The Heparin Binding Domain of S-Protein/Vitronectin Binds to Complement Components C7, C8, and C9 and Perforin from Cytolytic T-Cells and Inhibits Their Lytic Activities†

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ABSTRACT: S-Protein/vitronectin is a serum glycoprotein that inhibits the lytic activity of the membrane attack complex of complement, i.e., of the complex including the proteins C5b, C6, C7, C8, and $C9_n$. We show that intact S-protein/vitronectin or its cyanogen bromide generated fragments also inhibit the hemolysis mediated by perforin from cytotoxic T-cells at 45 and 11 μM, respectively. The glycosaminoglycan binding site of S-protein/vitronectin is responsible for the inhibition, since a synthetic peptide corresponding to a part of this highly basic domain (amino acid residues 348–360) inhibits complement- as well as perforin-mediated cytolysis. In the case of C9, the synthetic peptide binds to the acidic residues occurring in its N-terminal cysteine-rich domain (residues 101–111). Antibodies raised against this particular segment react 25-fold better with the polymerized form of C9 as compared with its monomeric form, indicating that this site becomes exposed only upon the hydrophilic–amphiphilic transition of C9. Since the cysteine-rich domain of C9 has been shown to be highly conserved in C6, C7, and C8 as well as in perforin, the inhibition of the lytic activities of these molecules by S-protein/vitronectin or by peptides corresponding to its heparin binding site may be explained by a similar mechanism.

The complement system lyses target cells by inserting into their membrane a pore-forming complex (membrane attack complex, MAC) consisting of the five proteins C5b, C6, C7, C8, and C9 [for reviews, see Podack and Tschopp (1984) and Müller-Eberhard (1984)]. Consequently, the membrane barrier is impaired and cell lysis ensues due to osmotic swelling and massive Ca^{2+} influx. In vivo, only lysis of cells bearing complement-activating molecules (for instance, antibodies or activators of the alternative pathway) on their surface are susceptible to this attack, although in vitro it is possible to induce lysis of bystanding cells that have themselves not initiated complement activation (Götze & Müller-Eberhard, 1970). The release of C5b–7 complexes from the activating particle, which are able to bind to C8 and C9, causes the lysis of innocent bystander cells. At least two serum proteins, i.e., lipoproteins and S-protein/vitronectin, can prevent this potentially devastating process by complexing nascent C5b–7 complexes, thereby rendering them inactive (Podack et al., 1978; Lint et al., 1977).

Recently, lysis of bystanding cells was described to occur under special circumstances in cytotoxic T-cell (CTL) mediated cytolysis (Fleischer, 1986). Cytoplasmic granules of CTL contain a pore-forming protein, perforin, which is released upon the interaction of effector (CTL) and the appropriate target cell (Henkart, 1985; Podack & Königsberg, 1984; Masson et al., 1985). Released perforin polymerizes in the presence of Ca^{2+} and forms pores of polyperforin in the target cell membranes morphologically similar to those of the MAC of complement (Podack et al., 1985; Masson & Tschopp, 1985). The similarity of these two lytic proteins goes beyond mere ultrastructural homology; C6, C7, C8α, C8β, and C9 have been shown to have sequence or antigenic homology with perforin (Tschopp et al., 1986b; Young et al., 1986; Zalman et al., 1986; Lowrey et al., 1987; Howard et al., 1987; Rao et al., 1987; Haefliger et al., 1987). The observed homology is particularly strong in a segment with high cysteine content that occurs at amino acid residues 78–115 in human C9. A highly homologous domain occurs as a repeat unit in the low-density lipoprotein (LDL) receptor (Südhof et al., 1985). One salient feature of this motif, apart from the stability conferred by the three intradomain disulfide bridges, is a cluster of negative charges at the C terminus (Stanley et al., 1985; DiScipio et al., 1984; Haefliger et al., 1987). Seven out of 12 residues are acidic in C9 (5 of 12 in C8β) at this location. A synthetic peptide covering this region was shown to influence the hemolytic activity of both perforin and the late complement components (Tschopp et al., 1986b), suggesting that this cysteine-rich domain plays a functional role in the assembly of the MAC or polyperforin.

Considering this structural homology, it is therefore not surprising that the activity of CTL perforin is sensitive to inhibitors known to abolish complement-mediated cytolysis. As in the case of complement, perforin or CTL-mediated lysis is abrogated or diminished in the presence of lipoproteins (Tschopp et al., 1986a). Negatively charged molecules, such as proteoglycans or suramin, or agents carrying high positive charges, such as protamine or polylysine (Baker et al., 1975; Tschopp & Masson, 1987), also are effective inhibitors of both lytic systems.

