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Complement inhibition by human vitronectin involves non-heparin binding domains

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SUMMARY

Vitronectin (complement S-protein), a multifunctional glycoprotein, inhibits complement-mediated cytolysis at two identified stages of terminal complement complex (TCC) formation: blocking of C5b-7 membrane binding, and prevention of C9 polymerization. However, the functional domain(s) of vitronectin involved in these reactions remains incompletely defined. In order to identify the complement inhibition site, a 12-kD heparin binding fragment and two other internal fragments (53 kD and 43 kD) of vitronectin were isolated after cyanogen bromide (CNBr) treatment of the native molecule. Potent inhibition of guinea pig erythrocyte (GPE) reactive lysis was demonstrated with native vitronectin, total CNBr digest and the 53-kD and 43-kD fragments, but only very poorly by the heparin binding 12-kD peptide. Similarly, the 43-kD fragment blocked the binding of C5b-7 to immobilized vitronectin, whereas the 12-kD fragment had no effect. These data localize the C5b-7 binding site to a 43-kD internal region. Further characterization of the fragments was carried out in an assay which detected C9 polymerization in the presence of C5b-8. Polymerized material was separated by PAGE, detected by autoradiography and quantified after excision from the gels. Results showed that polymerization did not occur in the presence of the 53-kD and 43-kD fragments. However, the 12-kD heparin binding fragment had no effect. It is proposed that prevention of C5b-8-induced C9 polymerization resides at a site in an internal region of the vitronectin molecule.

Keywords vitronectin complement inhibition terminal complement complex

INTRODUCTION

The components of the complement system interact to mediate a variety of biologically important consequences. Most of these interactions result from proteolytic cleavage of native components with assembly of generated fragments into enzymatically active complexes. The high turnover of individual components and their fragments [1] demands that the complement cascade is controlled by systems of inhibitors that are distributed throughout the two primary pathways and the terminal complement complex (TCC) (i.e. C5b-9). The functions of complement are both physiological and pathological, with the former mediated substantially by products of the C3 molecule [2]. In contrast, there is evidence that the components of the TCC contribute significantly to the mediation of tissue damage in several human and experimental disorders [3]. In an attempt to prevent this damage, interest has focused on natural complement inhibitors which reside in the plasma and on the cell surface.

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The multifunctional glycoprotein vitronectin has been identified as a component of the water-soluble component of the TCC (i.e. SC5b-9). This protein can divert nascent C5b-7 from binding to membranes, thus forming SC5b-9, which is haemolytically inactive. Evidence indicates that the ability of vitronectin to mask apolar surfaces on SC5b-7(-9) is essential in preventing the transformation of these hydrophilic complexes to the hydrophobic state of the membrane attack complex (MAC) [4,5]. Comparison of cDNA and genomic sequence data of vitronectin has placed it in the pexin gene family together with other proteins such as haemopexin. These molecules share a common repeat pattern of peptide motifs along the polypeptide chain as well as an unusual positioning of introns within the repeats. However, considerable structural modifications have occurred for some of the repeats to facilitate unique functional properties of the individual polypeptides [6]. Vitronectin is purported to participate in cell adhesion, coagulation and fibrinolysis [7] as well as its role in the TCC. The several biological functions which have been ascribed to this single polypeptide chain may be rationalized on the basis of the location of unique functional sequences in defined areas of the polypeptide, combined with the potential

for a number of secondary modifications and conformational effects.

Fragmentation studies with cyanogen bromide (CNBr) have established the domain structure of vitronectin [8], and the highly charged heparin binding region has been identified with complement inhibition [9]. It is proposed that a conserved highly acidic, cysteine-rich domain present in C6, C7, C8 and C9 can interact ionically with the heparin binding peptide. In particular, the effect of this region on C9 tubule formation has been examined. This indicates that unfolding of the C9 molecule during polymerization, with exposure of the acidic domain, facilitates binding of the heparin peptide and results in diversion of C9 polymerization and lysis. A conformational change within the vitronectin molecule resulting in accessibility of the normally masked heparin binding region was also identified [10].

