Human C4b-binding protein, C4bp

Chymotryptic cleavage and location of the 48 kDa active fragment within C4bp

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C4bp, a regulator of the classical pathway of complement system, is composed of 6–8 disulfide-linked subunit chains of 75 kDa. Upon incubation with chymotrypsin, C4bp was rapidly cleaved into a nicked C4bp, composed of disulfide-linked 48 kDa and 27 kDa fragments. Subsequent slow cleavage on the 27 kDa fragment resulted in the liberation of the active site-containing 48 kDa fragment from the nicked C4bp. The N-terminal amino acid sequence of the 48 kDa fragment was identical to that of the parent subunit chain of C4bp, indicating that the 48 kDa active fragment was released from the N-terminal side of the parent subunit chain. Based on these results, a possible gross structure of C4bp is proposed.

C4b-binding protein *Active fragment* *Limited proteolysis* *Subunit chain structure*

1. INTRODUCTION

C4b-binding protein, C4bp, is an important regulator of the C3 convertase, C4b,2a, of the classical pathway of complement system; it acts as a cofactor for the cleavage of C4b by C3b inactivator [1–3] and also acts as the decay-accelerator of the C3 convertase [4].

C4bp is known to be composed of 6–8 disulfide-linked polypeptide chains of 70–75 kDa [5–9]. Previously, we reported that chymotrypsin liberated a 48 kDa fragment having the cofactor activity from each subunit chain of C4bp and produced a 180 kDa core fragment composed of disulfide-linked 25 kDa fragments [10].

The authors in [11] examined the gross structure of C4bp by electron microscopy and obtained evidence suggesting that C4bp has a spider-like structure and is composed of 7 thin, elongated, and flexible subunits that are linked to a small central body and that C4b binds to the peripheral ends of each subunit resembling tentacles.

However, it has remained unclear whether the functional domain of C4bp is located in the N- or C-terminal side of the subunit chain. So, determination of the N-terminal amino acid sequence of the 48 kDa active fragment seemed to provide information concerning the gross structure of C4bp.

We here investigate the process of liberation of the 48 kDa active fragment from C4bp by chymotryptic cleavage and determine the chemical nature of the 48 kDa active fragment. Based on these results, a possible model of subunit chain structure of C4bp is proposed.

Abbreviations: C4bp, C4b-binding protein; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; DTT, dithiothreitol

2. MATERIALS AND METHODS

Human C4bp and its two major chymotryptic

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fragments, 180 kDa core and 48 kDa active fragments, were isolated as in [10]. SDS–PAGE was performed as in [12]. Amino acid analysis was performed as in [13]. The N-terminal amino acid sequence analysis of the carboxamidomethylated 48 kDa fragment was performed by automated Edman degradation in a JEOL JS-47K Sequence analyzer using 0.25 M Quadrol buffer in the presence of 3 mg Polybrene [14] and double-coupling program at the first step. PTH derivatives of amino acids were identified by HPLC in a Dupon Zorbax BP-ODS column (4.6 × 250 mm) using a Hitachi 655 liquid chromatograph system. Isoelectric focusing was performed as in [15], using 5.5% polyacrylamide gels and ampholines of pH 4–6 for the core fragment and pH 5–9 for the active fragment. Immunoelectrophoresis was performed using an antiserum against C4bp as in [6].

3. RESULTS

3.1. *Time course of chymotryptic cleavage of C4bp*

C4bp was incubated with chymotrypsin at 37°C and samples were periodically taken and subjected to SDS–PAGE before and after treatment with DTT (fig.1). SDS–PAGE of the unreduced samples showed that liberation of the 48 kDa fragment from C4bp was a slow reaction and became apparent after 30 min incubation with chymotrypsin under these conditions. On the other hand, SDS–PAGE of reduced samples showed that prior to the liberation of the 48 kDa fragment, the parent 75 kDa subunit chains were rapidly cleaved into two fragments of 48 kDa and 27 kDa and that parallel to the liberation of the 48 kDa fragment, the cleavage of the 27 kDa fragment into a 25 kDa fragment appeared to proceed. These results suggest that the liberation of the active fragment from C4bp by chymotrypsin is a two-step reaction; firstly, rapid cleavage occurs on a loop of the polypeptide chain bridged by a disulfide bond to yield a nicked C4bp and secondly, slow cleavage on the 27 kDa fragment splits the linkage between the two fragments and releases the 48 kDa fragment from the nicked C4bp.