We show here that S-protein/vitronectin efficiently blocks perforin activity. The recent cloning of S-protein/vitronectin cDNA (Suzuki et al., 1985; Jenne & Stanley, 1985) has permitted the identification of several distinct domains within this molecule (Figure 2). It contains the entire somatomedin B peptide at its NH_2 terminus, an Arg-Gly-Asp (RGD) sequence thought to act as a cell attachment site, and a very basic heparin binding domain close to the COOH terminus. This domain is involved in the functional role of S-protein/

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vitronectin as a potent heparin-neutralizing protein, thereby regulating the extent of inhibition of coagulation enzymes by antithrombin III–heparin (Preissner & Müller-Berghaus, 1987).

The function of S-protein/vitronectin as a complement inhibitor, as well as the inhibitory effect of basic molecules on the lytic systems in general, led us therefore to investigate the role of the heparin binding site of S-protein/vitronectin. Here, we not only report that this domain does indeed block the lytic activity of complement and perforin but also demonstrate that it does so, as shown in the case of C9, by interacting with a highly conserved, negatively charged cysteine-rich domain.

Experimental Procedures

Proteins and Peptides. Complement components C5b–6, C7, C8, and C9 were isolated according to published procedures (Podack et al., 1978; Podack et al., 1979; Kolb & Müller-Eberhard, 1976; Biesecker & Müller-Eberhard, 1980). The C9a and C9b domains of C9 were obtained by first cleaving C9 with α-thrombin (Biesecker et al., 1982). To obtain the fragments in pure form without sodium dodecyl sulfate (SDS) as originally described (Biesecker et al., 1982), the enzyme-treated mixture was loaded on a Mono P (Pharmacia) chromatofocusing column equilibrated in 0.025 M [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-tris/HCl), pH 7.0, containing 7 M urea. The fragments were eluted with polybuffer 74/HCl, pH 4.0, containing 7 M urea. The peak eluting first was nondissociated C9, the second was C9b, and C9a eluted last. Perforin was isolated from CTL line B6.1 (Masson & Tschopp, 1985). Purified S-protein was obtained from fresh human plasma (Preissner et al., 1985). In some instances, S-protein was further purified chromatographically by loading 1 mg of S-protein on a Mono Q strong-anion-exchange column (Pharmacia). The column was washed with 10 mL of 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, containing 150 mM NaCl. The protein was eluted, at a flow rate of 1 mL/min, with a linear gradient of 150–500 mM NaCl in the same buffer. CNBr-generated fragments of reduced and carboxymethylated S-protein were produced by incubating 1 mg of the purified protein dissolved in 70% formic acid with a 40-fold molar excess of CNBr (Fluka, Buchs, Switzerland) for 24 h at 20 °C (Suzuki et al., 1984). Protamine chloride and poly(L-lysine) (HBr salt; M_r 4000) were purchased from Sigma (St. Louis, MO). The peptide derived from the sequence of S-protein (residues 348–360) was synthesized by the Fmoc strategy on a semiautomatic peptide synthesizer (Bachem AG, Bubendorf, Switzerland). The peptide was purified by cation-exchange chromatography (Mono S, Pharmacia), and its purity was ascertained by amino acid analysis.

Antibodies. The antisera against peptides A and B of C9 have been described (Tschopp et al., 1986b), as well as the specificities of monoclonal antibodies Ae 11 (a gift of Dr. T. E. Mollnes, University of Oslo, Norway; Mollnes et al., 1985) and M3 (Cytotech Inc., San Diego, CA; Stanley et al., 1985). The monoclonal anti S-protein antibody was purchased from Cytotech Inc.

Hemolytic Tests. In order to test the inhibitory effect of the various molecules on the activity of perforin, C9, or C8, the latter proteins were adjusted to a concentration able to lyse approximately 80% of the sheep red blood cells.

The appropriate concentration of perforin in 100 μL of GVB (10 mM veronal buffer, pH 7.4, 142 mM NaCl, 0.1% gelatin, 1 mM Ca^{2+}, and 0.15 mM Mg^{2+}) plus 20 μL of the inhibitor was added to 300 μL of sheep erythrocytes (1.5×10^7 cells/mL) in GVB. The mixture was incubated for 15 min at 37 °C, the erythrocytes were spun down at 1500 rpm for 5 min, and the degree of hemolysis was measured by determining the hemoglobin release at 412 nm in a Gilford spectrophotometer. The hemolytic activity was calculated as the Z number [$Z = -\ln(1 - y)$, where y is the fraction of cells lysed]. The decrease in the Z number was then transformed into relative hemolytic activity.

C9 hemolytic assays were performed by diluting C9 to a concentration causing 80% lysis of the erythrocytes (usually 1–2 ng mL^{-1}). C9, inhibitors, and erythrocytes bearing complement components C1–C8 on their surface (1.5×10^7 cells/mL) were then mixed in a total volume of 400 μL of GVB and incubated for 30 min at 37 °C. The degree of lysis was determined as described above. C8 activity was carried out by incubating erythrocytes having C1–C7 bound (1.5×10^7 cells/mL) with inhibitors and 0.1 μg mL^{-1} C8 for 15 min at 37 °C in 400 μL of GVB. The cells were washed twice in GVB and resuspended in 400 μL of GVB containing 1 μg mL^{-1} C9. Incubation was continued for 30 min at 37 °C and the degree of hemoglobin release determined.