Our previous work [11] has shown that, *in vitro*, vitronectin predominantly promotes the formation of SC5b-7 and is far less effective at inhibiting C9 lytic pore formation. Furthermore, the vitronectin domain responsible for inhibition of C5b-7 membrane attachment did not appear to be the heparin binding site. In the present study we have examined the interaction of complement with vitronectin fragments obtained by CNBr cleavage in an effort to define further the role of the heparin and non-heparin binding regions.

MATERIALS AND METHODS

Purification of vitronectin

Vitronectin was purified from human plasma using a heparin–Sepharose affinity column (LKB-Pharmacia, Uppsala, Sweden) in the presence of 8 M urea [12]. The purified protein contained both the 75-kD and 65-kD forms and was homogeneous as shown by SDS–PAGE.

CNBr fragments of vitronectin

CNBr modification was carried out as described [8]. Reduced and carboxymethylated vitronectin was modified by incubation of 5 mg protein with 5 mg CNBr in 2 ml 70% HCOOH for 8 h or 24 h at 20°C. The samples were then diluted 1:10 with water and lyophilized.

Heparin–Sepharose chromatography of the digests was carried out as follows. The sample was dissolved in 2 ml 50 mM Tris–HCl pH 7·0 containing 8 M urea (starting buffer) and applied to a heparin–Sepharose column (1·5 × 5 cm). The column was washed with 50 ml starting buffer and the unbound fragments were collected. The bound material was eluted with 0·5 M NaCl in the same buffer. The unbound material was dialysed against PBS (dialysis tubing, 12 kD retention limit; Sigma, St Louis, MO). The heparin-bound material was dialysed in benzoylated dialysis tubing (2 kD retention limit; Sigma). Protein was measured by the Bradford procedure (Bio-Rad Laboratories, Hercules, CA).

Activity of the TCC

The complement proteins C7, C8 and C9 and bovine serum albumin (BSA)-EIA grade, were purchased from Sigma. Purified C5b-6 was isolated according to published procedures [13].

Studies of complement reactive lysis were carried out with 4% v/v guinea pig erythrocytes (GPE) in complement fixation diluent (CFD; Oxoid, Basingstoke, UK). Purified C5b-6 complex was used at a concentration that gave 50–80% lysis in the presence of C7 (0·57 nM), C8 (0·68 nM) and C9 (1·41 nM). The intermediate, GPE C5b-7 was prepared by incubation of GPE and C5b-6 and C7 for 15 min at 37°C. The cells were washed before centrifugation at 2500 *g* for 5 min. Reactive lysis was measured either after a further incubation for 30 min of intermediates with the remaining late components (C8 and C9), or after simultaneous incubation of all components with GPE at the concentrations previously indicated. Dose-response curves were plotted as per cent inhibition of lysis of GPE *versus* inhibitor concentration (μM), where total lysis was measured as the absorbance in the absence of inhibitor after subtraction of background cellular lysis.

N-terminal sequencing

Samples were transferred (electrophoretically) to polyvinylidene difluoride membranes and sequencing was carried out with an ABI 473A Protein Sequenator (Applied Biosystems, Foster City, CA) using standard phenyl isothiocyanate chemistry [14].

Competitive inhibition of C5b-7 vitronectin binding by CNBr fragments

Binding of C5b-6 and C7 to immobilized carboxymethylated vitronectin was carried out in 96-well microtitre plates. Vitronectin (20 μg/ml, 50 μl/well) was immobilized on wells for 16 h at 4°C. Excess sites were blocked by incubation at 37°C for 1 h with 3% v/v BSA in PBS 0·1% Tween. C5b-6 (150 ng) in PBS–0·1% BSA was reacted in the wells for 15 min at 37°C in the absence or presence of vitronectin and fragments. The wells were cooled to 4°C before the addition of C7 (50 ng) to take advantage of the temperature dependence of the kinetics of C5b-6 binding to C7, and the reaction was allowed to proceed at 37°C for 20 min, in a total volume of 40 μl. After additional washing in 20 mM Tris, 500 mM NaCl, pH 7·5 containing 1% BSA, binding of C5b-7 was quantified by reaction with polyclonal anti-C7 (ICN, Lisle, IL) (0·1 ml, 1:100 dilution) and detected with peroxidase-conjugated rabbit anti-goat IgG secondary antibody (Dako, Carpinteria, CA) (1:1000 dilution) with *o*-phenylene diamine/H_2O_2 as substrate, at 492 nm. Non-specific background absorbance, due to binding of C7 and vitronectin fragments in the absence of C5b-6 and an irrelevant MoAb (anti-influenza virus haemagglutinin) of the same isotype, was subtracted.

