

## Membrane cofactor protein (CD46) in seminal plasma is a prostasome-bound form with complement regulatory activity and measles virus neutralizing activity

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### SUMMARY

Human seminal plasma contains 0.55 µg/ml of membrane cofactor protein (MCP; CD46) of 60 000 MW. By ultracentrifugation, gel filtration and immunoelectron microscope methods, we found that the MCP in seminal plasma was associated with prostasomes. The functional properties of the prostasome-bound MCP were assessed in comparison with a recombinant soluble form, γMCP1, which is composed of four short consensus repeats (SCR), type C of the serine/threonine-rich domain (ST<sup>C</sup>), and unknown significance (UK). The MCP in seminal plasma, although demonstrably bound to prostasomes, behaved more like the soluble form of MCP. In the absence of detergent it, together with factor I, degraded the fluid-phase ligand, methylamine-treated C3 [C3(MA)], which is insensitive under no-detergent conditions to the membrane form of MCP and factor I. Moreover, C3dg fragment was generated as a final product instead of C3bi during the incubation, indicating that the prostasomal MCP and proteases may be responsible for the C3dg generation. The prostasomes neutralized measles virus (MV) infectivity, while γMCP1, for the most part, did not. These results, taken together with the CD59 concentration on the prostasomes, suggest that the prostasomes are potential immunomodulators for complement activation, providing the C3- and C9-step inhibitors. The present report also reinforces the idea that there are two different forms of MCP in semen. One is located in the inner acrosomal membrane of spermatozoa, which appears through acrosomal reaction and spermatoon-egg interaction. The other is a prostasome-bound form maintaining activities sufficient to regulate complement activation and, probably, MV infection.

### INTRODUCTION

Immunosuppressive activity was found in human seminal plasma by Stites & Erickson in 1975.<sup>1</sup> E series prostaglandins were thereafter identified as molecules responsible for this activity, which was associated with prostasomes.<sup>2</sup> The prostasomes are pentalaminar or multilaminar vesicles secreted by the prostate. Although the physiological roles of these immunosuppressive substances are unknown, they would contribute to local modulation of the immunological environment and successful fertilization.

Recently, Rooney *et al.*<sup>3</sup> reported that CD59, an inhibitor of the membrane attack complex (MAC) of complement (C), resides on the prostasomes. In addition, a C3-step C regulatory protein, decay-accelerating factor (DAF; CD55), is also partly

present on the prostasomes.<sup>4,5</sup> Both are glycosyl-phosphatidylinositol (GPI)-anchored proteins.<sup>6</sup> Thus, the prostasomes may also be responsible for C-mediated immune responses and their regulation by C-regulatory proteins.

Membrane cofactor protein (MCP; CD46) is a C3b–C4b binding glycoprotein that possesses cofactor activity for factor I-mediated proteolytic inactivation of C3b and C4b.<sup>7</sup> Recently, MCP was found to serve as a measles virus (MV) receptor.<sup>8,9</sup> Although MCP was first identified<sup>10</sup> and then cloned<sup>11</sup> in leucocytes or its cell lines, we and others have recently found this molecule in human semen.<sup>12–14</sup> A spermatozoon MCP having a molecular mass of 43 000 MW (spermatozoa membrane MCP; smMCP) is localized on the spermatozoa inner acrosomal membranes<sup>12–16</sup> and is expressed during acrosomal reaction at spermatozoon-egg interaction, suggesting its importance in sustaining the binding of spermatozoa to oocytes.<sup>15–17</sup>

We first described a 60 000 MW soluble form of MCP (seminal plasma soluble MCP; ssMCP) abundant in seminal plasma, and this protein was also recognized by antibodies to

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MCP.<sup>13,18</sup> The structural and functional properties, and the roles, of ssMCP are as yet unknown. We presumed the primary role of this protein is to protect alloantigenic spermatozoa from local complement attack in the female genital tract,<sup>13,19</sup> since both numerous alloantibodies against sperm and C are contained within the general tract.<sup>20</sup> In fact, in other tissues including placental trophoblasts, MCP protects the semi-allogeneic fetoplacental unit from the maternal immune system.<sup>21</sup>

In this study, we focused on the localization and characterization of ssMCP.