Alternatively, C1–C7- or C1–8-bearing erythrocytes were incubated for 15 min at room temperature with various concentrations of inhibitors, but in the absence of C8 and C9, or C9, respectively. The cells were washed twice in GVB, and only then were the lytic proteins C8 and C9 added. This protocol was used to test the capacity of the inhibitory molecules to interact with cell-bound C5b–7 and C5b–8, respectively.

The influence of S-protein or different S-protein-derived peptides on native C8, C9, or perforin was determined by incubating C8, C9, or perforin at a concentration of 10 μg/mL with various concentrations of the inhibitors. After 15 min at room temperature, the lytic proteins were diluted 100–10 000-fold (thereby diluting out the inhibitors) and their remaining lytic activity was assayed as described.

Biochemical and Immunological Techniques. Polyacrylamide electrophoresis (PAGE) in the presence of SDS (SDS–PAGE) was performed according to the method of Lämmli (1970), and Western blot and dot–blot analyses were performed according to the method of Towbin et al. (1979). The nitrocellulose was rinsed 3 times with Tris-buffered saline (TBS) and incubated with the first antibody for 4 h in the presence or absence of the various inhibitors. The blot was stained by using protein A–peroxidase conjugates (Sigma) and 4-chloro-1-naphthol and H_2O_2 as substrates.

Affinity Chromatography. Purified S-protein peptide (5 mg) was coupled to CNBr-activated Sepharose (1 mL; Pharmacia) according to the manufacturer's instructions. Polylysine–Sepharose beads were purchased from Pharmacia. Monomeric C9, Zn^{2+}-polymerized C9 (Tschopp, 1984), C9a, or C9b was dialyzed against 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and loaded onto the columns equilibrated in the same buffer at room temperature. The column was washed with the equilibration buffer (10 mL), proteins were eluted with the same buffer containing either 0.5 M NaCl or, alternatively, 4 M guanidine hydrochloride (10 mL each), and fractions of 1 mL were collected. The eluted proteins were assayed with the Coomassie blue binding assay of Bradford (1976).

Results

S-Protein has been reported to inhibit MAC activity at least at two stages during MAC assembly; first, by binding to nascent C5b–7 (Podack et al., 1978) and, second, by inhibiting C9 polymerization (Dahlbäck & Podack, 1985), the process

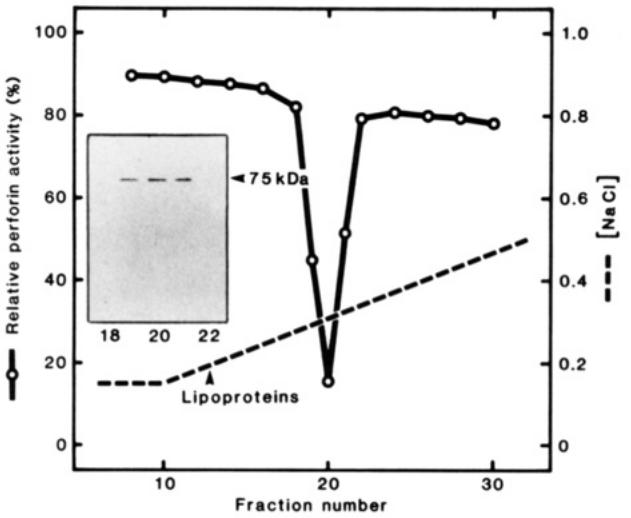

FIGURE 1: Inhibition of the lytic activity of perforin by S-protein. Purified S-protein/vitronectin (see Experimental Procedures) was loaded onto a Mono Q anion-exchange column and eluted by increasing the NaCl concentration (---). Individual fractions were tested for inhibitory activity on perforin-mediated sheep red cell hemolysis. The inset shows a Western blot analysis of the inhibitory fractions using a monoclonal anti-S-protein/vitronectin antibody. It reveals a 75-kDa molecule corresponding to S-protein/vitronectin. In an independent experiment, the ionic strength condition at which high-density lipoproteins (HDL) eluted from the Mono Q column was determined.

that leads to the formation of ultrastructurally distinct tubules. Since C9 and perforin not only share the propensity to polymerize into ultrastructurally similar pores but also exhibit sequence homology, we tested whether S-protein was also able to abolish perforin activity. Isolated perforin was therefore mixed with purified S-protein or S-protein cleaved by cyanogen bromide, and its lytic activity was tested on sheep red blood cells. As a control, C8 and C9 activity was tested on C5b–8- and C5b–7-bearing red blood cells, respectively. The results of the experiment are shown in Table I. C9 and C8 activity was reduced by half at S-protein concentrations of 1.3 and 1.6 μM, respectively. A similar concentration of S-protein is effective on purified perforin and is also required for the inhibition of poly(C9) formation (Podack et al., 1984). Surprisingly, CNBr-treated S-protein shows approximately 3 times more inhibitory activity, indicating that the integrity of the protein's tertiary structure is not a prerequisite for its activity and that, in contrast, amino acid sequences normally buried in the native protein may be involved in this interaction.