Radiolabelling of C9

C9 was labelled with ^{125}I by the lactoperoxidase method [15], and separated from free ^{125}I by gel filtration on Sephadex G-10 equilibrated in PBS–0·1% BSA. The specific radioactivity of the labelled protein was $4{\cdot}8 \times 10^6$ ct/min per μg protein.

Polymerization of C9

C9 polymerization was induced by incubation with the C5b-8 complex. C5b-6 (2 μg) was mixed with C7, C8 and C9 (incorporating 2 ng ^{125}I C9) in a molar ratio of 1:1:1:12. The mixture was incubated for 30 min at 37°C in the absence or presence of vitronectin fragments. The reaction was terminated by heating (5 min, 100°C) in SDS buffer under reducing conditions. Polymerized material was separated by electrophoresis in 4–15% gradient slab gels and detected by

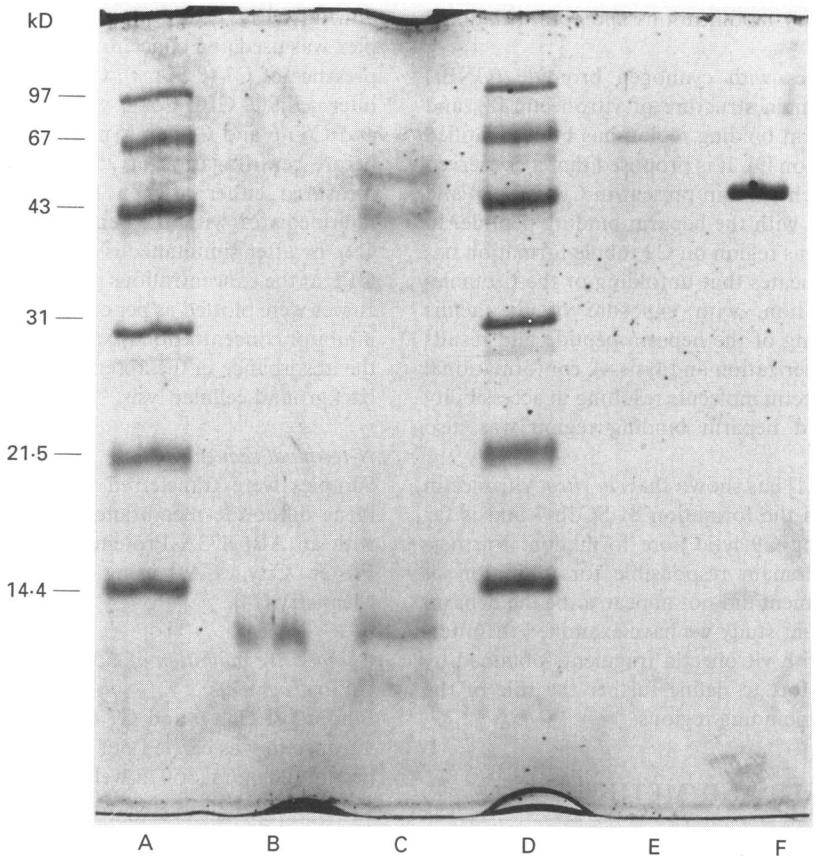

Fig. 1. Cyanogen bromide (CNBr) fragments of vitronectin analysed by SDS–PAGE on a 15% acrylamide gel. Lanes A and D, molecular weight markers; lane B, 12-kD peptide purified by heparin–Sepharose chromatography; lane C, total cleavage products from an 8-h CNBr digest; lane E, purified vitronectin; lane F, purified 43-kD fragment of vitronectin. The molecular weight standards used were: phosphorylase 6 (97 kD), bovine serum albumin (BSA; 67 kD), ovalbumin (43 kD), carbonic anhydrase (31 kD), trypsin inhibitor (21·5 kD) and lysozyme (14·4 kD).

autoradiography. Polymerized C9 bands were quantified by measuring the radioactivity present after excision from the gel.

