RESEARCH COMMUNICATION

Limited tryptic cleavage of complement factor H abrogates recognition of sialic acid-containing surfaces by the alternative pathway of complement

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The potency of complement factor H (H) in accelerating the decay of the alternative pathway C3 convertase, C3b,Bb (decay-accelerating activity), was used as a measure of the affinity of native versus trypsin-treated H for the complement protein C3b bound to surfaces. When about 99 % of H was cleaved at the primary tryptic cleavage site 34 kDa from the *N*-terminus, its decay-accelerating activity on C3b,Bb on sheep erythrocytes fell about 60-fold, whereas the trypsin-treated H was only 3–4 times less potent than native H in dissociating C3b,Bb on Sepharose 4B. The residual decay-accelerating activity, remaining after the primary cleavage, was not affected by secondary cleavage at a site 120 kDa from the *N*-terminus, as shown with H preparations cleaved to different degrees. Because cell surface sialic acid is known to be responsible for the high affinity of H for C3b bound on sheep erythrocytes, the results strongly suggest that the integrity of the primary tryptic cleavage site of H is essential for the recognition of sialic acid-containing surfaces by the C3b–H complex.

INTRODUCTION

Complement factor H (H) is a single-chain plasma glycoprotein of molecular mass 155 kDa, consisting of 20 structurally similar repeating units of about 60 amino acids, the so-called short consensus repeat (SCR) units, characteristic of proteins having affinity for fragments of the complement proteins C3 and C4 [1]. H is a complement regulatory factor that binds to the C3 activation fragment C3b via its *N*-terminal region and thereby competes with factor B for binding to C3b. It also functions as a cofactor of the serine protease I in the cleavage inactivation of C3b and accelerates the spontaneous decay of the C3 convertase of the alternative pathway (AP) of complement (known as decay-accelerating activity) [2–5]. All of these activities of H tend to inhibit the activation of the AP by preventing the formation of and dissociating the AP C3 convertase, C3b,Bb, which consists of a metal-ion-stabilized complex between C3b and the activation fragment Bb of factor B. This regulation by H is, however, largely bypassed when C3b binds through complement activation to surfaces preventing efficient binding of H to the surface-bound C3b [3,6–8]. As a result, large amounts of AP C3 convertase can accumulate on such a surface in serum, i.e. the surface is an activator of the AP. Activating surfaces are generally nearly neutral or positively charged. Examples of AP activators are the polysaccharide materials zymosan and Sephadex/Sepharose [7,9,10], as well as many micro-organisms [11]. Typical surfaces that do not activate the AP are cell membranes containing large amounts of surface sialic acid, such as those of human and sheep erythrocytes. Sialic acid on a surface has been shown to prevent activation of the AP by increasing the affinity of H for C3b bound to the surface. Along with the increased affinity for C3b, the I-cofactor activity of H and its decay-accelerating activity are also potentiated by sialic acid [3,7,8,12].

A limited tryptic cleavage of H has been shown to lead to a loss

of affinity for C3b bound to sheep erythrocytes [13]. Because very low affinities are difficult to measure directly, the decay-accelerating activity of H was used in this study as a measure of affinity to test whether the loss of affinity for sheep erythrocyte-bound C3b caused by trypsin might be due to a loss of the sialic acid-induced affinity enhancement and, if so, which tryptic cleavage site in H is crucial for this loss. Evidence will be presented here that cleavage of H at the primary tryptic cleavage site about 34 kDa from the *N*-terminus produces a preparation which has the same decay-accelerating activity on C3b,Bb assembled on sheep erythrocytes and Sepharose 4B.

MATERIALS AND METHODS

Buffers, chemicals and proteins

The following buffers were used in assays with particle-coupled C3b: 0.01 M-sodium 5,5'-diethylbarbiturate/0.15 M-NaCl/0.02 % NaN₃, pH 7.3 (Veronal-buffered saline; VBS); VBS/0.2 % BSA (AVB); AVB/1 mM-NiCl₂ (AVB-Ni); and AVB/2 mM-EDTA (AVBE). Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden), and Na¹²⁵I was from Amersham International. Trypsin [diphenylcarbamoyl chloride (DPCC)-treated, from bovine pancreas] and soybean trypsin inhibitor were purchased from Sigma (St. Louis, MO, U.S.A.). All chemicals were of reagent grade.

The complement proteins C3, B, H and D were isolated as described [14]. Proteins were labelled on ice with ¹²⁵I using the Iodogen method [15] to specific radioactivities of 1–3 µCi/µg.

Trypsin treatment of H

H was treated with trypsin for the times indicated in the text at a concentration of 0.9 mg/ml in VBS at 37 °C using an enzyme/substrate ratio of 1:100 (w/w), after which a 4-fold

Abbreviations used: AP, alternative pathway of complement; C3b and Bb, activation fragments of complement proteins C3 and B respectively; C3b,Bb, alternative pathway C3 convertase; DPCC, diphenylcarbamoyl chloride; H, complement protein H; SCR, short consensus repeat; VBS, Veronal-buffered saline (for composition, see the text); AVB, VBS containing 0.2 % BSA; AVB-Ni, AVB containing 1 mM-NiCl₂; AVBE, AVB containing 2 mM-EDTA.

weight excess of soybean trypsin inhibitor (relative to trypsin) was added.

