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Effect of sodium chloride concentration on fluid-phase assembly and stability of the C3 convertase of the classical pathway of the complement system

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The assembly of the classical-pathway C3 convertase from C4 and I_2-treated C2 by the action of $C\bar{1}s$ is an Mg^{2+}-dependent reaction. The Mg^{2+} concentration necessary for the assembly of C3 convertase in the fluid phase was found to be dependent on NaCl concentration. In the absence of NaCl more than 5 mM-$MgCl_2$ was found to be required, whereas 0.5 mM-$MgCl_2$ was adequate for the assembly of C3 convertase in the presence of 150 mM-NaCl. The C3 convertase assembled in a low-ionic-strength buffer was extremely labile compared with that assembled in buffer of physiological ionic strength, and the stability of C3 convertase was improved with the increase in NaCl concentration. It was found that the stabilizing effect of NaCl on C3 convertase was due to inhibition of the dissociating activity of C2b, which was formed during the assembly of C3 convertase. In addition to the dissociation-accelerating effect, C2b inhibited the assembly of C3 convertase in low-ionic-strength buffer, and this effect also was diminished with increase in NaCl concentration. An increase in NaCl concentration to more than 200 mM resulted in a decrease in the assembly of C3 convertase. This effect was not due to the lability of the assembled C3 convertase but due rather to the inhibition of C2 cleavage by $C\bar{1}s$. Purified C3 convertase itself is stable in dilute medium or high-ionic-strength medium such as 500 mM-NaCl, suggesting that the interactions between C4b and C2a are hydrophobic. In these respects C2b seemed to be functionally similar to C4bp, but C2b failed to act as a cofactor for the Factor I-catalysed C4b cleavage.

INTRODUCTION

The complement system is a humoral host defence composed of about 20 plasma proteins (Lachmann & Hughes-Jones, 1985). A central reaction in the complement system is the activation of C3 convertase, which cleaves C3 into C3a anaphylatoxin and nascent C3b (Reid & Porter, 1981). The nascent C3b is capable of binding to hydroxy residues on receptive surfaces such as immune complexes and micro-organisms (Law & Levine, 1977). The foreign materials with bound C3b are processed by polymorphonuclear leucocytes and macrophages through C3b receptors (Griffin, 1988).

There are two types of C3 convertases, which are generated by two separate enzyme cascade systems, the classical and alternative pathways. The classical-pathway C3 convertase is generated by limited proteolysis of C4 and C2 by $C\bar{1}s$ (Müller-Eberhard *et al.*, 1967). This activation seems to consist of two sequential proteolytic reactions. Initially, C4 (200 kDa) is cleaved by $C\bar{1}s$ into C4a (9 kDa) and C4b (190 kDa). C4b generates a stable binding site for C2 (110 kDa) and forms an Mg^{2+}-dependent precursor complex, C4b–C2, the C2 portion of which is subsequently cleaved by $C\bar{1}s$ into C2a (70 kDa) and C2b (35 kDa) (Vogt *et al.*, 1982). C2a remains bound to C4b and exerts the C3-cleaving activity. The C3 convertase, C4b–C2a, thus assembled is a labile serine proteinase and undergoes a rapid loss of activity with a half-life of 2 min at 37 °C due to the temperature-dependent dissociation of C2a (Kerr, 1980). Pretreatment of human C2 with dilute I_2 solution has been generally used as an effective method for the preparation of stabilized C3 convertase with a half-life of 30 min at 37 °C (Polley & Müller-Eberhard, 1967). Although the exact mechanism of the stabilization of C3 convertase has not yet been clarified, the I_2 treatment modifies a thiol residue in the C2a portion and thus stabilizes the non-covalent interaction between C2a and C4b (Parkes *et al.*, 1983). The dissociation of C3 covertase is accelerated by serum and cellular C4b-binding proteins, such as C4bp (Gigli *et al.*, 1979), CR1 (Iida & Nussenzweig, 1983) and DAF (Nicholson-Weller *et al.*, 1982). These regulatory proteins are known to contain tandem repeats of an identical sequence of 60 amino acid residues in length, which are generally termed short consensus repeats (Reid & Day, 1989).

