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Complement C9 is inserted into membranes in a globular conformation

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Complement component C9 undergoes a major conformational change during its insertion into a biological membrane from a globular to an extended form. At 0°C a single C9 binds but a membrane attack complex (MAC) is not formed. We show that the C9 bound at 0°C is accessible to the intracellular space and sensitive to trypsin digestion, suggesting that C9 inserts in its globular state and requires an elevated temperature in order to change conformation.

Complement; C9; Membrane assembly; Cytotoxicity

1. INTRODUCTION

The insertion of complement component C9 into a biological membrane depends on the prior formation in the membrane of a stoichiometric C5b-C6-C7-C8 complex (C5b–8). One or more C9 molecules are able to bind to this receptor and insert into the bilayer to form a stable pore. During C9 insertion the molecule undergoes a rearrangement from an aqueous, globular protein into an elongated molecule of about twice the length with the properties of an integral membrane protein (review [1]). Understanding this process could be of importance for the post-translational insertion of other membrane proteins as well as complement action, yet few of the steps have been described in molecular detail.

Studies by Boyle et al. [2] showed that a single C9 molecule could bind to C5b–8 at 0°C and that trypsin digestion of this complex at 0°C could prevent subsequent MAC formation on warming to 37°C. If the complex assembled at 0°C was warmed to 37°C, even for short times, before digestion with trypsin the sensitivity of MAC formation to proteolysis was lost. This result was interpreted as being due to initial binding of globular C9 to an extracellular binding site on C5b–8 followed by shielding of the C9 with lipids after elevating the temperature to 37°C. We have now used a monoclonal antibody to C9 to show that the trypsin-sensitive C9 is accessible to intracellular antibodies, suggesting that globular C9 is inserted deep into the lipid bilayer and that the temperature-dependent step in MAC formation is the transition from globular to extended C9.

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2. EXPERIMENTAL

2.1. *Iodination of C9*

10 μg human C9, purified by the method of Morgan et al. [3], was labelled with 0.5 mCi ^{125}I by the Iodogen method [4] giving an incorporation of 0.6 atoms per molecule.

2.2. *Immunoprecipitation of C9*

Mouse monoclonal antibodies (2 μg IgG) were added to ^{125}I-labelled C9 (5 ng, 100000 cpm) in 500 μl PBS containing 1% Triton X-100, 0.1% SDS, 0.1% deoxycholate and 0.1% bovine serum albumin (RIPA buffer). Mouse IgG was then adsorbed onto sheep anti-(mouse Ig) immunoadsorbant at 0°C overnight and after 2 washes in RIPA buffer, 1 wash in RIPA buffer containing 0.5 M NaCl and 2 washes in 10 mM Tris-HCl (pH 7.4),

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the immunoprecipitates were dissolved in sample buffer containing 4% SDS. After boiling for 5 min the samples were electrophoresed on 12.5% polyacrylamide gels which were dried and autoradiographed.

2.3. *Erythrocyte ghost experiments*

Rabbit erythrocyte ghosts, with or without entrapped monoclonal antibodies, were prepared from 100-μl aliquots of packed erythrocytes as previously described for pigeon erythrocytes, but with 7 final washes [5,6]. Ghosts were coated with C5b–8 by incubation for 5 min at 37°C with C9-depleted human serum diluted 1 : 5 in PBS. The ghosts were washed twice with medium A (140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$ and 10 mM Tes, pH 7.4) and resuspended in 100 μl of the same medium at 0°C. ^{125}I-labelled C9 (5 ng, 100000 cpm) was added for 2 min at 0°C and after washing twice with cold medium A, the ghosts were incubated in 100 μl medium A for a further 5 min at 0 or 37°C. The ghosts were again washed twice with cold medium A, resuspended in 100 μl of the same medium and incubated for 1 h at 0°C with trypsin (0.5 mg/ml). Soybean trypsin inhibitor (0.5 mg/ml) and PMSF (40 μg/ml) were added for 5 min at 0°C and after 2 further washes ghosts were lysed in 500 μl RIPA buffer and used for immunoprecipitation as described above.