## MATERIALS AND METHODS

### Samples, cells and antibodies

Semen was obtained from healthy volunteers and the seminal plasma was recovered by centrifugation (1600g, 10 min) after liquefying for 30 min at room temperature. The prostasomes were prepared from the seminal plasma by centrifugation at 200 000g for 20 hr at 4°. The soft pellet was resuspended in phosphate-buffered saline (PBS). Thus, the prostasome samples were prepared within 25 hr of ejaculation.

CHO cells producing human soluble MCP were provided as described previously.<sup>22</sup> The MCP molecules produced in the supernatants were of the serine/threonine-rich domain (STC)/long cytoplasmic tail (CYT) phenotype covering 1–279 amino acids.

The monoclonal antibodies (mAb) against human MCP (M177)<sup>23</sup> and human CD59 (5H8)<sup>24</sup> were produced and purified from murine ascites as described previously. A mAb against MV H protein was a gift from Dr S. Ueda (Osaka University, Japan).<sup>25</sup>

### SDS-PAGE and immunoblotting

Electrophoresis was performed under non-reducing conditions by the methods of Laemmli<sup>26</sup> using 10% acrylamide gels, and the samples were electrophoretically transblotted onto a nitrocellulose sheet.<sup>27</sup> MCP and CD59 in the samples were then detected with respective mAb, 2000-fold diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA) and a Konica Immunoblotting Kit (Konica Co., Tokyo, Japan) as described previously.<sup>28</sup>

### Gel filtration

Seminal plasma was dialysed against 50 mM NaCl/20 mM sodium phosphate, pH 7.2, at 4° overnight, and then applied onto a Sephadex G-100 column or a Superose fast protein liquid chromatography (FPLC) column (data not shown) equilibrated with 50 mM NaCl/20 mM sodium phosphate. This buffer was also used as an eluant. The protein concentrations in each fraction were evaluated by measurement of A280 nm. The elution profile of CD59 was monitored as a marker of the prostasomes. The apparent quantities and cofactor activity of MCP in each fraction were also assessed by immunoblotting and fluid phase factor I-cofactor assay (see below).

The gel filtration analysis was performed with different buffer systems, PBS, pH 7.4, and Krebs-Ringer HEPES buffer, pH 7.2, and similar results were obtained (data not shown).

### Factor I-cofactor activity

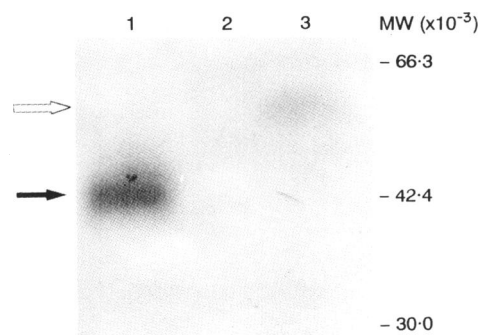
Cofactor assay for detecting degradation products of C3 was

performed as described previously<sup>28,29</sup> with slight modifications. Methylamine-treated fluorescent labelled C3 [f-C3(MA)] was used as a substrate;<sup>29</sup> the fluorescence was incorporated into the SH residue exposed secondary to the opening of the thioester bond in the C3d portion.<sup>29</sup> Briefly, after dialysis against 50 mM NaCl/20 mM sodium phosphate, pH 7.2, at 4° overnight, 10 µg of f-C3(MA), 0.8 µg of factor I<sup>30</sup> and 100 µl of prostasome solution were incubated for 2–10 hr at 37° in 50 mM NaCl, 20 mM phosphate buffer with or without 10 µg of complement factor H.<sup>31</sup> The samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions by adding 2% 2-mercaptoethanol. Cofactor activity was evaluated by measuring diminution of the 120 000 MW  $\alpha$  chain and evolution of its cleavage products, the 75 000 MW  $\alpha_1$  fragment and the 41 000 MW fluorescent fragment, putative C3dg, using a spectrofluorometer (Hitachi F2000; Hitachi Co., Tokyo, Japan).<sup>29,31</sup>