Coupling of C3b to particles: assays for binding of H to C3b and for the decay-accelerating activity

C3b was coupled to both Sepharose 4B and sheep erythrocytes by catalysis with the AP C3 convertase using two sequential C3b deposition steps as described previously [16]. A small amount of ^{125}I -labelled C3 was regularly included in coupling together with unlabelled C3 to estimate coupling efficiency, which varied within the limits 140–210 mg/ml of packed particles.

Binding of ^{125}I -labelled H to sheep erythrocyte-bound C3b was assayed by incubating the cells with agitation for 30 min at room temperature as a 5% (v/v) suspension in 120 μl of AVB containing the radiolabelled ligand, after which the bound and free ligand were separated by centrifuging (700 g , 1 min) the cells through 600 μl of 20% (w/v) sucrose in AVB in 1 ml conical centrifuge tubes (Fisher Scientific, Pittsburgh, PA, U.S.A.). About one-third of the supernatant was removed, the tube was filled with buffer and emptied leaving the cells at the bottom, after which the radioactivity of the tube was counted. When Sepharose 4B was used as the solid phase, the assay was performed as above except that a microtitre-format filter plate was used for both the incubation and separation steps as described previously [16], using AVB for rinsing of the particles. Particles without C3b were used as controls in the assays. The unspecific binding to control particles, subtracted to obtain specific binding values, was regularly less than 2% of the total radioactivity.

The decay-accelerating activity of native and trypsinized H on the Ni^{2+} -stabilized C3b,Bb complex was measured as follows. C3b was coupled to Sepharose 4B or sheep erythrocytes as above, after which C3b,Bb was formed by adding ^{125}I -labelled factor B (10^6 c.p.m./100 μl of packed particles) plus factor D (about 1 $\mu\text{g}/\text{ml}$ final concn.) to a 50% (v/v) suspension of the particles in AVB-Ni. The mixture was agitated for 4 min at 30 $^\circ\text{C}$, followed by addition of ice-cold AVBE to give a 10% (v/v) suspension. Aliquots of the mixture (60 μl) were added to 60 μl of AVBE containing different concentrations of the H preparation, the mixtures were agitated for 30 min at room temperature and the radioactivity bound on the particles was counted after removing the free fraction as described above for the binding assays.

SDS/PAGE and densitometry

SDS/PAGE was run using the buffer system of Laemmli [17] on 10% polyacrylamide gels in the Mini-Protein II electrophoresis cell of Bio-Rad (Richmond, CA, U.S.A.). Samples were heated for 4 min at 95 $^\circ\text{C}$ in the presence of 1% SDS and 0.025 M-dithiothreitol and run at room temperature at 200 V. Gels were stained with Coomassie Brilliant Blue. As molecular mass standards, the LMW kit of Pharmacia was used.

Quantities of tryptic fragments of H in stained SDS/PAGE gels were estimated by scanning with the 5301 Video Densitometer (Pharmacia). To avoid error due to differences in staining properties, the percentage of total stain contributed by each fragment after various times of trypsinization was extrapolated to zero time, thus giving an estimate of the relative staining intensity compared with the theoretical value based on molecular mass. The value obtained was then used as a correction factor to calculate the contribution of the fragment to total protein.

RESULTS

When H was treated with trypsin for 10–55 min, run on

SDS/PAGE in reduced form and stained, a characteristic pattern of bands was obtained. The band corresponding to native H quickly disappeared with trypsinization, concomitantly with the appearance of two major bands with apparent molecular masses of 34 and 120 kDa, plus two minor bands of 45 and 79 kDa. These split products, which are held together by disulphide bonds under non-reducing conditions, have been characterized previously. Although the reported molecular masses vary slightly, it is clear from previous reports that the 34 and 120 kDa fragments represent the N- and C-terminal parts of H respectively, and the 45 and 79 kDa fragments are produced from the 120 kDa fragment by a secondary cleavage [5,13,18].

To analyse the effects of the main tryptic cleavages on function without major interference from unsplit H, preparations trypsinized for 20 and 55 min (H-tr./20 and H-tr./55, respectively) were used for experiments. SDS/PAGE of these preparations is shown as an inset to Fig. 1. Densitometry of SDS/PAGE gels showed that roughly 99% of H was cleaved into smaller fragments in 20 min. H-tr./20 contained 85% of the 34 kDa fragment and 41% of the 120 kDa fragment originally present. The corresponding values for H-tr./55 were 76% (34 kDa) and 6% (120 kDa).