RESULTS

Fragmentation of vitronectin by CNBr

Cleavage of vitronectin by incubation for 8 h and 24 h with CNBr resulted in a distinct set of fragments which were comparable to those described previously [8]. After heparin–Sepharose chromatography of the vitronectin digests and subsequent dialysis, the fragments were identified by a 15% SDS–PAGE (Fig. 1). Non-heparin binding material from the 8 h incubation with CNBr consisted of major bands of molecular weight 53 kD and 43 kD (Fig. 1, lane C), while the major band after 24 h digestion was 43 kD (Fig. 1, lane F). The fraction which bound to the heparin–Sepharose column was also separated by SDS–PAGE and consisted of peptide material of molecular weight of approximately 12 kD (Fig.1, lane B). Further estimation of the size of the 12-kD fragment carried out on a 20% acrylamide SDS–PAGE using the Pharmacia Phast system confirmed the estimated size, in that it ran between 8 kD and 14 kD (gel not shown). The origin of this peptide was confirmed by N-terminal sequence analysis. The sequence was in agreement with previously published data [8] for the CNBr heparin binding peptide of vitronectin (Table 1).

Inhibitory action of vitronectin and CNBr fragments in reactive lysis

Each of the vitronectin fragments caused dose-dependent inhibition in the reactive lysis assay system. Fifty percent inhibition was achieved at a concentration of 0·09 μM by intact vitronectin. Modification and cleavage of the protein did not adversely influence the activity as the 8 h total digest, as well as the non-heparin binding fragments, all showed similar inhibitory activity (50% in the range 0·014–0·022 μM;

Table 1. NH_2-terminal amino acid sequence of the peptide fragment of vitronectin isolated by heparin–Sepharose chromatography

Cycle number	1	2	3	4	5	6	7	8
Expected*	A	P	R	P	S	L	A	K
Found	A	P	R	P	S	L	A	K
Yield†	135	75	81	68	30	38	10	14

* Expected amino acid according to [7].
† Yields are given in pmol.

Table 2. Inhibition of reactive lysis by vitronectin or cyanogen bromide (CNBr) fragments

Fragment	Concentration (μM)
Unmodified vitronectin	0·09 ± 0·01
Total CNBr digest	0·022 ± 0·007
53-kD + 43-kD fragments	0·014 ± 0·003
43-kD fragment	0·021 ± 0·005
Heparin binding fragment	3·4 ± 0·45

The concentration of inhibitor (μM) which produced 50% inhibition of total lysis was calculated from dose response curves of guinea pig erythrocyte (GPE)-reactive lysis assays. Assays were performed three times in duplicate, and results are expressed as mean ± s.e.m.

see Table 2). However, the heparin binding peptide was less active, requiring 3·4 μM to achieve 50% inhibition. The data suggest that the major inhibitory site(s) is located within the 43-kD component.

Site of action of the 43-kD vitronectin fragment in reactive lysis

Further information on the role of vitronectin fragments in the lysis system was obtained by examining inhibition during GPE C5b-7 formation and comparing this with the activity on addition of C8 and C9 to preformed GPE C5b-7. The heparin binding peptide and the 43-kD fragment were used in these assays. The data indicate that the major activity of the 43-kD fragment takes place during GPE C5b-7 formation, with 50% inhibition at 0·024 μM compared with 0·016 μM for the CNBr total digest mixture. The activity of the purified heparin binding fragment was much less (50% inhibition at 2·9 μM). All of the fragments were significantly less active inhibitors of lysis in the steps after membrane attachment of C5b-7. However, the fragments which did not contain the heparin binding peptide were approximately two-fold more active at inhibition of C8- and C9-mediated lysis of preformed GPE C5b-7 than the total digest (Fig. 2).

