgG) and substrate. Lane 1, untreated PMN MCP; lane 2, leucocyte elastase-treated PMN MCP; lane 3, cathepsin G-treated PMN MCP; lane 4, 42 kDa and 30 kDa fragments of PMN MCP. The arrowhead indicates the fragment derived from the leukocyte elastase- or cathepsin G-treated PMN MCP. Molecular size markers are also shown.

generation of $iC3_{MA}$ was assessed by SDS/PAGE and a fluorescence spectrophotometer. On a weight basis, the cofactor activity of the 56–80 kDa PMN MCP for factor I-mediated $C3_{MA}$ cleavage was greater than that of PBMC MCP. Intact PMN MCP appeared to be more active as a factor I cofactor than the MCP from the two leukaemia cell lines HSB2 and K562, or the MCP from PBMC (Fig. 4*c*). The 42 kDa fragment of PMN MCP was still able to serve as a factor I cofactor, and its potency was similar to that of PBMC MCP (Fig. 4*b*). Cofactor activity for factor I-mediated $C4_{MA}$ cleavage was also investigated and a similar tendency was observed (results not shown).

Proteolytic liberation of PMN MCP fragments

The 42 kDa and 30 kDa fragments of PMN MCP were released during solubilization (Fig. 5). The difference in the MCP phenotypes characteristic of PBMC MCP and platelet MCP in any one donor did not affect the size and amount of the liberated fragments of PMN MCP (Fig. 5*a*). The fragments disappeared unless the solubilized PMN were treated with an inhibitor cocktail. We therefore tested the effect of the inhibitors on fragment liberation (Fig. 5*b*). The proteolytic fragments were detected only if the solubilization buffer contained PMSF. Other combinations containing IAA and EDTA did not affect the degradation of PMN MCP, nor did acidic conditions accelerate fragment release (results not shown). Although long-term incubation with PMSF resulted in an increase in the amount of fragments, the degradation of intact MCP was not accelerated even without the inhibitor cocktail (results not shown), suggesting the relative resistance of intact MCP to most PMN proteases. Hence the sensitivity of MCP to proteases increased on conversion of native PMN MCP to the 42 kDa/30 kDa fragments.

We next treated PMN MCP with purified PMN proteases. Neither the 42 kDa nor the 30 kDa fragment was released by cathepsin D, cathepsin G or human leucocyte elastase (Fig. 6). The latter two are serine proteases, which digested PMN MCP into small fragments, one of 28 kDa (somewhat smaller than the 30 kDa fragment) which was detected with mAb M177. This unstable 28 kDa intermediate was subsequently degraded, which is inconsistent with the results obtained with limited proteolysis of MCP under solubilization, during which the 42 kDa and 30 kDa fragments were generated. These results were confirmed

in other experiments in which the incubation times or enzyme concentrations were varied (results not shown). Other proteases tested (thrombin, plasmin, C1s, plasma kallikrein and pepsin) did not cleave MCP at all (results not shown).

DISCUSSION

Trophoblast–lymphocyte cross-reactive antigen (TLX), HuLy-m5 and MCP have all been included in CD46 (Purcell *et al.*, 1990*b*). Studies on TLX and HuLy-m5 (Stern *et al.*, 1986; Purcell *et al.*, 1990*b*) have focused on the relationship of these antigens to human leucocyte histocompatibility antigen of lymphocytes. In our MCP study it was found that PMN also possess an MCP, which differs from the previously reported MCP/CD46 in that it binds very weakly to C3b–Sepharose and does not appear as a doublet (a characteristic of MCP) on SDS/PAGE (Seya *et al.*, 1988). The distinct properties of PMN MCP may be a factor in its not having been identified until quite recently.

Normal mature cells possess less MCP than tumour cells (Seya *et al.*, 1990*a*). This has made it difficult to obtain sufficient amounts of purified normal cell MCP and has prevented us from investigating its structure and function in normal blood cells. In the present study, we used an mAb (M177) against MCP for purification from several tumour cell lines. The MCP purified in this manner retained full factor I cofactor activity compared with MCP purified by the conventional method. PMN MCP and PBMC MCP were subsequently purified, allowing for analysis of these proteins for the first time.

Recently, Post *et al.* (1990) and Purcell *et al.* (1991) isolated several distinct MCP cDNAs from human tumour cell lines and placenta. The cDNAs that were cloned and sequenced possess the same four *N*-terminal SCRs but differ in the Ser/Thr-rich region, transmembrane domain and cytoplasmic tail. These authors suggested that alternative splicing of the MCP exons determined the MCP phenotype. Therefore cell-specific size variation of MCP phenotypes may be explained by the difference in primary structure of the Ser/Thr-rich region, transmembrane region and cytoplasmic tail, or by a difference in glycosylation.