Although C2b is not a component of the C3 convertase, there is increasing evidence suggesting that the C2b portion of the C2 molecule may play an important role in the assembly of C3 convertase. Nagasawa & Stroud (1977) reported the binding of C2b to C4b–Sepharose and showed the formation of a C4b–C2b complex by native disc gel electrophoresis. Also, Kerr (1980) has separated a C4b–C2b complex by gel filtration of dissociated C3 convertase. More recently, Oglesby *et al.* (1988) have prepared a monoclonal antibody to C2b that inhibits the assembly of C3 convertase. They have proposed that the C2b domain is implicated in the assembly of C3 convertase, possibly at the stage of formation of a precursor complex, C4b–C2. Just like other short-consensus-repeat-containing C4b-binding proteins, C2b has been shown to contain three short consensus repeats at its *N*-terminus (Bentley, 1986) and acts as a dissociation-accelerator of C3 convertase (Nagasawa *et al.*, 1985).

This information suggests that at least two C4b-binding sites, one on C2b and the other on C2a, are implicated in the assembly of C3 convertase. However, little is known about the mechanism of interaction between C4b and the two C4b-binding sites on C2.

The assembly of C3 convertase has been shown to require bivalent ions such as Mg^{2+} and Ni^{2+} (Fishelson & Müller-Eberhard, 1982). When we investigated the metal-ion-dependency for the assembly of C3 convertase, we observed that the Mg^{2+} concentration necessary for the assembly of C3 convertase was dependent on the ionic strength of the buffer. The present studies were instigated by the need to clarify this observation. It

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was found that the physiological ionic strength is a critical factor for the assembly and stabilization of fluid-phase C3 convertase. This effect is due to the restriction at higher ionic strength of the ability of C2b to accelerate the dissociation of C3 convertase. The data also suggest that the interaction between C4b and C2b is ionic, whereas that between C4b and C2a is non ionic, possibly hydrophobic.

MATERIALS AND METHODS

Human complement proteins

C4 and Factor I (Nagasawa *et al.*, 1980), C2 (Nagasawa & Stroud, 1977), $C\bar{1}s$ (Takahashi *et al.*, 1975) and C4bp (Nagasawa *et al.*, 1982) were purified by the respective reported methods. C4b or C2 fragments were prepared by incubating C4 or C2 with $C\bar{1}s$ at a substrate/enzyme weight ratio of 50:1 for 60 min at 37 °C, and the fragments were purified by size-exclusion h.p.l.c. on a TSK G3000SW column (7.8 mm × 300 mm) (HG type; Tosoh, Tokyo, Japan).

Protein concentrations were estimated from the absorbance at 280 nm, assuming an A_{280} absorption coefficient value of 0.1% solution = 1.0. Complement proteins were used after dialysis against 20 mM-Tris/HCl buffer, pH 7.4.

Purification of C3 convertase

To obtain a stabilized C3 convertase, C2 was treated with I_2 according to the method of Polley & Müller-Eberhard (1967) and dialysed against 20 mM-Tris/HCl buffer, pH 7.4. A mixture containing C4b (17 μg) and C2 (24 μg) in 100 μl of 20 mM-Tris/HCl buffer, pH 7.4, containing 10 mM-$MgCl_2$ and 150 mM-NaCl was incubated with $C\bar{1}s$ (2 μg) for 5 min at 37 °C. The incubation mixture was subjected to size-exclusion h.p.l.c. on a TSK G3000SW column (7.8 mm × 300 mm; HG type). Elution of proteins was performed with 0.1 M-sodium phosphate buffer, pH 6.8, and monitored by measuring the absorbance at 220 nm. The peak fraction corresponding to about 300 kDa was collected and used as purified C3 convertase (Nagasawa *et al.*, 1985).

The analysis of assembly and stability of C3 convertase was also performed by size-exclusion h.p.l.c. on the TSK G3000SW column and elutions were always performed with 0.1 M-sodium phosphate buffer, pH 6.8. The effect of additives on the assembly of C3 convertase was assessed by comparing the amounts of C3 convertase formed during 5 min incubation at 37 °C. On the other hand, the effect of additives on the stability of C3 convertase was assessed by comparing the amounts of C3 convertase remaining after 30 min incubation at 37 °C.

