Evidence that a nicked C4b, C4b′, is a functionally active C4b derivative

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Factor I-catalyzed C4b cleavage is a regulatory reaction for the classical pathway of the complement system. Although the reaction was shown to be a two-step reaction, (i) production of a nicked form of C4b, C4b′, as an intermediate cleavage product and (ii) subsequent splitting of C4b′ into C4c and C4d, it is not known which of the two steps represents the inactivation of the C4b function in the assembly of C3 convertase, C4b,2a. We have purified C4b′ and assessed the ability of C4b′ to assemble C3 convertase with C2 by utilizing size exclusion high performance liquid chromatography. Evidence was obtained demonstrating that C4b′ still retains the function of C4b to assemble C3 convertase. Thus, the substantial step for the inactivation of the C4b function appears to be the second cleavage reaction, that is, the cleavage of C4b′ into C4c and C4d.

Complement *C3 convertase* *nicked C4b* *HPLC* *Molecular assembly*

1. INTRODUCTION

The fourth component of human complement, C4, is a glycoprotein of 200000 Da composed of three disulfide-linked polypeptide chains, α (94 kDa), β (75 kDa), and γ (35 kDa) [1]. The role of C4 in the complement system is to assemble C3 convertase, which is a labile complex composed of C4b and C2a, C4b,2a [2]. The activation of C3 convertase consists of the following three steps; initial cleavage of C4 with $\overline{C1}$s into C4a (9 kDa) and C4b (190 kDa), binding of C4b with C2 in the presence of Mg^{2+}, and subsequent cleavage of C4b-C2 complex by $\overline{C1}$s into C4b,2a and C2b [2–5]. The cleavage of C4 by $\overline{C1}$s occurs on the α chain and generates on C4b molecule at least two different binding sites; one is a labile binding site to immune complexes [6,7] and another is a stable binding site for C2 [2–5]. The function of C4b to assemble C3 convertase is controlled by two plasma proteins, C4b-binding protein (C4bp) [8] and factor I [9]. C4bp binds with C4b to induce I-catalyzed C4b-cleavage which occurs on the α' chain and yields two functionally inactive fragments, C4c (140 kDa) and C4d (45 kDa) [8]. We previously reported that a nicked form of C4b, C4b′, with the same molecular mass as C4b was generated as an intermediate cleavage product [11]. Although the formation of nicked C4b was later confirmed by several groups [11,12], it still remained to be determined whether C4b′ still retains the functional activity to generate C3 convertase. Evidence will be presented demonstrating that the nicked C4b is an active C4b derivative. Thus, in contrary to C3b cleavage, in which formation of a nicked C3b represents the inactivation of C3b [12,13], the cleavage of C4b′ into C4c and C4d represents a true inactivation step of the C4b function.

2. MATERIALS AND METHODS

Human complements, such as C2 [14], C4b [14], C3 [13], C4bp [15], I [10] and $\overline{C1}$s [16], were prepared as previously described. Nicked C4b was purified following the method described in [10]; in

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brief, C4b was incubated with I and C4bp at 37°C so as to achieve almost 50% conversion of C4b into C4c and C4d, as judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently subjected preparatively to disc-PAGE. One gel was stained, and the unstained gels were sliced by referring to the stained gel. The gel segments corresponding to C4b, which showed the same mobility on disc-PAGE as C4b, were collected and extracted with 40 mM Tris-HCl buffer, pH 7.4, at 37°C for 16 h. This method is based on evidence that when the C4b cleavage reaction goes up to almost 50% cleavage, the remaining C4b is already converted into the nicked form, C4b′ [10]. Fig.1 shows SDS-PAGE of the purified C4b′, which was composed of four bands; α'-1 fragment (75 kDa), β (75 kDa), γ (33 kDa) and α'-2 fragment (16 kDa). The ability of C4b′ to assemble C3 convertase was determined by measuring the amounts of C3 convertase (270 kDa), which was separated by size exclusion HPLC using TSK G3000 SW column (Toyo Soda Mfg, Japan) [14]. In addition, the enzymatic activity of C3 convertase to cleave C3 into C3a and C3b was determined by measuring the formation of C3b by disc-PAGE, in which C3b migrates more to the anodal side than C3. The percent cleavage of C3 was determined by den-