Perforin-mediated lysis is highly sensitive to inactivation by lipoproteins. It is necessary to exclude contaminating lipoproteins from the S-protein preparation, since relative to lipoproteins, a 1000× higher concentration of S-protein is required for perforin inhibition. Thus, purified S-protein was

Table I: Inhibition of the Lytic Activity of Perforin and Late Complement Components by Intact S-Protein/Vitronectin and CNBr-Generated S-Protein/Vitronectin Fragments[a]

	S-protein/ vitronectin (μM)	S-protein CNBr-generated fragments (μM)
C9 activity	1.3	0.5
C8 activity	1.6	0.4
perforin	1.0	0.3
poly(C9) formation	2.6[b]	nd[c]

[a] Purified S-protein/vitronectin or a mixture of S-protein/vitronectin fragments generated by CNBr was tested for inhibitory activity and C8, C9, and perforin activity (see Experimental Procedures). Molar concentrations of either intact S-protein/vitronectin or the fragmented protein (assuming in each case a M_r of 75000) that reduce the lytic activities by half are given. The assays were performed under limiting concentrations of the lytic proteins, allowing lysis of approximately 80% of the added red blood cells, in the absence of the inhibitor. [b] Data taken from Podack et al. (1984). [c] nd, not determined.

applied to a Mono Q anion-exchange column and subsequently eluted with increasing salt concentrations. Each fraction was tested for its inhibitory activity on perforin (Figure 1). The profile of inhibition followed the profile of S-protein elution exactly. Since, in addition, no apoprotein AI/AII or B could be detected with specific antibodies (data not shown), we consider the possibility of contaminating lipoproteins in the S-protein preparation as very unlikely.

The fact that S-protein-derived peptides are even more potent inhibitors than the native protein demonstrated that the native structure is not essential for the inhibitory activity. In order to characterize the inhibition peptide, CNBr-cleaved S-protein was applied to heparin-Sepharose (Suzuki et al., 1984). While no inhibitory activity was detected in the breakthrough fraction, it could be found when the ionic strength was increased to 0.65 M NaCl. When analyzed by SDS–PAGE, a major 12-kDa fragment plus two minor bands (data not shown) were found in the 0.65 M NaCl eluate. Using the identical protocol for the heparin chromatography, the 12-kDa peptide has been previously described (Suzuki et al., 1984) to correspond to the heparin binding fragment of S-protein/vitronectin (residues 341–403).

The possible involvement of this positively charged domain in the inhibition of the lytic protein is in agreement with the observation that other positively charged molecules like protamine are also good inhibitors (Tschopp & Masson, 1987). Thus, a peptide corresponding to the most basic amino acid residues (348–360) of the heparin binding fragment of S-protein/vitronectin, in which 7 out of 13 residues are positively charged, was synthesized to test this hypothesis (Figure 2). The synthetic peptide inhibited the lytic activity of perforin as well as C8- and C9-mediated hemolysis in a concentration range of 6–18 μM peptide/mL (Figure 3). In addition, 35

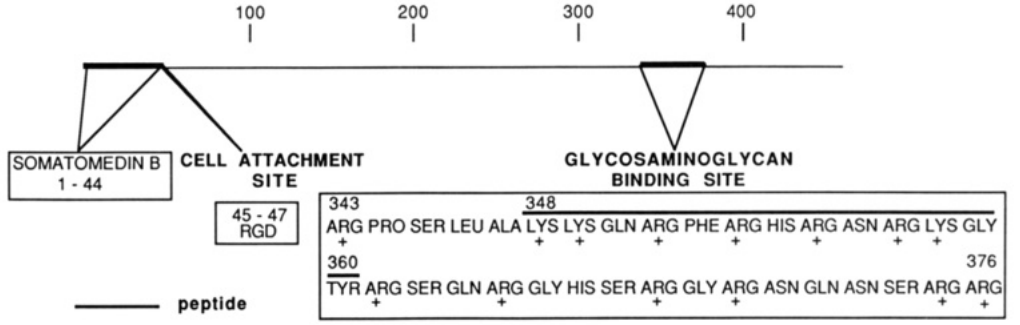

FIGURE 2: Schematic representation of the multidomain structure of the S-protein/vitronectin molecule. Amino acid residues 1–44 correspond to somatomedin B, and 45–47 contains the sequence Arg-Gly-Asp (RGD) known to be important in the cell adhesion properties of S-protein/vitronectin. Between residues 343 and 376, almost half of the amino acids are basic, thus serving as the glycosaminoglycan binding site. The sequence of the peptide synthesized is indicated by a solid line above it.