Assay of the cofactor activity in Factor I-catalysed C4b cleavage

The cofactor activity of C4bp and C2b was determined by measuring the decrease in the C4b band (190 kDa) on SDS/PAGE as described previously (Nagasawa *et al.*, 1980). In routine assays mixtures containing 12 μg of C4b, 1 μg of Factor I and different amounts of C4bp or C2b in 50–100 μl of 20 mM-Tris/HCl buffer, pH 7.4, were incubated for 4 h at 37 °C and subjected to SDS/PAGE according to the method of Fairbanks *et al.* (1971). The amount of C4b remaining was determined by densitometric scanning of the stained gels at 550 nm.

RESULTS

Requirement for Mg^{2+} for the assembly of fluid-phase C3 convertase

Pryzdial & Isenman (1986) have reported that the requirement for Mg^{2+} in the assembly of fluid-phase alternative-pathway C3 convertase is not absolute. We attempted to re-evaluate the requirement for Mg^{2+} for the assembly of classical-pathway C3 convertase in the fluid phase. C4b and C2 in 20 mM-Tris/HCl buffer, pH 7.4, were mixed in the presence and in the absence of 10 mM-$MgCl_2$ and incubated with $C\bar{1}s$ at 37 °C. After 5 min, portions were subjected to size-exclusion h.p.l.c. on a TSK G3000SW column. As shown in Fig. 1, a sharp peak corresponding to C3 convertase was observed when the assembly was performed in the presence of Mg^{2+}, whereas no peak corresponding to C3 convertase was detected with the Mg^{2+}-free sample. This result demonstrates that, in contrast with the alternative-pathway C3 convertase, the classical-pathway C3 convertase requires Mg^{2+} for its assembly.

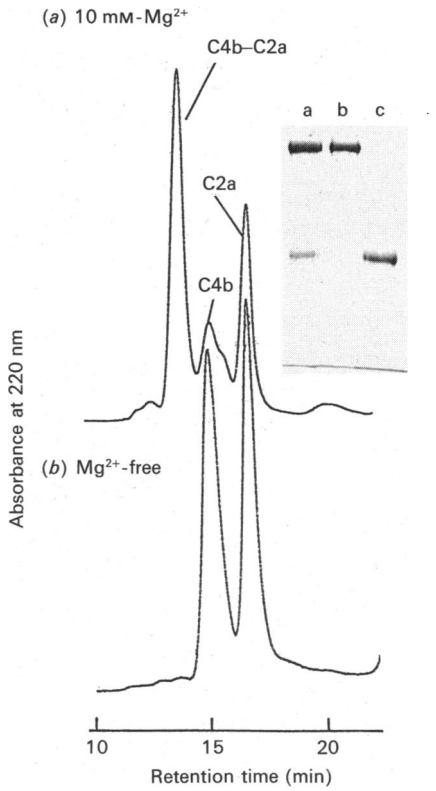

Fig. 1. Separation of C3 convertase by size-exclusion h.p.l.c.

C4b (2 μg) and C2 (4 μg) were mixed in 20 mM-Tris/HCl buffer, pH 7.4, and incubated with $C\bar{1}s$ (0.5 μg) in the presence (*a*) and in the absence (*b*) of 10 mM-$MgCl_2$. After 5 min at 37 °C the incubation mixture was subjected to size-exclusion h.p.l.c. as described in the Materials and methods section. To confirm that the sharp peak eluted at 14.5 min represents C3 convertase, the peak fraction was pooled, dialysed against water, freeze-dried and subjected to SDS/PAGE on a 7.5% acrylamide gel under non-reducing conditions (Laemmli, 1970). The gel was stained with Coomassie Blue stain and is shown in the inset: lane a, the peak fraction, 5 μg; lane b, C4b, 3 μg; lane c, C2a, 3 μg.