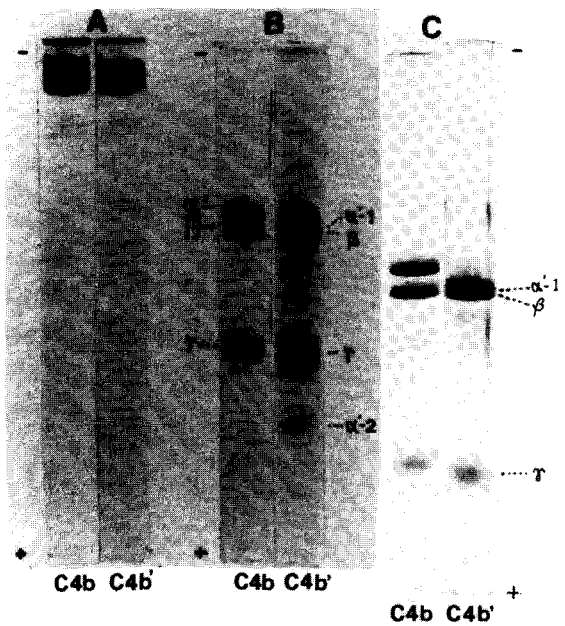

Fig.1. SDS-PAGE of C4b and nicked C4b, C4b′. C4b and C4b′ were subjected to SDS-PAGE for 60 min at 8 mA per tube using 5.6% acrylamide gels before (A) or after (B) treatment with 2-mercaptoethanol. The bands of α'-1 and β chains of C4b′ were overlapping each other to give a broad band in track B. The overloading of C4b′ (30 μg) in track B was to show the α'-2 band, which is α'16 fragment from the carboxy-terminal end of the α' chain and is only faintly stained by the Coomassie blue-staining method. Since the overloading of C4b′ in track B made assessment of the absence of C4b in the C4b′ preparation difficult, prolonged SDS-PAGE was performed for 90 min at 8 mA per tube with proper amounts of reduced C4b′ (6 μg) (C). Track C showed that C4b was absent in the C4b′ preparation. However, the α'-2 band of C4b′ was not stained in track C, because of low amount of applied C4b′.

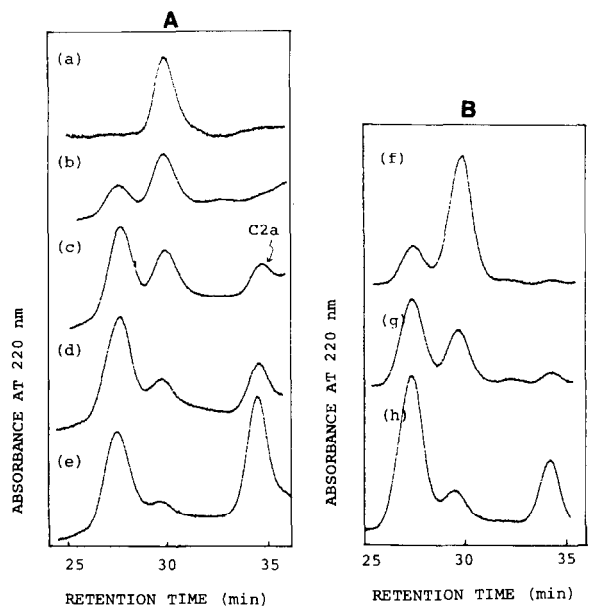

Fig.2. Demonstration of assembly of C3 convertase by HPLC. 2 μg of C4b or C4b′ and different amounts of oxy C2 were incubated with C1s in 50 μl of 20 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ for 5 min at 37°C and subjected to HPLC using TSK G3000SW column equilibrated with 0.1 M phosphate buffer, pH 6.8, at 20°C. Elution of proteins was determined by measuring the absorbance at 220 nm. A. C4b control (a). The molar ratios of C4b to oxy C2 were 1:1 (b), 1:2 (c), 1:4 (d) and 1:6 (e). B. The molar ratio of C4b to oxy C2 were 1:1 (f), 1:2 (g) and 1:4 (h). The amounts of C1s were adjusted to 1:10 (w/w) of the amounts of oxy C2. The peak of C2b which was eluted broadly between 40 and 45 min was omitted from the chart.

sitometrically scanning the Coomassie blue-stained gels at 550 nm. For the recovery of stable C3 convertase, iodine-treated C2, oxy C2 [2], was used. Protein concentrations were estimated from the absorbance at 280 nm, assuming an absorption coefficient value of 0.1% solution = 1.0. SDS-PAGE and disc-PAGE were performed as in [17] and [18], respectively.

3. RESULTS

Previously, we reported that HPLC using TSK G3000SW column was effective for the analysis of activation of C3 convertase; it clearly separates C3 convertase from C4b, C1s, C2a and C2b [14]. Then, the ability of C4b′ to assemble C3 convertase was determined by measuring the amounts of C3 convertase separated by HPLC and compared with that of intact C4b. C4b or C4b′ was mixed with different amounts of oxy C2 in the presence of 10 mM $MgCl_2$ incubated with $\overline{C1s}$ for 5 min at 37°C, and subjected to HPLC. Fig.2 shows the elution patterns of proteins. The peak eluted at 27 min represents the elution of C3 convertase and was found to increase with the increase of amounts of oxy C2. Conversely, the peak eluted at 30 min, which corresponds to C4b or C4b′ decreased with the increase of C3 convertase. When the percent conversion of C4b′ into C3 convertase was compared with that of intact C4b, the two curves were almost overlapping (fig.3). These results demonstrate that the nicked form of C4b still retains the ability to assemble C3 convertase as intact C4b.

We next assessed whether the C3 convertase assembled from the nicked C4b was indeed functionally active complex. Then, two C3 convertase preparations assembled from intact C4b and nicked C4b were isolated by HPLC and incubated with C3. As shown in fig.4, the C3 cleaving activities of the two C3 convertase preparations were found to be similar. Thus, it was shown that the nicked C4b was not an inactive derivative of C4b but an active intermediate produced during the I-catalyzed C4b cleavage reaction.