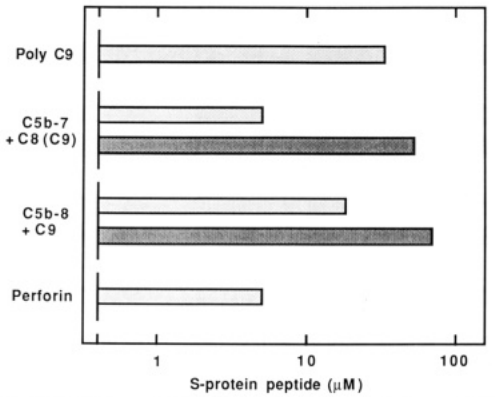

FIGURE 3: Inhibition of C8, C9, and perforin lytic activities and poly(C9) formation by the S-protein-derived peptide. Poly(C9) formation was assessed by SDS–PAGE as measured by the decrease of the amount of high molecular weight closed tubular poly(C9). C8, C9, and perforin lytic activities were measured either by adding the peptide together with the lytic molecules to sheep red blood cells bearing the appropriate receptor molecules (light bars) or by adding the peptide to the red blood cells, washing them, and then adding the lytic proteins (dark bars; appropriate only for C8 and C9, since no receptor molecule is required for perforin).

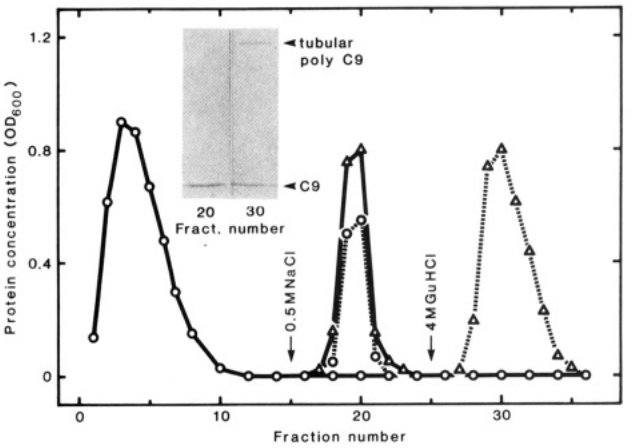

FIGURE 4: Binding of monomeric C9 and poly(C9), respectively, to S-protein peptide/poly(lysine) coupled to Sepharose. A total of 200 μg of Zn^{2+}-polymerized C9 (△) containing a heterogeneous mixture of $C9_n$ oligomers, with n varying from 2 to 18 (and open and closed tubules), and monomeric C9 (○) was applied onto a S-protein peptide–Sepharose (---) or polylysine column (—) and subsequently eluted as indicated. Fractions 20 and 30 eluted from the peptide–Sepharose column were analyzed by SDS–PAGE (2.5–10% gradient gel) for their content of high molecular weight, SDS-resistant, closed tubular poly(C9).

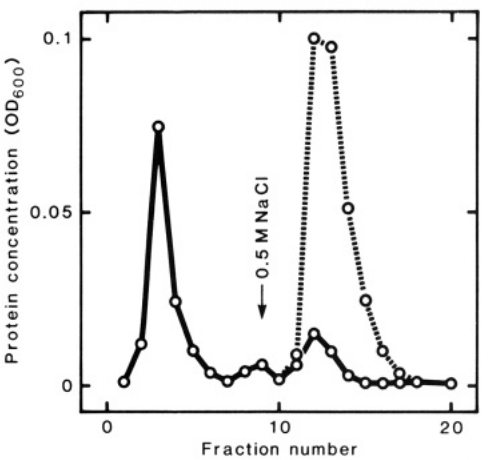

FIGURE 5: Binding of C9 domains to the synthetic S-protein peptide column. Monomeric C9 was cut by α-thrombin into two distinct domains, C9a and C9b. The isolated domains (200 μg each) were subjected to affinity chromatography. The N-terminal C9a fragment (---) remained bound to the S-protein peptide–Sepharose column, while the C9b fragment (—) was in the breakthrough fractions.

μM peptide also inhibited poly(C9) formation. Approximately 10-fold more peptide was required to reduce C8 and C9 activity when, after addition of the peptide to C5b–7- and C5b–8-bearing erythrocytes, the cells were washed to remove unbound peptides. This experiment suggests that the peptide exerts its activity by binding to cell-bound C5b–7 and C5b–8 and not to monomeric C8 or C9, since preincubation of the monomeric form of the lytic proteins with the peptide is without inhibitory effect (data not shown). Two other cationic molecules, polylysine and protamine, were tested. While 10-fold more polylysine was required to inhibit the lytic activity of C8 and C9 (data not shown), thus excluding a simple charge effect, protamine was highly active at 0.07 μM (Tschopp & Masson, 1987).