3. RESULTS

M34 and M36 are two monoclonal antibodies raised against globular C9 which have a similar dissociation constant [3], but which show a different inhibition of ^{14}C-labelled sucrose efflux from erythrocyte ghosts. M36 inhibits efflux when added externally, but M34 only inhibits efflux when included with the label inside the ghosts [5].

Fig.1 shows that monoclonal M36 has a similar epitope to M34 by Western blot of trypsin fragments of C9. Both antibodies were able to bind to identical fragments, indicating that their epitopes are located within the same proteolytically stable domains. The first trypsin cleavage occurs between residues 391 and 392 of human C9 [7], giving rise to an amino-terminal fragment of approx. 48 kDa in unreduced gels which is positive with both antibodies (lanes 5,8). The 21 kDa fragment released by trypsin digestion from the carboxy-terminus is not detected by either antibody. Further digestion with trypsin results in a second cleavage in the centre of the molecule close to the α-thrombin cleavage site. The fragment released by this cleavage (approx. 30 kDa on unreduced gels) is also positive (fig.1, lanes 6,9) showing that both M34 and M36 bind to the amino-terminal (C9a) half of the molecule.

M34 and M36 were also checked for their ability

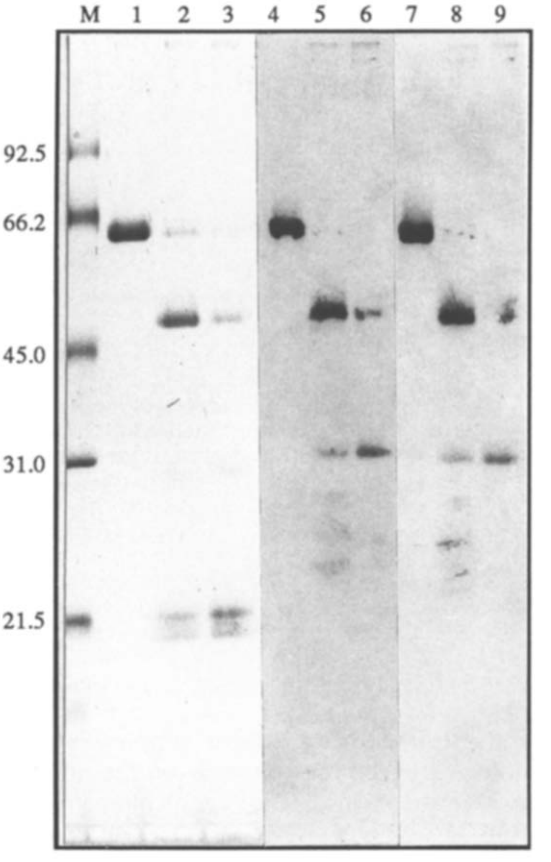

Fig.1. M34 and M36 have epitopes in the amino-terminal half of C9. Purified human complement component C9 was digested with TPCK-treated trypsin and run on a 10–15% non-reducing polyacrylamide gel. Lanes: (1–3) Serva brilliant blue stain; (4–6) monoclonal M34 immunoblot; (7–9) monoclonal M36 immunoblot. Lanes: (1,4,7) 2 μg undigested C9; (2,5,8) 2 μg C9 incubated with 100 ng trypsin for 30 min at 25°C; (3,6,9) 2 μg C9 incubated with 500 ng trypsin for 30 min at 25°C.

to immunoprecipitate C9. Fig.2a shows that the recovery of labelled C9 was similar using both M34 and M36, but that after trypsin digestion of the C9 for 60 min at 0°C little immunoprecipitable material remains.