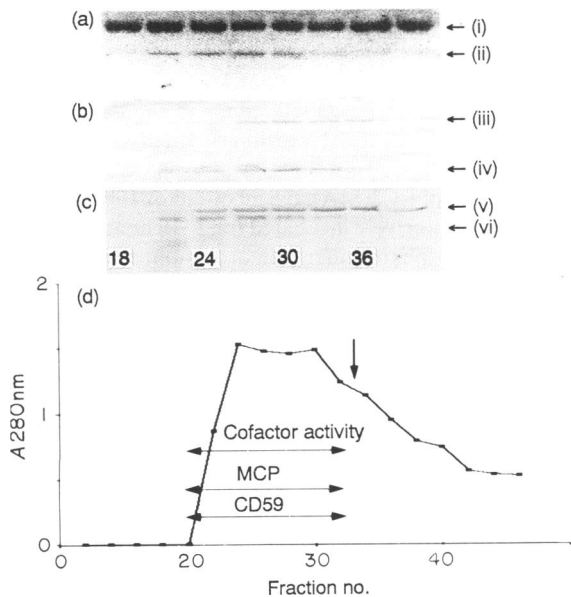
In some experiments, 1% Nonidet P-40 (NP-40) was used as a solubilizer, and cofactor activity of relevant MCP, either a soluble or a membrane form, was determined as described above. The effect of the solubilizer on cofactor activities of soluble and membrane forms were examined comparatively.

### Immunoelectron microscope

Immunoelectron microscope study was conducted by a modification of the method of Watts *et al.*<sup>32</sup> Seminal plasma was placed on formvar-coated nickel grids for 1 min at room temperature and the grids were pretreated with PBS containing 2% normal goat serum, 0.5% bovine serum albumin (BSA) and 0.1% gelatin. After two washes with PBS, they were incubated with the first antibody (20 µg/ml) for 1 hr at 37°. The grids were again washed and treated for 1 hr at 37° with a goat anti-mouse IgG [F(ab')<sub>2</sub>] (1/40 dilution) second antibody conjugated to 10 nm gold particles (Bio Cell RL, UK). The grids were then washed a final time with PBS. Mouse ascites fluid with a similar protein concentration was used as a negative control. The grids, thus prepared, were counterstained by 3% uranyl acetate in 50% ethanol for 30 seconds and observed under a JEOL100 CX (JEOL Co., Japan) electron microscope operated at 80 KV.



**Figure 1** Western blot of MCP in semen. Solubilized spermatozoa (lane 1), the supernatant (without prostasome; lane 2) and the precipitate (with prostasome; lane 3) of seminal plasma after ultracentrifugation were electrophoresed and blotted onto a nitrocellulose sheet. The sheet was stained using M177 (anti-MCP), goat anti-mouse IgG F(ab')<sub>2</sub>, and a Konica immunostaining kit. The closed arrow indicates smMCP of 43 000 MW and the open arrow the ssMCP of 60 000 MW.



**Figure 2.** Elution profile of MCP in the seminal plasma from a Sephadex G100 column. Cofactor activity (a), antigens reacted with 5H8 (b) and M177 (c) are shown. The latter two were the result of immunoblotting, while the former was a fluorogram using f-C3(MA). The positive fraction ranges of these activities or antigens are shown in (d). The protein elution profile was read at A280 nm. The closed arrow indicates the elution peak of BSA (66 000 MW). Cofactor activity and antigenicity of MCP and CD59 were found in the same fractions. Arrows in (a–c) indicate: (i) C3(MA); (ii) C3(MA)i or C3bi; (iii) and (v) a non-specific binding protein; (iv) CD59; (vi) ssMCP. One of the three experiments is shown. Although not shown in the figure, similar results were obtained with a Superose FPLC column.