We focused our attention on the C9–S-protein/vitronectin interaction to elucidate in more detail the mechanism of interaction of S-protein with the various lytic proteins, since C9 can be obtained in reasonable amounts and can be transformed from the monomeric form into the polymerized form by the simple addition of Zn^{2+} ions. In contrast, C8 requires the presence of C5b–7 for activation. Since the synthetic S-protein-derived peptide inhibited poly(C9) formation (Figure 3), C9 was expected to bind to the peptide coupled to Sepharose. When monomeric C9 was passed through this affinity column at normal ionic strength, it was retained. By increasing the salt concentration to 0.5 M NaCl, monomeric C9 was again eluted (Figure 4). The affinity of Zn^{2+}-polymerized C9 for synthetic peptide–Sepharose was higher, since poly(C9) was not eluted at NaCl concentrations as high as 3 M. Only denaturing conditions (4 M guanidine hydrochloride) allowed the detachment of poly(C9). A polylysine–Sepharose column was used to estimate the contribution of the charge–charge effect within the interaction of poly(C9) with the basic peptide. Figure 4 shows that, in contrast to monomeric C9, poly(C9) binds to the polylysine column at normal ionic strength conditions and can be eluted at 0.5 M NaCl, indicating a lower affinity to polylysine relative to the synthetic peptide.

The same peptide–Sepharose column was used to define the segment within C9 accounting for this interaction. Monomeric C9 is cleaved by α-thrombin into two fragments designated C9a (amino acid residues 1–244) and C9b (245–537). Only the C9a fragment was retained by the S-protein-derived peptide–Sepharose column, suggesting that the peptide interacts with a domain localized in C9a, but not in C9b. At 0.5 M NaCl, C9a can be eluted from the affinity column (Figure 5).

C9a contains a sequence rich in acidic amino acids. This cluster (amino acid residues 101–112) is localized at the C terminus of the cysteine-rich domain. It is conserved throughout evolution within C6, C7, C8, C9, and perforin. This cluster of negative charges is therefore a likely candidate for the interaction with the positively charged S-protein peptide. To elucidate this hypothesis, we used an antiserum that specifically recognizes this particular region of C9. It was raised against a synthetic peptide corresponding to amino acid residues 101–111, i.e., exactly to the highly acidic region, and was initially called antipeptide B (Tschopp et al., 1986b). Figure 6 shows the reactivity of this antibody. When different quantities of poly(C9) and monomeric C9 were applied onto nitrocellulose, the polymeric form is detected down to 40 ng, whereas 25 times more monomeric C9 is required to obtain the same reactivity. The surface exposure of a second present in this cysteine-rich domain was tested by using an antiserum

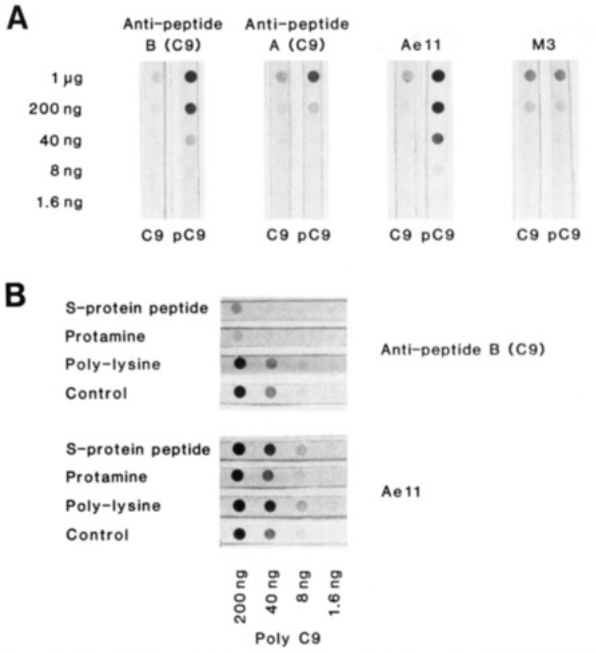

FIGURE 6: Dot blot analysis of monomeric C9 and poly(C9). (A) Different quantities of the two forms of C9 were spotted onto nitrocellulose and revealed by using antiserum against peptide A (amino acid residues 74–86 of C9), peptide B (corresponding to residues 101–111), monoclonal antibody Ae 11 recognizing a neoantigen present only in the polymeric form of C9, and monoclonal antibody M3. (B) Inhibition of antipeptide B–poly(C9) interaction by S-protein peptide, protamine, and polylysine (60 μM). As a negative control, inhibition of the interaction between Ae 11 and poly(C9) was tested.

against peptide A (corresponding to residues 74–87 of C9). Only a slightly better reactivity toward poly(C9) was detected (Figure 6A). Controls included antibodies Ae 11, which detects a neoantigen exposed only in poly(C9), and M3, which reacts with an epitope exposed in both conformations of C9.