We herein show that there is a size polymorphism in PMN MCP, consisting of several distinct bands on SDS/PAGE clearly detected by M177. The size heterogeneity appears to reflect differences in *O*-glycosylation, since it diminished greatly after *O*-glycanase digestion, and the molecular size of the deglycosylated PMN MCP was nearly identical to those of PBMC and HSB2 MCPs (Fig. 2). This molecular size heterogeneity of PMN MCP, therefore, does not reflect a difference in primary structure but is largely attributable to the *O*-linked carbohydrate content. The unusual structural features of PMN MCP are, therefore, caused mainly by post-translational *O*-glycosylation. A recent report showing that the MCP of epidermis and cultured keratinocytes give a single broad band of ~ 60 kDa which is smaller than the conventional MCP (Sayama *et al.*, 1991) may also be explained by *O*-linked sugar polymorphism.

It is likely that a functional domain in MCP is liberated from PMN during solubilization and purification. Proteolysis, and not alternative splicing, is responsible for the short form of PMN MCP with full cofactor activity, since PMN do not express an mRNA corresponding to the MCPs of low molecular masses (Purcell *et al.*, 1991), and the longer that the PMN are incubated, the more 42 kDa/30 kDa MCP is released. The fact that no MCP fragment liberation was observed in human cells except for PMN is consistent with previous studies (Cole *et al.*, 1985; Stern *et al.*, 1986; Purcell *et al.*, 1990*a*). A variety of PMN proteases may be crucial in fragment liberation.

MCPs possess *N*-linked sugars in the *N*-terminal SCRs. The finding that the 42 kDa and 30 kDa fragments possess *N*-linked sugars but not *O*-linked sugars reinforces the idea that these MCP moieties are proteolytic products that contain *N*-terminal SCRs but lack the Ser/Thr-rich region, the transmembrane domain and the cytoplasmic tail. Because the 42 kDa fragment can still serve as a cofactor for factor I and react with M177, which blocks MCP cofactor activity, the functional domain responsible for MCP cofactor activity must be located within the SCRs. Indeed, in spite of the variation in *O*-linked sugars, MCPs of tumour cell origin, PBMC MCP and the 42 kDa fragment, which share the same SCR domains, exhibit identical cofactor activity. However, the relatively high cofactor activity of PMN MCP remains to be explained.

Cole *et al.* (1985) and Ballard *et al.* (1988) reported that PMN MCPs were scarcely recovered from surface-labelled solubilized PMN by the C3-affinity method, even when a sufficient amount could be recovered by immunoprecipitation. This present study, together with our previous findings, provides three possibilities for this discrepancy. (1) Unstimulated PMN usually express a very low number of MCPs (Seya *et al.*, 1990*a*); in this case the surface-labelling method is inefficient for detection of membrane MCP. (2) In PMN, the predominant C3b-binding protein is not MCP but CR1, so that co-existent CR1 may engage the ligand C3b, resulting in an inhibition of MCP binding to C3b–Sepharose. (3) PMN MCP and/or its fragments yielded during isolation have less affinity for C3b than the conventional MCP, resulting in a lack of recovery using the C3-affinity method. In fact, our preliminary data suggest that the PMN MCP preparation contains the fragments poorly bound to $C3_{MA}$–Sepharose. This point could be confirmed using recombinant forms of MCP. It is most likely that PMN MCP is susceptible to proteases during purification, yielding the fragments which have less affinity for C3b. The low affinity of the fragments for C3b may cause the difference in potency of cofactor activity between PMN MCP and its fragments. If this is the case, certain protease-sensitive domains in PMN MCP must influence C3b binding and cofactor function.

The present study points to the possibility that soluble forms of MCP are released from native PMN MCP by granulocyte-specific proteases. However, since use of the known granulocyte proteases did not result in 42 kDa/30 kDa-fragment release, the protease responsible for limited MCP proteolysis remains unidentified. Interestingly, CR1 (Ripoche & Sim, 1986) and DAF (Seya *et al.*, 1987) are also protease-sensitive: CR1 is released by plasma serine proteases and DAF is converted into a soluble form of 55 kDa with decay-accelerating activity by certain leucocyte enzymes. One of these may be a protease, as the size of the DAF fragment is identical to that derived by papain. As with the MCP fragments, these DAF fragments are soluble, losing the membrane anchor plus a hydrophobic stretch of amino acids. In addition, several reports have demonstrated that adhesion proteins or receptors such as MEL-14 (Kishimoto *et al.*, 1989), phosphatidylinositol-linked FcR (Huizinga *et al.*, 1988) and tumour necrosis factor receptor (Porteu & Nathan, 1990) are released by stimulation of PMN. Although further studies are needed to determine the relationships among these phenomena, proteolytic clipping-out of the functionally active fragments by putative granulocyte proteases may be important in modulating inflammatory responses, including complement activation *in vivo*.

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