3.2. *Characterization of the 48 kDa active fragment*

Table 1 gives the amino acid composition of the

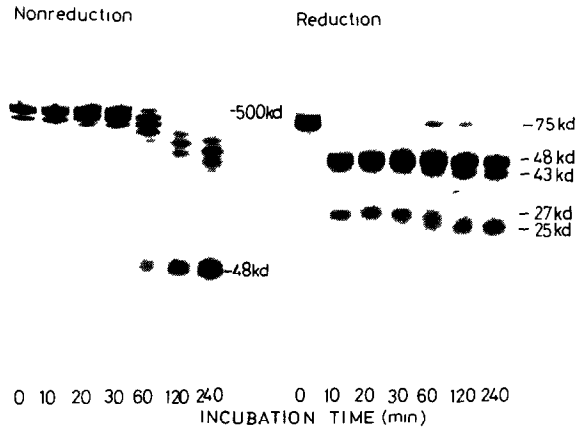

Fig.1. Time course of chymotryptic cleavage of C4bp. C4bp was incubated with chymotrypsin (C4bp/chymotrypsin, 150:1, w/w) at 37°C for indicated periods. Aliquots of the incubation mixture were removed, mixed with 2 mM DFP, and subjected to SDS–PAGE before and after treatment with 10 mM DTT. SDS–PAGE of reduced and nonreduced samples were performed with 7.5 and 3.5% polyacrylamide gels, respectively.

Table 1
Amino acid compositions of the 180 kDa and 48 kDa fragments of C4bp[a]

Amino acid	Residues/100 residues	
	180 kDa	48 kDa
Asp	8.6	8.9
Thr	3.9	7.8
Ser	5.8	8.6
Glu	18.2	9.8
Pro	8.9	9.9
Gly	7.3	9.8
Ala	4.5	4.1
Val	6.9	5.5
Met	1.7	0.3
Ile	3.5	5.0
Leu	8.9	4.9
Tyr	4.0	3.5
Phe	0.9	4.3
His	0.7	3.5
Lys	7.0	5.2
Arg	3.9	5.4
Cys/2[b]	5.5	4.8

[a] Samples were hydrolyzed in 5.7 N HCl for 24 h at 110°C under vacuum. The values are the average of triplicate analyses

[b] Half-cystine was estimated as cysteic acid

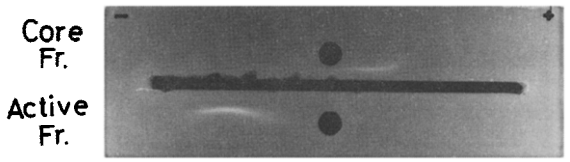

Fig.2. Immunoelectrophoresis of two major chymotryptic fragments of C4bp. The 180 kDa and 48 kDa fragments were subjected to electrophoresis on 1% agar gel and diffused against anti-C4bp antiserum. Anode was on the left.

48 kDa active fragment and, for comparison, of the 180 kDa core fragment. The core fragment is rich in Glu, Leu, and Lys, compared to the active fragment, while the active fragment is rich in Thr, Phe, His, and Arg, in comparison with the core fragment.

Fig.2 shows immunoelectrophoresis of the two fragments. The core fragment migrates toward the anodal site, while the active fragment migrates toward the cathodal site. The isoelectric points of the core and active fragments were found to be 4.7 and 7.5, respectively. These results indicate that the two domains characteristically differ from each other in amino acid composition and surface charges.