Since we conjectured that the negatively charged region covered by peptide B binds to the basic S-protein peptide, competition experiments were performed to abolish antipeptide B–poly(C9) interaction. When the S-protein peptide was added to the incubation mixture at a concentration known to inhibit poly(C9) formation (60 μM), binding of the antipeptide B antibody was decreased (Figure 6B). The same was true for protamine (at even lower concentrations, i.e., 2 μM), whereas polylysine (60 μM) was not inhibitory. In no case was the interaction of the monoclonal antibody Ae 11 with poly(C9) inhibited, indicating the specificity of the inhibition of the antipeptide A–poly(C9) interaction.

Discussion

Recent studies have shown that the lytic pore-forming proteins of the complement cascade exhibit not only ultrastructural but also sequence homology with perforin, the protein mediating, at least in part, cytolytic T-cell- and NK-cell-induced cell lysis. In addition, these proteins share a variety of natural and artificial inhibitors, including lipoproteins and positively and negatively charged molecules. It has been proposed that the main functional role of S-protein within the complement cascade is to inactivate in serum potentially harmful MAC in order to avoid bystander lysis. Our report shows that the plasma protein S-protein/vitronectin not only is a potent physiological inhibitor of the complement membrane attack complex assembly but also exerts its activity on perforin.

In activated cytolytic T-lymphocytes, perforin is sequestered in cytoplasmic granules in an inactive site. Upon interaction with the appropriate target cell, the contents of the granules are released into the contact zone between effector and target cells. In the presence of Ca^{2+}, perforin polymerizes and inserts into the attacked cell, resulting in transmembrane channel formation and consequent cell lysis. Such a lytic mechanism comprises two pitfalls. First, it is feasible that autolysis could occur; i.e., perforin inserts into the cell from which it has originally been released. However, this phenomenon has not been described, and it has been suggested (Tschopp & Conzelmann, 1986) that self-killing may be inhibited by proteoglycan structures that are present in the granules (Schmidt et al., 1986), which are known to inhibit perforin activity (Tschopp & Masson, 1987). Second, perforin may diffuse out of the contact zone and kill other innocent cells. Again, this has not been observed except under certain circumstances (Fleischer, 1986). The absence of bystander cell lysis may be attributed to S-protein/vitronectin or, as reported earlier, to lipoproteins. These proteins may represent an important control mechanism of perforin-mediated cytolysis.

S-Protein binds to and blocks the activity of C7, C8, C9, and perforin, presumably interacting with a domain common to all of these molecules. Our results strongly suggest that S-protein binds to the cysteine-rich domain, which is conserved in all these proteins, clearly explaining the apparent nonspecificity of S-protein inhibition. This cysteine-rich domain is highly negatively charged, particularly in the region of amino acids 101–112, in which segment seven amino acids are acidic. Thus, it can serve as an ideal acceptor site for the extremely basic segment of the S-protein–heparin binding site.

The sequence of the S-protein duplicated by the synthetic peptide has a predicted β-sheet structure. Thus, from Lys^{349} to Arg^{357}, five positive charges are aligned on one side of the sheet, forming an extremely high local concentration of basic amino acids. Inhibition of the lytic activities by this peptide still requires rather high peptide concentrations (6–18 μM). However, the S-protein concentration in serum is also high (approximately 4 μM). Compared with CNBr-fragmented S-protein (half-inhibitory activity observed at approximately 0.4 μM), the peptide is 15–45-fold less active. This difference may indicate that the segment required for optimal inhibition in larger than the one covered by the peptide. Interestingly, protamine is far more potent than S-protein (0.07 μM) and represents the most efficient inhibitor for the lytic proteins found to data (Tschopp & Masson, 1987). Salmon sperm protamine (32 amino acids, 21 basic residues) has most of the basic residues present in the complete glycosaminoglycan binding site of S-protein (34 amino acid residues, 15 basic residues) conserved. That the inhibition is not only a matter of basic charges is apparent, since polylysine is approximately 1000- and 10-fold less active than protamine or the synthetic S-protein peptide, respectively.

S-Protein interaction with the late complement components is limited to the activated protein, i.e., to the proteins in the complexed form. Hence, in the native monomeric serum form the interaction site must be occluded, as shown schematically for C9 in Figure 7. On the basis of the study with the antibody-recognizing amino acid residues 101–111, we propose that these acidic residues as well as the hydrophobic domains of C9 are initially buried. In the presence of Zn^{2+} (or C5b–8), C9 undergoes major conformational changes and folds up around its hinge region, thereby doubling its length. This transition does not require conformational rearrangements of secondary structures of C9, since C9a and C9b may conserve their initial conformation and just move around the hinge region. Indeed, only small secondary structure changes have