M36 was then entrapped in ghosts and ^{125}I-labelled C9 bound at 0°C as described in section 2. Cells were either maintained at 0°C (fig.2b, lane 1) or warmed to 37°C (fig.2b, lane 2) for 5 min and then cooled and digested with trypsin for 60 min at 0°C. It can be seen that raising the temperature to 37°C for 5 min renders the C9 insensitive to trypsin, thus increasing its recovery in the immunoprecipitation. When this experiment was

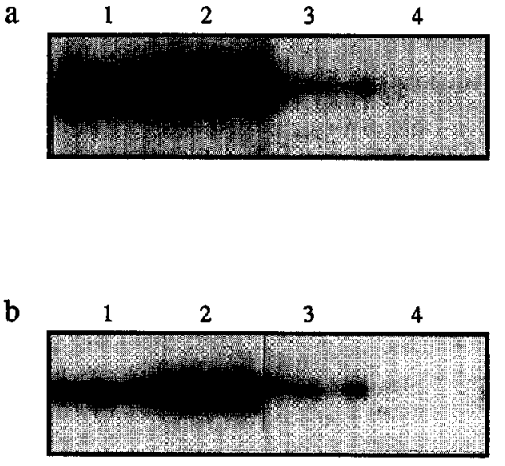

Fig.2. M34 but not M36 is able to prevent the molecular rearrangement of C9 by binding to C9 from the cytoplasmic compartment during insertion. (a) Control immunoprecipitation of 5 ng human C9 by 2 μg monoclonal antibodies M34 (lanes 1,3) and M36 (lanes 2,4). Lanes: (1,2) before digestion of C9; (3,4) after digestion with 0.5 mg/ml trypsin for 60 min at 0°C. (b) Immunoprecipitation of C9 in rabbit erythrocyte ghosts as described in section 2 with (lanes 2,4) or without (lanes 1,3) an incubation for 5 min at 37°C before trypsin digestion at 0°C. Lanes 1,2, intracellular M36; 3,4, intracellular M34.

repeated with M34 no protection of the C9 against trypsin digestion was observed (fig.2b, lanes 3,4). This antibody is therefore able to prevent the transition in C9 which normally renders it trypsin insensitive after warming to 37°C. Only membrane-bound C9 can be present in immunoprecipitates, since the ghosts are washed before solubilization of the ghosts can release the antibody and also because the concentration of trypsin used would have completely degraded the extracellular C9 (fig.2a).

4. DISCUSSION

Morgan et al. [5] showed that one monoclonal antibody raised against C9 (M34) had the interesting property of being able to inhibit the efflux of ^{14}C-labelled sucrose when induced with the label inside erythrocyte ghosts, but not when added extracellularly. M34 was subsequently shown to bind to the amino-terminal portion of C9 after cleavage with trypsin or α-thrombin [7], but does not react with reduced protein fragments or short segments of polypeptide expressed in *E. coli* (not shown), suggesting a discontinuous epitope involving cysteine-rich regions within the amino-terminal half of C9. Taken together, these two results implied that the amino-terminal portion of C9 is transiently exposed to the cytoplasmic space during insertion into the membrane [8]. One molecular interpretation of this conclusion is that C9 inserts into the membrane in its globular conformation and only in a second step refolds into a membrane-interacting extended conformation [1,8]. We have tested this model by investigating whether the C9 which is capable of interacting with M34 from the intracellular space is trypsin sensitive, a characteristic of globular C9.

The experiments that we have presented confirm the original observation by Boyle et al. [2] that C9 bound at 0°C undergoes a transition on raising the temperature to 37°C that renders it resistant to trypsin digestion. We also find that this transition may be prevented by intracellular monoclonal antibody M34 showing that: (i) the epitope of M34 on the amino-terminal half of C9 has access to the intracellular compartment during insertion into erythrocytes; (ii) C9 inserts into the membrane in its trypsin-sensitive conformation; and (iii) M34 locks C9 in its globular conformation preventing the transition to a trypsin-resistant form.

The earlier terminal components C7, C8α and C8β have all been shown to have a C9-like sequence [9] which allows a change from globular to extended forms on insertion into the membrane [10]. Even a single C9 molecule could therefore be stabilised in its extended form when C5b–9 is raised from 0 to 37°C, giving a proteolytically stable protein complex. Such complexes have also been shown to make stable channels of a size suggesting the contribution of components other than C9 to the transmembrane pore [11]. Thus, the transition from trypsin-sensitive to -insensitive C9 in these experiments is most likely to represent the conformational rearrangement of C9 from globular C9 bound to C5b–8 to its extended conformation. From the present data, this transition occurs after insertion into the membrane and requires an elevated temperature.

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