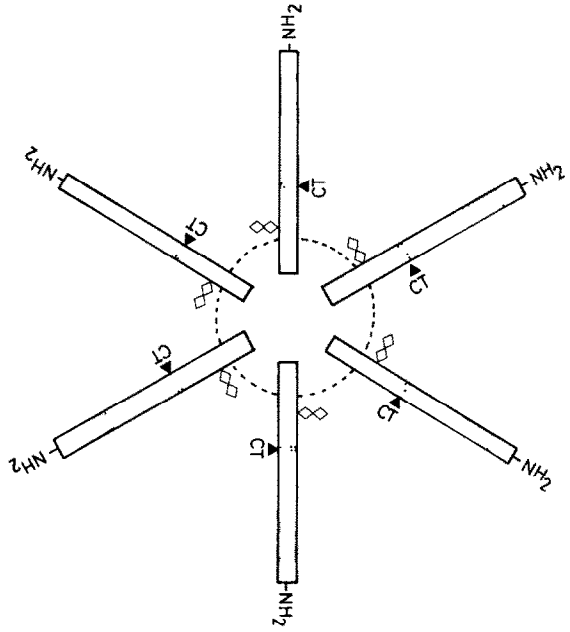

Fig.3. A possible model of the subunit chain structure of C4bp. C4bp is tentatively shown as a 6-subunit chain structure. CT, chymotrypsin; ◇◇, represents carbohydrate chains. Shadow zone and dotted line denote the active domain of C4bp and disulfide linkage, respectively.

Twenty steps of the Edman degradation of the 48 kDa active fragment with an average repetitive yield of 98% established a single unambiguous sequence as:

Asn–Cys–Gly–Pro–Pro–Pro–Thr–Leu–Ser–
Phe–Ala–Ala–Pro–Met–Asp–Ile–Thr–Leu–
Thr–Glu

where Cys is *S*-carboxamidomethylated cysteine.

This amino acid sequence is identical to the N-terminal amino acid sequence of C4bp reported in [7], indicating that the 48 kDa active fragment has been derived from the N-terminal portion of the parent 75 kDa subunit chain of C4bp. The 16th amino acid that has remained undetermined by the authors in [7] was identified as isoleucine.

4. DISCUSSION

Chymotryptic cleavage of C4bp revealed that each subunit chain of C4bp contained a specific peptide bond which located on a loop of the polypeptide chain cross-linked by a disulfide bond and was highly susceptible to chymotryptic cleavage. Chymotrypsin rapidly cleaves this site and produces a nicked C4bp, composed of disulfide-linked 48 kDa and 27 kDa fragments. Subsequent slow cleavage on each 27 kDa fragment allows liberation of the 48 kDa active fragment from the nicked C4bp. It remains unknown whether a third cleavage also occurs on the 48 kDa fragment for the liberation of the 48 kDa fragment from the nicked C4bp. If the third cleavage does not occur on the 48 kDa fragment, the 48 kDa fragment released from the nicked C4bp will have a disulfide-linked 2 kDa fragment derived from the 27 kDa fragment. However, the purified 48 kDa fragment gave a single unambiguous sequence. So, it seems possible that a third cleavage occurs at the vicinity of the C-terminus of the 48 kDa fragment and splits off a peptide fragment containing a cysteinyl residue through which the 48 kDa fragment is linking to the 27 kDa fragment. SDS–PAGE of reduced samples also showed further cleavage of the 48 kDa fragment into a

43 kDa fragment upon prolonged incubation with chymotrypsin.

The N-terminal amino acid sequence of the 48 kDa active fragment was found to be identical to that of the parent subunit chain of C4bp. A possible gross structure of C4bp is shown in fig.3. Six or eight subunit chains of C4bp are cross-linked together by disulfide bonds at their C-terminal side to form a core domain, which would correspond to the central body observed by electron microscopy [11]. Chymotrypsin sequentially splits at least two peptide bonds of each subunit chain and liberates an N-terminal derived 48 kDa fragment from each subunit chain of C4bp. The C4b-binding domain is located on the N-terminal derived 48 kDa fragment, while the carbohydrate chains are located on the C-terminal derived core domain [10].

Authors in [11] reported that C4b bound to the peripheral ends of the subunit chains of C4bp. It has been reported [7] that removal of the N-terminal 40 residue by trypsin did not affect the cofactor activity of C4bp. This result suggests that C4b-binding site would be located not at the N-terminus of each subunit chain but on a slightly inner portion from the N-terminal side.

To characterize the C4b-binding domain in more detail, we are attempting to prepare lower molecular mass active fragments by further proteolysis of the 48 kDa active fragment. Although 40 kDa and 25 kDa fragments were obtained by tryptic and chymotryptic cleavage of the 48 kDa fragment respectively, these fragments hardly showed cofactor activity (not shown). However, characterization of these fragments may provide valuable information concerning the critical region for cofactor activity, i.e., C4b-binding site, of C4bp.

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