#### *Inhibition of infective activity of MV*

Vero cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (DMEM/5% FCS) and

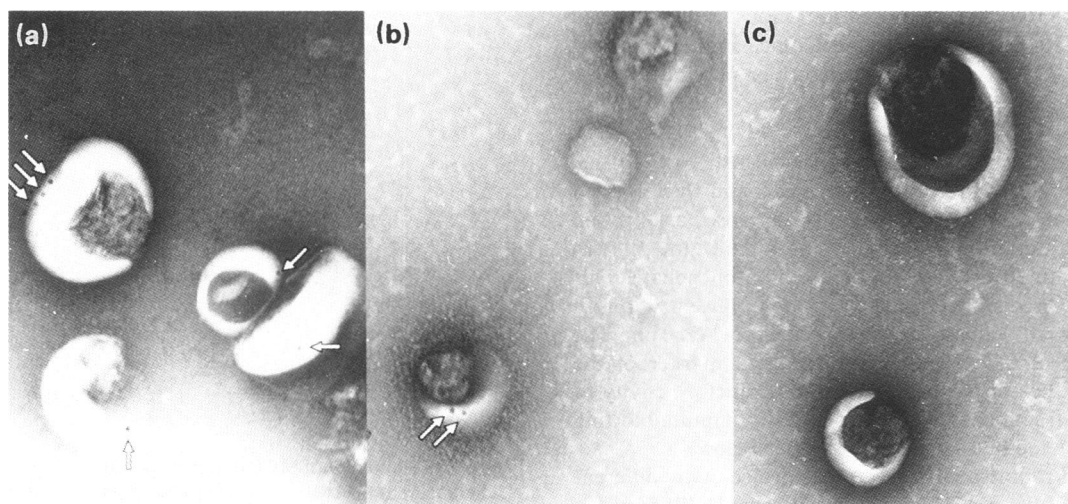
antibiotics. The Nagahata strain of MV was a gift from Dr S. Ueda (Osaka University, Japan). MV ( $10^{-10^4}$  plaque-forming units; PFU) was treated with a soluble form of MCP ( $\gamma$ MCP1), intact seminal plasma, seminal plasma containing no prostasomes, or prostasomes ( $50 \mu\text{l}$  of a 25% solution). At 3-hr intervals, a monolayer of Vero cells was incubated with the pretreated MV at  $1-10^3$  PFU/well in a 24-well plate. After cells had been cultured for 3–4 days, the cytopathic effect was evaluated under a Nikon inverted microscope. Production of MV H protein was confirmed by the immunofluorescent method using anti-H mAb, as described previously.<sup>25</sup> The experiments were performed three times in duplicate.

## RESULTS

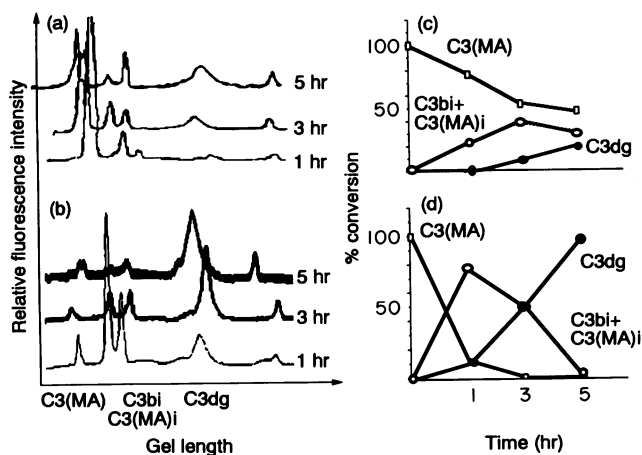
### Association of ssMCP with the prostasomes

Three methods were utilized to examine the relationship between ssMCP and the prostasomes. First, the prostasomes were separated from spermatozoa and seminal plasma by centrifugation and analysed by Western blotting using anti-MCP, M177 (Fig. 1). As we have described elsewhere,<sup>13</sup> MCP in seminal plasma was recognized as a single protein of 60 000 MW in the prostatic fraction, which was bigger than smMCP of 43 000 MW in solubilized spermatozoa, and no positive band for MCP was found after removing prostasomes in seminal plasma. Anti-CD59 (5H8) was used as a monitoring marker for the prostasomes,<sup>3,4</sup> and we confirmed the presence of ssMCP together with CD59 using the same blotting sheet (data not shown).