Effect of Mg^{2+} concentrations on the assembly and stability of C3 convertase

Next, we attempted to assess the minimum concentration of Mg^{2+} needed for the assembly of the classical-pathway C3 convertase. Fig. 2 showed that the Mg^{2+} requirement for the assembly of C3 convertase was markedly affected by NaCl concentration; at low ionic strength (20 mM-Tris/HCl buffer, pH 7.4) 10 mM-$MgCl_2$ is required for the maximum formation of C3 convertase, whereas at physiological ionic strength (150 mM-NaCl 20 mM-Tris/HCl buffer, pH 7.4) the assembly of C3 convertase reached a plateau at physiological Mg^{2+} concentration.

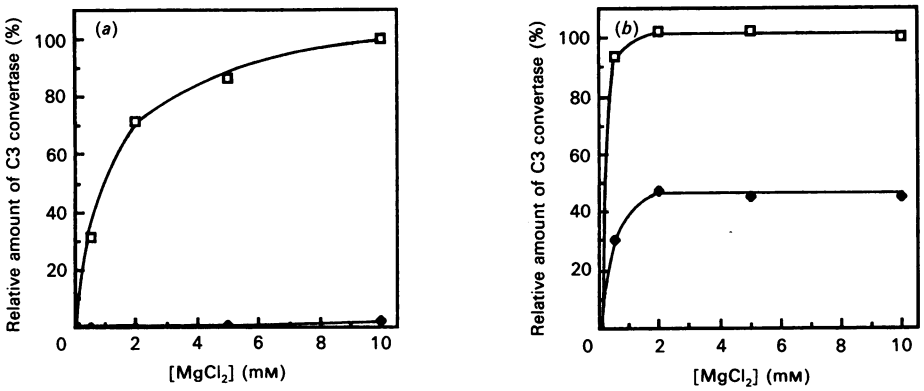

Fig. 2. Effect of Mg^{2+} concentration on the assembly and stability of C3 convertase

The indicated concentrations of $MgCl_2$ were included in a mixture of C4b and C2 in 20 mM-Tris/HCl buffer, pH 7.4 (*a*) or 20 mM-Tris/HCl buffer, pH 7.4, containing 150 mM-NaCl (*b*). Assembly of C3 convertase was started by addition of $C\bar{1}s$. After 5 min (□) and 30 min (◆) incubation at 37 °C, the incubation mixtures were subjected to size-exclusion h.p.l.c. The amounts of C3 convertase were estimated from the peak area of the h.p.l.c. chromatogram. The proportions of C4b, C2 and $C\bar{1}s$ were the same as those in Fig. 1. Results are expressed as percentages of the amount of C3 convertase assembled in the presence of 10 mM-$MgCl_2$ and 150 mM-NaCl.

These results suggested that physiological NaCl concentration is required for the activation of C3 convertase at physiological Mg^{2+} concentration. In addition, the stability of C3 convertase was found to be affected by ionic strength. In the absence of NaCl more than 90% of C3 convertase was dissociated after 30 min at 37 °C. On the other hand, in the presence of 150 mM-NaCl about 50% of C3 convertase still remained after 30 min. The stability of C3 convertase in low-ionic-strength buffer was not improved by the addition of 10 mM-$MgCl_2$.

Effect of ionic strength upon the assembly of C3 convertase

The above results suggest that, in addition to Mg^{2+} concentration, ionic strength is a critical factor for the fluid-phase assembly of C3 convertase. Therefore we examined whether the low yield of C3 convertase assembly in the presence of 0.5 mM-$MgCl_2$ would be improved by increasing NaCl concentration. Fig. 3 showed that the amounts of C3 convertase assembled in the presence of 0.5 mM-$MgCl_2$ gradually increased with increase of NaCl concentration and reached a maximum at 150 mM-NaCl. Increase of NaCl concentration above 150 mM in the buffer resulted in inhibition of the assembly of C3 convertase. Fig. 4 shows SDS/PAGE of the C2 cleavage by $C\bar{1}s$ at different NaCl concentrations. C2 cleavage by $C\bar{1}s$ was found to be inhibited with increase in NaCl concentration. These results suggest that the inhibition of C3 convertase assembly at high ionic strength was due to inhibition of C2 cleavage by $C\bar{1}s$.