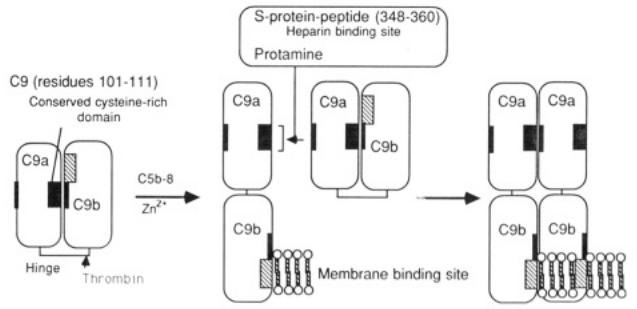

FIGURE 7: Hypothetical model of C9 polymerization. In the monomeric, globular form of C9, the acidic amino acid residues 101–111 and the membrane interaction site are occluded. Upon interaction with Zn^{2+} or C5b–8, C9 undergoes a transition into an extended form, thereby exposing both domains. Whereas the membrane binding site (located between residues 245 and 347 of C9b, unpublished results) inserts into the lipid bilayer, the cysteine-rich domain forms a new interaction site for the subsequent globular C9. This process is repeated and leads to the oligomerization of C9. The blockage of this newly exposed receptor site with the basic heparin binding site of S-protein/vitronectin or protamine inhibits polymerization.

been measured by circular dichroism during the transition (Tschopp et al., 1982). After unfolding, both the conserved cysteine-rich domains residing in C9a and the membrane-interacting site would be exposed at the surface. Unfolded C9 recognizes and binds to a new monomeric C9 via, at least in part, the acidic region of the cysteine-rich domain. The nascently bound C9 then unfolds and binds to yet another C9 molecule. This oligomerization step ceases when either the pool of monomeric C9 is exhausted or polymerizing C9 closes the tubule to form closed tubular poly(C9).

The cluster of newly exposed negative charges in unfolded C9 may not only serve as a binding site for another C9 but can also bind to the basic segment of S-protein causing the inhibition of the lytic event. Once S-protein has bound via this electrostatic interaction to poly(C9), binding may be further stabilized, possibly via an as yet undefined hydrophobic domain of S-protein that could cover the membrane interaction site of C9. This renders the complex water soluble and hence inactive.

There exists other evidence that the initial interaction of C9–C9 or C9–S-protein/vitronectin is a charge–charge interaction. First, several anionic or cationic molecules have been described to inhibit C9 activity. Second, deoxycholate, an anionic detergent, dissociates S-protein from the isolated SC5b–9 complex (Podack & Müller-Eberhard, 1980), whereas nonionic surfactants have no effect. Third, C9 polymerization mediated by Zn^{2+} does not occur at high ionic strength (>1 M NaCl; Tschopp, 1984).

Their structural homology suggests that a similar transition and inhibition mechanism may occur in C6, C7, C8, and perforin. In this respect it is interesting to recall the purification procedure of C5b–6. A polylysine–Sepharose column is used to affinity purify C5b–6. The same affinity procedure, however, does not purify monomeric C5 or C6 (Podack et al., 1978), possibly because the cysteine-rich domain is not exposed in native C6.

The increased inhibitory activity of fragmented S-protein suggests that the heparin binding site within S-protein/vitronectin is not completely exposed in native S-protein. Barnes (1985) has shown that at neutral pH most of the S-protein does not bind to heparin–Sepharose, but at lower pH or by the addition of chaotropic agents, binding of S-protein to heparin is promoted. Likewise, CNBr-fragmented S-protein/vitronectin has been shown to be significantly more active in the abrogation of heparin-mediated inhibition of factor Xa by antithrombin III (Preissner & Müller-Berghaus, 1987).

Interestingly, S-protein/vitronectin is found in plasma in native as well as in a partially proteolyzed form (Preissner et al., 1985), in which the polypeptide chain is cleaved at Arg^{402}, right after the heparin binding site (Dahlbäck & Podack, 1985). The ratio of native and cleaved forms varies in plasma or in purified protein preparations (Preissner et al., 1985; Dahlbäck & Podack, 1985; Akama et al., 1986), implying a possible mechanism for the different functional activities of S-protein. In addition, S-protein has been shown to be complexed to the thrombin–antithrombin III complex (Ill & Ruoslahti, 1985; Jenne et al., 1985; Podack et al., 1986; Preissner et al., 1987) to form a ternary complex with M_r ~350 000. This complex formation may eventually lead to the exposure of the heparin binding site.

In conclusion, we show that the activity of not only the membrane attack complex but also perforin can be regulated by serum concentrations of S-protein/vitronectin. It does so by binding via its basic heparin binding site to a cluster of negative charges contained within a cysteine-rich domain of the lytic components. This segment is cryptic in the monomeric inactive proteins but becomes exposed only upon complex formation. Since it binds to the heparin binding site, it may be considered as functionally analogous to heparin. Indeed, preliminary experiments show that other heparin binding proteins also interfere with the lytic activities of the complement system.

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Registry No. C7, 80295-57-4; C8, 80295-58-5; C9, 80295-59-6; heparin, 9005-49-6.

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