Second, the prostasomes were separated by a molecular sieve column, and the elution profiles of CD59 (prostatic marker) and ssMCP were compared by immunoblotting (Fig. 2). The prostasomes were eluted around the void volume, as shown in the blot stained with anti-CD59. ssMCP eluted in parallel with CD59. There was a contaminating protein of 75 000 MW that non-specifically reacted with the



**Figure 3** Immunoelectron microscopic features of the prostasomes stained with anti-CD59 and anti-MCP. The prostasomes were reacted with 5H8 (a), M177 (b) or mouse ascites fluid (c), and then a secondary antibody bearing gold particles (10 nm in diameter). The samples were observed under an electron microscope. Arrows indicate gold particles conjugated with goat anti-mouse IgG, which reacted with anti-CD59 and anti-MCP. MCP was found on the prostasomes as in CD59.



**Figure 4.** Evaluation of the products generated by factor I and the prostasomes. Factor I-cofactor assay was performed using the substrates f-C3(MA) or C3(MA)i (prepared by the addition of factor H). The fragments carrying fluorescence (i.e. the C3 d portion) were analysed by SDS-PAGE (under reducing conditions) and a spectrofluorometer. (a) Fluorescence scanning of the electrophoretogram. The substrate f-C3(MA) was incubated with protease factor I and the prostasomal fraction containing MCP (see Fig. 2). At timed intervals, the reaction was stopped and the samples were resolved by SDS-PAGE. (b) The substrate f-C3(MA)i (containing factor H) was incubated with the same amounts of factor I and the prostasomal fraction as in (a). The fluorescent peaks corresponding to the C3 derivatives are indicated at the bottom of the panel, based on the mobility of each band. (c) and (d) Percentages of conversion and generation of each component were calculated from the data in (a) and (b) on the computer. An almost complete conversion of C3(MA) to C3dg was observed when C3(MA)i was used as a substrate (b and d). Although not mentioned in the text, we observed a 68 000 MW fluorescent peak, which reflected the generation of C3bi (b). Prostasomal enzymes may be concerned with C3bi generation. Open square, C3(MA); open circle, C3bi and C3(MA)i; closed circle, C3dg.

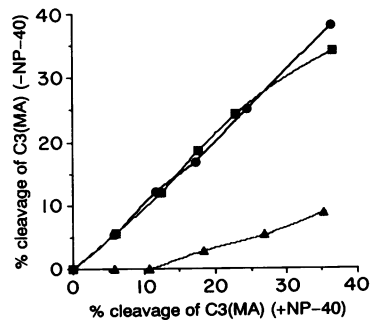
HRP-labelled second antibody, and ssMCP (60 000 MW) showed a faster retention time than this 75 000 MW protein. The ssMCP peak also preceded the albumin (66 000 MW) peak. These findings support the idea that ssMCP forms a complex with the prostasomes.

Third, we performed immunoelectron microscopic analysis. M177 was used as a primary antibody for detection of MCP. MCP molecules were identified on a prostasomal membrane, as indicated by the gold particles at the periphery of the vesicles. Anti-CD59 was used as a positive control. The MCP molecule appeared on the prostasomes, as in the case of CD59, since gold particles were seen on the prostasomal membranes in both cases (Fig. 3).

Finally, we could not detect any MCP in the seminal plasma from which the prostasomes were removed, using a quantitative ELISA assay (capable of detecting 5 ng/ml of MCP). Hence, prostasome-unbound forms of MCP should be minimal, less than 5 ng/ml, if any.

#### Functional properties of the prostasome-bound ssMCP

A factor I-cofactor assay was performed in the fluid phase using the C3 d portion-labelled substrate, f-C3(MA). The prostasome fractions had cofactor activity enough to convert C3(MA) to



**Figure 5.** Influence of detergent (NP-40) on the cofactor activity of MCP from various sources. Variable amounts of the membrane and soluble forms of MCP were incubated with constant amounts of factor I and f-C3(MA) in buffer containing 0.5% NP-40 (x-axis) or buffer without NP-40 (y-axis). Triangle, the membrane MCP isolated from CHO cells; circle, the soluble form isolated from the prostasomes in seminal plasma; square, the recombinant soluble form,  $\gamma$ MCP1.