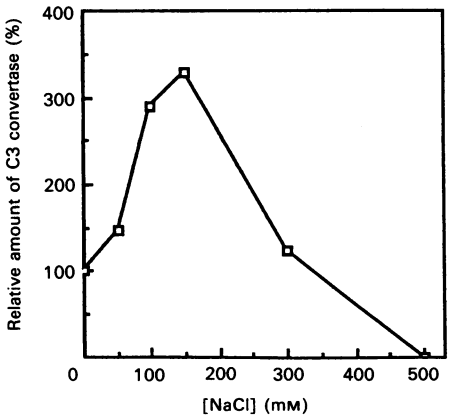

Fig. 3. Effect of NaCl concentration on the assembly of C3 convertase

Assembly of C3 convertase was performed in 20 mM-Tris/HCl buffer, pH 7.4, containing 0.5 mM-$MgCl_2$ and the indicated concentrations of NaCl. After 5 min at 37 °C the amounts of C3 convertase assembled were assessed by size-exclusion h.p.l.c. Results are expressed as percentages of a control assembled in 0.5 mM-$MgCl_2$.

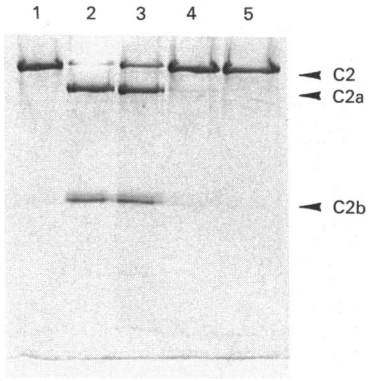

Fig. 4. Effect of NaCl concentration on C2 cleavage by $C\bar{1}s$

C2 (2.5 μg) was incubated with 0.1 μg of $C\bar{1}s$ in 20 mM-Tris/HCl buffer, pH 7.4, containing different concentrations of NaCl for 2 min at 37 °C and subjected to SDS/PAGE on a 10% acrylamide gel under non-reducing conditions (Laemmli, 1970). The gel was stained with Coomassie Blue. Lane 1, control C2; lane 2, 0 mM-NaCl; lane 3, 150 mM-NaCl; lane 4, 500 mM-NaCl; lane 5, 1 M-NaCl.

Effect of ionic strength upon stability of purified C3 convertase

The apparent increase in C3 convertase assembly with increase in NaCl concentration suggested that the stability of C3 convertase itself might be influenced by ionic strength. To test this possibility, C3 convertase was purified by h.p.l.c. and allowed to dissociate in buffer containing various concentrations of NaCl. As shown in Fig. 5, the dissociation of purified C3 convertase was not affected by the ionic strength of the buffer. This result suggests that the non-covalent interaction between C4b and C2a is mediated not by ionic interaction but rather by non-ionic hydrophobic interaction.

Thus the improved assembly of C3 convertase at physiological ionic strength could not be explained by a direct effect of NaCl

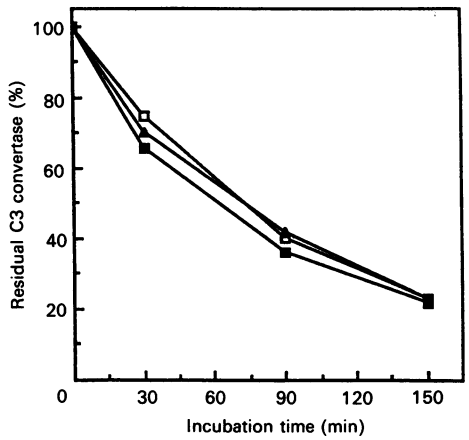

Fig. 5. Dissociation of purified C3 convertase in buffers of different ionic strengths

C3 convertase that had been purified by size-exclusion h.p.l.c. was dialysed against 20 mM-Tris/HCl buffer, pH 7.4, and adjusted to the following NaCl concentrations: □, 0 mM; ▲, 150 mM; ■, 500 mM. After incubation at 37 °C for the indicated periods, the C3 convertase remaining was assessed by size-exclusion h.p.l.c.