When H-tr./20 and H-tr./55 were labelled with ^{125}I and their binding to C3b coupled to sheep erythrocytes and Sepharose 4B was assayed, no specific binding of trypsinized H to C3b on either solid phase could be demonstrated. In contrast, $13.6 \pm 1.2\%$ and $3.7 \pm 0.1\%$ (mean $\pm$ S.D. of triplicates) of ^{125}I -labelled native H bound specifically to C3b on sheep erythrocytes and Sepharose 4B respectively, under the same conditions, using H/C3b molar ratios of 0.016 (sheep erythrocytes) and 0.021 (Sepharose 4B).

To evaluate the affinity of trypsin-treated H for C3b, the indirect method of assaying for the decay-accelerating activity of H on the AP C3 convertase was used next, based on the

Fig. 1. Decay-accelerating activity of native and trypsin-treated H

The decay-accelerating activity of native and trypsinized H on C3b,Bb assembled on sheep erythrocytes and Sepharose 4B was assayed as described in the Materials and methods section. The x-axis shows the molar ratio of H to solid-phase C3b in the reaction mixture, and the y-axis shows the ratio of the radioactivity remaining on the solid phase after 30 min of incubation in the presence of H to that in its absence. The solid phases and H preparations were as follows: $\circ$, sheep erythrocytes, native H; $\triangle$, sheep erythrocytes, H-tr./20; $\square$, sheep erythrocytes, H-tr./55; $\bullet$, Sepharose 4B, native H; $\blacktriangle$, Sepharose 4B, H-tr./20; $\blacksquare$, Sepharose 4B, H-tr./55. The points are means $\pm$ S.D. of triplicate determinations. The inset shows SDS/PAGE of native versus trypsinized H. Molecular masses (kDa) of standards are indicated on the right, and the time of incubation with trypsin (min) is given above the lanes.

assumption that this activity is directly correlated to the affinity of H for C3b. Fig. 1 shows the decay-accelerating activity of native H versus that of H-tr./20 and H-tr./55 on C3b,Bb on Sepharose 4B and sheep erythrocytes. It can be seen that (1) on sheep erythrocytes, about 60-fold more of trypsinized than of native H was needed for an equivalent effect, whereas the decay-accelerating activity on C3b,Bb formed on Sepharose 4B fell only 3–4-fold as a result of trypsinization, and (2) the decay-accelerating activities of H-tr./20 and H-tr./55 were essentially identical and did not depend on the solid phase used.

DISCUSSION

It is shown here that cleavage of the complement protein H at the primary tryptic cleavage site 34 kDa from the *N*-terminus makes the protein refractory to the enhancement of affinity for surface-bound C3b by sialic acid on the surface. This is based on the following evidence: (1) sialic acid is known to be responsible for the high affinity of H for C3b on sheep erythrocytes [7,12], and (2) an H preparation which had been cleaved by about 99 % at the primary tryptic cleavage site 34 kDa from the *N*-terminus, but only by 60 % at the secondary site 120 kDa from the *N*-terminus, did not discriminate between activating and non-activating surfaces, as determined by decay-accelerating activity, and a much more extensive cleavage at the latter site did not alter this activity.

The 60-fold decrease in the decay-accelerating activity of H on sheep erythrocyte-bound C3b,Bb, caused by the trypsin treatment, roughly corresponds to a lowering of the association constant from $(1-2) \times 10^7 \text{ M}^{-1}$, the value previously reported for the association of H with sheep erythrocyte-bound C3b, to $(2-3) \times 10^5 \text{ M}^{-1}$, which agrees well with the previously estimated association constant for the binding of H to C3b on zymosan [7,19]. The fluid phase association constant of H for C3b has been reported to be as high as $4.4 \times 10^6 \text{ M}^{-1}$ at 20 °C [20], and it has been shown that a limited tryptic cleavage of H, like that used in this study, does not lower the fluid phase affinity, as measured by I-cofactor activity [13]. Therefore the present results imply that the complex of C3b and trypsinized H recognizes both sheep erythrocytes and Sepharose 4B equally as activators of the AP, i.e. the binding of trypsinized H to C3b is restricted by both surfaces, as is that of native H by activating surfaces only.

The finding that trypsinization of H apparently abolished its ability to bind to C3b not only on sheep erythrocytes but also on Sepharose 4B, when assayed by the direct method of measuring the binding of ^{125}I -labelled H, must be related to a previous observation that a high density of C3b on a surface in itself

creates some high-affinity H binding sites [16]. This C3b density effect thus appears to be also abrogated when H is trypsinized, which may explain the 3–4-fold decrease in decay-accelerating activity on Sepharose-bound C3-convertase.

A fundamental question that arises from the present findings is by what mechanism the cleavage of H with trypsin at the primary site abolishes recognition of sialic acid by the C3b–H complex. This site may be directly involved in sialic acid recognition, but allosteric mechanisms cannot be ruled out. Answering this question is important to improve our understanding of the activation of the AP.

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