C3(MA)i. Since longer incubation and the addition of factor H [for preparation of C3(MA)i] revealed the possibility of further cleavage of f-C3(MA), the cleavage profiles of C3(MA) and C3(MA)i were chased, as shown in Fig. 4. Based on the MW, the smallest fluorescent fragment of C3(MA) corresponded to C3dg (which is 41 000 MW). As with the soluble form, an addition of NP-40 did not increase any cofactor activity of ssMCP, suggesting that ssMCP resides outside the prostasomes (consistent with the electron microscopic data) and functionally behaves as a soluble form (Fig. 5).

A recombinant soluble form of MCP ( $\gamma$ MCP1; 30  $\mu$ g) and the prostasomes with ssMCP (50  $\mu$ l of the 25% solution) were incubated with MV ( $10^4 \sim 10$  PFU) and the infectious doses of the treated MV solutions were determined using Vero cells. A representative one is shown in Table 1, in which numbers of the syncytia formed were evaluated under a microscope. No blocking activity for MV infection was observed with 30  $\mu$ g of  $\gamma$ MCP1. In contrast, strong inhibition of MV infection could be

**Table 1.** Effect of the prostasomes on MV infectivity

Preincubation of MV with	MV (PFU)			
	$10^3$	$10^2$	10	1
Seminal plasma (intact)	0/0	0/0	0/0	0/0
Seminal plasma (without prostasomes)	8/4	2/1	0/0	0/0
Prostasomes	0/1	0/0	0/0	0/0
$\gamma$ MCP1 (soluble form)	12/9	3/3	0/0	0/0
Buffer (PBS)	5/3	1/0	0/0	0/0

Intact seminal plasma, the seminal plasma from which the prostasomes were removed by centrifugation, the prostasomes, recombinant soluble MCP, or buffer only was incubated with  $10^4$  PFU of MV, then the mixtures 10-fold diluted. The serially diluted MV sources were poured over the Vero cell monolayers in the 24-well plate in duplicates. Three days later, the number of syncytia formed in the Vero cells was calculated under a microscope. The experiments were performed three times, and a representative one is shown.

accomplished by the prostasomes. Similar inhibitory profiles of MV infection were observed with seminal plasma, but the inhibitory function was abrogated if the prostasomes were removed (Table 1).

## DISCUSSION

In this study, we have demonstrated that MCP in human seminal plasma is bound to the prostasome, according to the results of ultracentrifugation, gel filtration and electron microscopic analyses, and by its antigenicity and cofactor activity. The results have allowed us to conclude that ssMCP is not a proteolytic product derived from membrane forms. The soluble MCP in human seminal plasma, now prostasomal MCP, expressed factor I-cofactor activity comparable to that in recombinant soluble MCP in the absence of NP-40. The result suggested that the molecules were mostly expressed on the prostasome surface, to function in seminal fluid. This issue is consistent with the report of 5' nucleotidase on the prostasomes.<sup>33</sup> Taken together with reports on the prostasome-bound DAF and CD59,<sup>3-5</sup> this suggests that the prostasomes appear to show a potential C-regulatory profile by expressing MCP, DAF and CD59.

Since the prostasomes have a pentalaminar, or occasionally multilaminar, membrane architecture,<sup>34</sup> the prostasome MCP must have a membrane-form structure. A characteristic feature of MCP is the use of alternative splicing to produce three commonly expressed ST domains, namely ST<sup>C</sup>, ST<sup>BC</sup> and ST<sup>ABC</sup>, and four forms of CYT, CYT1-4,<sup>6,35</sup> which cause genetic and organ-specific phenotypic heterogeneity of MCP.<sup>35-38</sup> Prostasomal MCP is particular because it does not reflect the individual phenotypes: all samples tested to date have exhibited a 60 000 MW single band on SDS-PAGE.<sup>13</sup> Although soluble forms of MCP are present in some body fluids,<sup>18,39</sup> their structures are probably distinct from the prostasomal MCP.