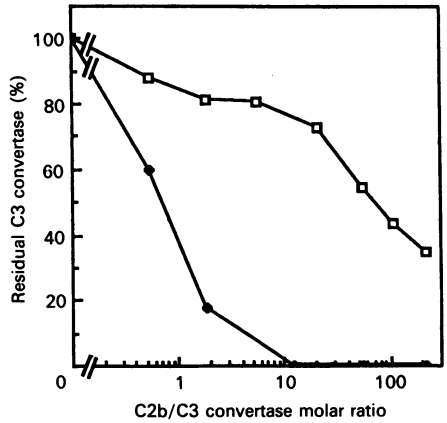

Fig. 6. Effect of C2b on the decay of C3 convertase

Purified C3 convertase was mixed with the indicated amounts of C2b in the presence (□) and in the absence (◆) of 150 mM-NaCl. After incubation for 30 min at 37 °C, the C3 convertase remaining was assessed by h.p.l.c. Results are expressed as percentages of a control in which C3 convertase in 150 mM-NaCl was incubated in the absence of C2b.

on the stability of C3 convertase. We have previously reported that C2b has the ability to accelerate the dissociation of C3 convertase (Nagasawa *et al.*, 1985). So it seemed likely that the effect of 150 mM-NaCl in improving C3 convertase assembly is to protect C3 convertase from the dissociating action of C2b, which was formed simultaneously with C3 convertase assembly. As shown in Fig. 6, addition of increasing amounts of C2b to purified C3 convertase in 20 mM-Tris buffer resulted in a decrease in the amount of C3 convertase remaining after 30 min. However, the C2b-induced lability of C3 convertase was effectively diminished in the presence of 150 mM-NaCl, suggesting the dissociating effect of C2b was sensitive to ionic strength. Therefore we incubated purified C3 convertase with a 10-fold molar excess of C2b in buffer containing various NaCl concentrations and the C3 convertase remaining was assessed by h.p.l.c. As shown in Fig. 7, C3 convertase was dissociated completely by a

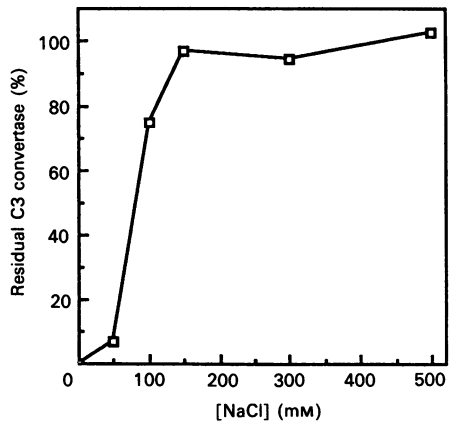

Fig. 7. Effect of NaCl concentration on C2b-induced dissociation of C3 convertase

Purified C3 convertase was mixed with 10-fold molar excess of C2b in the presence of the indicated concentrations of NaCl in 20 mM-Tris/HCl buffer, pH 7.4. After incubation for 30 min at 37 °C, the C3 convertase remaining was assessed by size-exclusion h.p.l.c. Results are expressed as percentages of a control in which C3 convertase was incubated in the absence of C2b.

10-fold molar excess of C2b in the absence of NaCl during 30 min at 37 °C. On the other hand, the dissociation-accelerating effect of C2b was gradually inhibited by increases in NaCl concentration and almost completely inhibited at NaCl concentration close to the physiological concentration.

Effect of C2b upon the assembly of C3 convertase

Next we attempted to assess a possibility that C2b might also interfere with the assembly of C3 convertase through inhibition of the assembly of proconvertase. As shown in Fig. 8, the addition of C2b to a mixture of C4b and C2 resulted in inhibition of the assembly of C3 convertase. This effect of C2b was depressed by the addition of 10 mM-$MgCl_2$ or 150 mM-NaCl.