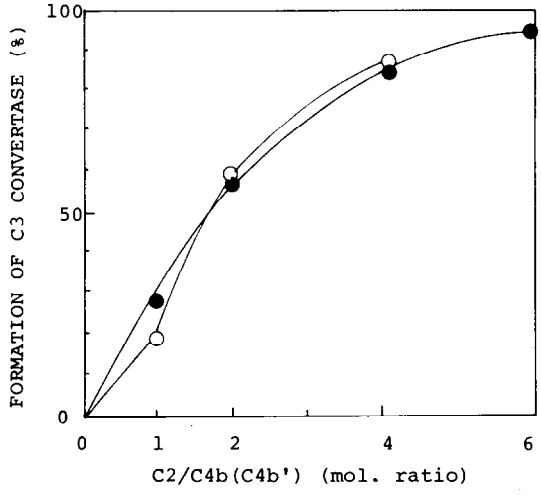

Fig.3. Comparison of the C3 convertase-forming ability of C4b′ with that of intact C4b. The percent conversion of C4b (○) or C4b′ (●) into C3 convertase in the presence of different amounts of oxy C2 was estimated from the ratio of peak-area of C3 convertase to the sum of the peak-areas of C4b or C4b′ and C3 convertase of fig.2.

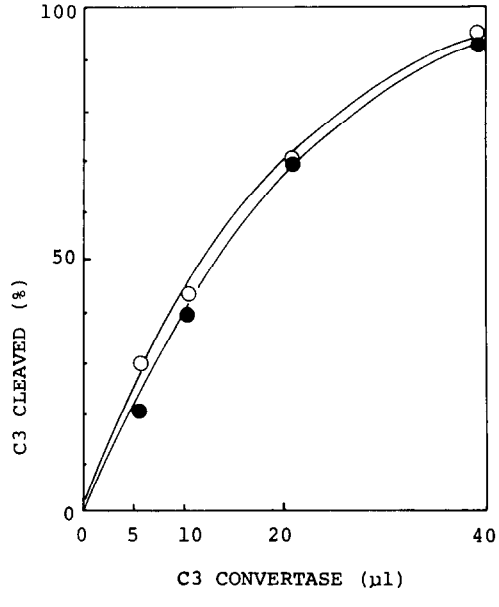

Fig.4. Comparison of C3-cleaving activity of the two C3 convertases assembled from C4b or C4b′. C3 convertase was assembled from C4b or C4b′ and isolated by HPLC as in fig.3. The C3 convertase fractions were adjusted to a protein concentration of approx. 1 μg/ml. Then, 25 μg of C3 were incubated with different amounts of C3 convertase assembled from C4b (○) or C4b′ (●) for 30 min at 37°C and subjected to disc-PAGE without reduction. The percent conversion of C3 into C3b was estimated by densitometric scanning of the Coomassie-blue stained gels at 550 nm.

4. DISCUSSION

C4b and C3b are constituents of C3 convertase of the classical and alternative pathways of the complement system, respectively, and the cleavages of C4b and C3b by factor I are critical reactions for the regulation of activation of the classical and alternative pathway C3 convertase. The I-catalyzed C3b cleavage requires cofactor proteins, such as factor H, C4bp, or CR1 and produces a nicked C3b, iC3b, which has the same molecular mass as C3b but consisted of the three disulfide-linked polypeptide chains, two α' chain fragments and intact β chain [9,13]. The cleavage of α' chain of C3b is responsible for the inactivation of C3b [9,13]. iC3b is a stable product of I-catalyzed C3b cleavage and is cleaved further into C3c and C3dg only when iC3b was incubated with factor I in the presence of CR1 [12]. In the case of C4b cleavage, nicked C4b was so rapidly cleaved into C4c and C4d that it was difficult to isolate C4b′ and to assess whether C4b′ is functionally active or inactive C4b derivative. The present paper demonstrates that C4b′ still retains the ability to assemble C3 convertase with C2 as intact C4b. This seems to be compatible with evidence that C4b′ is not a stable intermediate as iC3b but is rapidly cleaved into C4c and C4d.

In the case of C3b, the I-catalyzed cleavage of the α' chain is accompanied by the loss of the hydrophobic region as well as the functional activity to assemble C3 convertase with factor B [19]. It remains obscure whether the I-catalyzed C4b cleavage might induce conformational change. However, it seems that even if the conversion of C4b into C4b′ might be accompanied by a conformational change, the C2-binding site which appears on C4b through a conformational change is kept unchanged. In addition, it may be that the C2-binding site is located apart from the first cleavage site on the α' chain.

Although the classical pathway C3 convertase is a labile enzyme which decay-dissociates with a half-life of 5 min at 37°C [4], the C3 convertase assembled with oxy C2 is a stable enzyme with a half-life of 400 min at 24°C [14]. The present data also showed that HPLC using TSK G3000SW column and oxy C2 would provide a practical tool for the assessment of the function of C4b derivatives to assemble C3 convertase with C2.

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