The roles and functions of the prostasome have been unclear.<sup>34</sup> Rooney *et al.*<sup>3</sup> suggested that the prostasomes selectively carry GPI-linked proteins such as DAF and CD59 and that they have the capacity to transfer the GPI-anchored proteins to spermatozoa.<sup>3,40</sup> MCP, however, is a type 1 transmembrane protein, so the prostasomes cannot just be their carrier or reservoir.

There are two forms of MCP in semen: ssMCP and smMCP.<sup>13,41</sup> MCP, presumably smMCP, was cloned from a human testis cDNA library,<sup>42</sup> and had a homologous amino acid sequence to those of peripheral blood cells, but was devoid of sugars.<sup>13,41</sup> The lack of sugars may be convenient for cell-cell attachment since the sugars are generally charged and interfere with the contact between the membranes. In addition, smMCP is expressed after acrosome reaction on the inner acrosomal membrane.<sup>12,13,16</sup> Thus, smMCP might be specially differentiated for spermatozoon-oocyte interaction.<sup>17</sup> The two forms of MCP in semen, which are different in their source, structure and function, are probably essential for physiological spermatozoan factors.

According to other reports, the prostasomes have membrane-linked ectoenzyme systems, Mg<sup>2+</sup>- and Ca<sup>2+</sup>-dependent ATPase, protein kinases, and zinc-dependent peptidase activities.<sup>34,43</sup> These factors may modulate the signalling pathways and confer the forward mortality of the

spermatozoa.<sup>34</sup> MCP is an SCR protein,<sup>11</sup> most of which serves as cell adhesion<sup>44</sup> and signal-transducing receptors.<sup>45</sup> Unlike membrane MCP, the MCP on prostasomes serves as a factor I-cofactor for the C3b, which is not on the same membrane; i.e. prostasome MCP is functionally different from the conventional membrane forms. An intriguing idea is that the prostasome MCP functions as an adhesion molecule for facilitating the interaction of the prostasome ectoenzymes with substrates on other cells, or serves in conjunction with other ectoenzymes as a modulator for the spermatozoa mortality.

C3dg is a product yielded secondarily by factor I and CR1,<sup>29</sup> or factor I, factor H and plasma kallikrein.<sup>31,46</sup> Membrane-bound MCP, however, has been reported to have a cofactor activity in converting C3b to C3bi.<sup>7,28</sup> If we regard ssMCP as a membrane molecule, we could demonstrate a novel cofactor activity of MCP. However, naturally, the prostasomes contain various proteases,<sup>34</sup> and they might be responsible for the generation of C3dg. To explore what is responsible for the C3dg generation is important since, unlike C3bi, C3dg is a physiologically active substance, increasing vascular permeability,<sup>31</sup> inducing leucocyte chemotactic activity, and suppressing T-cell proliferation.<sup>31,46</sup>

We first demonstrated that the prostasomes could inhibit viral activity, probably via their MCP, while the recombinant soluble form could not in the Vero cell system. A possible interpretation for these data is that the prostasome functions like a 'mock cell' and, by taking up the virus, renders it unable to infect another cell. In contrast, soluble forms of MCP bind to the MV H protein but are insufficient for blocking viral infection due to the lack of membrane architecture. If so, the prostasome facilitates a 'safe' microenvironment for spermatozoa by acting as a 'trap' for viruses or activated complements via binding with the prostasomal MCP. It will be important to examine the prostasomes for any internal system that might respond to such external stimuli.

In summary, the soluble form of MCP in semen is a prostasome-bound form with full functions for membrane MCP, C inactivation and probably MV adsorption. It additionally expresses C regulatory activity, even in the absence of detergents, enough to inhibit C3b in the fluid phase. These properties are different from other membrane C regulatory proteins, DAF<sup>47</sup> and CR1.<sup>48</sup> The necessity of this form of MCP in the semen will be an important issue in investigating fertilization, local immunity in the female genital tract, and the signalling for spermatozoa activation.

## ACKNOWLEDGMENTS

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