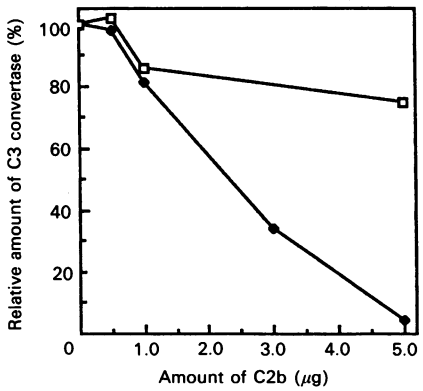

Fig. 8. Effect of the C2b on the assembly of C3 convertase

To a mixture of C4b (2 μg) and C2 (1 μg) in 50 μl of 20 mM-Tris/HCl buffer, pH 7.4, containing 5 mM-$MgCl_2$ were added the indicated amounts of C2b in the presence (□) and in the absence (◆) of 150 mM-NaCl and incubated with $C\bar{1}s$ (0.3 μg) for 5 min at 37 °C. The amounts of C3 convertase assembled were assessed by size-exclusion h.p.l.c. Results are expressed as percentages of a control in which activation of C3 convertase was performed in the absence of C2b.

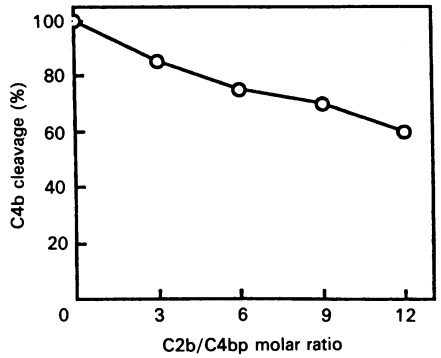

Fig. 9. Effect of C2b upon Factor I-catalysed C4b cleavage in the presence of C4bp

To a mixture containing 12 μg of C4b, 1 μg of Factor I and 1.5 μg of C4bp in 50 μl of 20 mM-Tris/HCl buffer, pH 7.4, were added the indicated amounts of C2b, and the mixtures were incubated for 4 h at 37 °C and samples subjected to SDS/PAGE. The percentage cleavage of C4b cleavage was determined by densitometric scanning of Coomassie Blue-stained gels.

Effect of C2b upon Factor I-catalysed C4b cleavage

The results presented so far indicate that C2b is a dissociation-accelerator of the classical-pathway C3 convertase. Natural dissociation-accelerators such as C4bp and CR1, but not DAF, also act as a cofactor for the Factor I-catalysed C4b-cleavage reaction. Consequently we next examined whether C2b, like C4bp, is such a cofactor. C2b, however, did not show cofactor activity. Rather, it was found that C2b inhibited the cofactor function of C4bp. As shown in Fig. 9, the Factor I-catalysed cleavage of C4b with C4bp as a cofactor was progressively suppressed upon addition of increasing amounts of C2b. The C4b-cleavage reaction was suppressed by 40% when a 12-fold molar excess of C2b over C4bp was present. Since C4bp had at least seven identical subunits, each having a domain for the cofactor activity, the molar ratio of 12:1 would correspond to 2:1 on the basis of equivalent weight. These results suggested that C2b can interfere with the formation of the C4b–C4bp complex, which is prerequisite for the Factor I-catalysed C4b-cleavage reaction.

DISCUSSION

Conditions for the assembly of the classical-pathway C3 convertase in the fluid phase have been investigated by Polley & Müller-Eberhard (1967), Vogt *et al.* (1982), Kerr (1980) and Villiers *et al.* (1985). However, most of these studies have been done at physiological ionic strength, and little attention has been paid to the effect of ionic strength on the assembly of the C3 convertase. In the present studies we found that ionic strength is a critical factor for the assembly and stability of C3 convertase.

The assembly of C3 convertase is known to be a metal-ion-dependent reaction, and 0.5 mM-$MgCl_2$ has generally been used. In the present studies we found that the physiological concentration of Mg^{2+} (0.5 mM) is not adequate when the assembly of C3 convertase was attempted in buffer of low ionic strength. More than 5 mM-Mg^{2+} is required for the assembly of C3 convertase in NaCl-free buffer, whereas 0.5 mM-Mg^{2+} is adequate for the assembly of C3 convertase at physiological ionic strength. Villiers *et al.* (1985) have reported that the formation of proconvertase, C4b–C2, is a metal-ion-dependent reaction. So it seems that a relatively high concentration of Mg^{2+} is required for the formation or stabilization of proconvertase under low-ionic-strength conditions.

In addition to the low yield of C3 convertase, the C3 convertase assembled in NaCl-free buffer was extremely labile compared with the C3 convertase assembled at physiological ionic strength. The stability of C3 convertase in NaCl-free buffer was not improved by the addition of 10 mM-$MgCl_2$. Thus physiological ionic strength seems to be an important factor for the stability as well as assembly of C3 convertase in the fluid phase.

On the other hand, the stability of C3 convertase that was isolated by size-exclusion h.p.l.c. was found to be independent of ionic strength, suggesting that the apparent lability of C3 convertase assembled in NaCl-free buffer was caused by components present in the incubation mixture. In a previous paper we reported that C2b acts as the dissociation-accelerator for C3 convertase (Nagasawa *et al.*, 1985). In the present studies we showed that the dissociation-accelerating action of C2b is regulated by ionic strength; increase of ionic strength to physiological strength resulted in inhibition of the dissociation-accelerating effect of C2b. So the apparent lability of C3 convertase assembled at low ionic strength seems to be due to C2b, which is a by-product of C3 convertase assembly. The reason for the decrease in the dissociation-accelerating effect of C2b due to increase of ionic strength is unknown at present. Kerr (1980) has reported that the C4b–C2b complex was detectable by gel filtration at 50 mM-NaCl, but not at 150 mM-NaCl, suggesting that C4b–C2b interaction is sensitive to ionic strength. So it appears that the interaction between C4b and C2b is ionic and that an increase in ionic strength prevents the interaction between the two components. The failure of 10 mM-$MgCl_2$ to stabilize C3 convertase at low ionic strength suggests that Mg^{2+} does not protect C3 convertase from the dissociating action of C2b.

The effect of ionic strength on the assembly of C3 convertase was found to be dual. Increase in ionic strength above physiological strength resulted in a decrease in the assembly of C3 convertase. This was due to the inhibition of C2 cleavage by $C\bar{1}s$. This inhibition of C2 cleavage is not due to inactivation of $C\bar{1}s$, because the esterolytic activity of $C\bar{1}s$ did not change in the presence of 500 mM-NaCl (results not shown). Once C3 convertase was assembled, its stability was not affected by increasing ionic strength to 500 mM-NaCl, suggesting that the forces between C4b and C2a are hydrophobic. Evidence that C2b interferes with the assembly of C3 convertase provides an additional proof that the C2b in the C2 molecule plays an important role in the assembly of C3 convertase. Ba is a fragment released during the assembly of alternative-pathway C3 convertase and is known to have three short consensus repeats, similarly to C2b (Morley & Campbell, 1984). Pryzdial & Isenman (1986) have reported that the assembly of the alternative-pathway C3 convertase is inhibited by Ba, but the rate of dissociation of alternative-pathway C3 convertase is not accelerated by Ba (Pryzdial & Isenman, 1987).

C4bp is an intrinsic regulator of the classical-pathway C3 convertase; it binds to C4b to accelerate the dissociation of C3 convertase and induce the Factor I-catalysed C4b cleavage. Although C2b is capable of accelerating the dissociation of C3 convertase, C2b fails to act as the cofactor for the Factor I-catalysed C4b cleavage (Gigli *et al.*, 1979; Nagasawa *et al.*, 1980). This suggests that a conformation change induced by the binding of C2b to C4b is enough to decrease the affinity between C4b and C2a but not enough to expose the cryptic site to Factor I. It seems that additional reinforcing interaction between C4b and C4bp may play an important role in causing the Factor I-catalysed C4b cleavage to occur. The binding site on C4b for C2b is not known. We have obtained a monoclonal antibody that recognizes a neoantigen on C4b and interferes with the binding of C4bp and C2 to C4b (Ichihara *et al.*, 1986). In the present study C2b was found to interfere with the function of C4bp to act

as a cofactor of Factor I-catalysed C4b cleavage. These results also seem to suggest that C2b and C4bp bind to a common site or closely related site on C4b.

It has been proposed that C2 binds to C4b through two different sites, one in the C2b portion and the other in the C2a portion (Oglesby *et al.*, 1988; Pryzdial & Isenman, 1987). Although interaction between C2b and C4b is labile in physiological ionic strength, the additional binding through the C2a portion to C4b may stabilize the C4b–C2 complex.

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