Binding of C5b-7 to vitronectin

The complement inhibitory effect of vitronectin suggested a key role for the 43-kD fragment in preventing C5b-7 membrane attachment. To substantiate this, direct binding of vitronectin fragments to nascent C5b-7 in cell-free solution was measured. A comparative study of the effects of the 43-kD fragment and the heparin binding peptide was carried out by adding either component to microtitre wells in the presence of a constant amount of C5b-6 and C7. Under these conditions the 43-kD fragment competed in a dose-dependent manner with immobilized vitronectin with approximately 70% decreased binding of C5b-7 at the plateau of the inhibition curve, while the heparin binding peptide had little effect (see Fig. 3).

C9 polymerization

The ability of vitronectin fragments to prevent C9 tubule formation was monitored by separation of monomer and

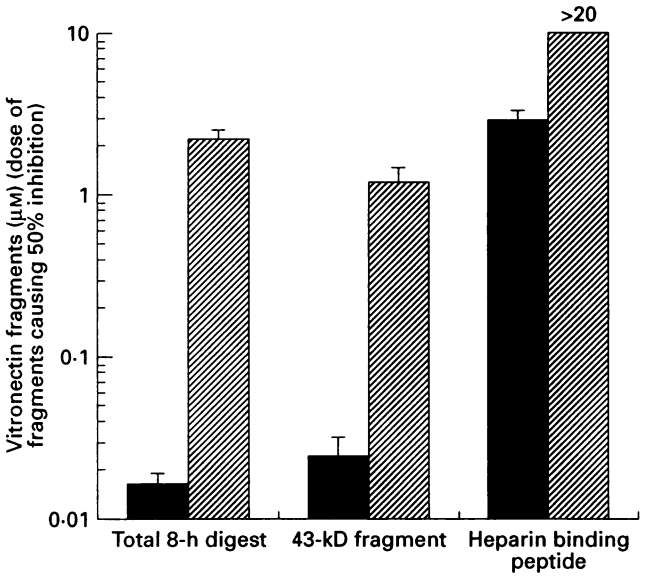

Fig. 2. Inhibition of guinea pig erythrocyte (GPE)-reactive lysis by vitronectin fragments at two stages of GPE C5b-9 formation. First (■), GPE were incubated with limiting concentrations of C5b-6 together with C7, C8, C9 and vitronectin fragments. Second (▨) an identical batch of GPE was preincubated with limiting concentrations of C5b-6 and C7, washed, and then C8, C9 and vitronectin fragments were added. The concentration (μM) of fragments which produced 50% inhibition from dose-response curves is shown in each case. This shows that all of the fragments were less active inhibitors of lysis in the steps after membrane attachment of C5b-7.

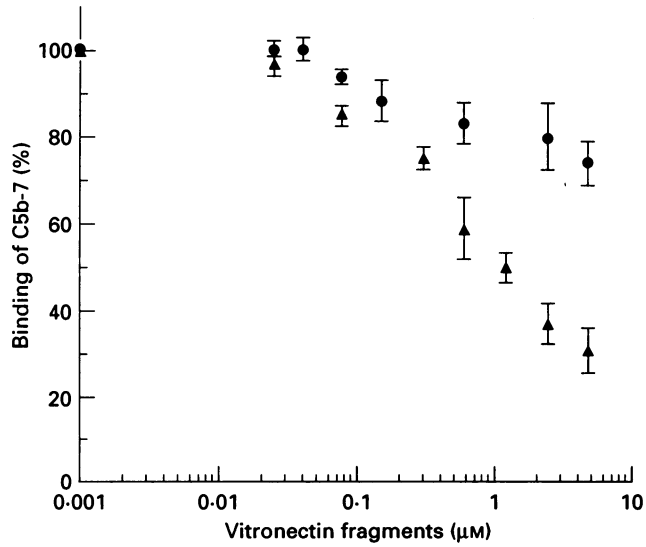

Fig. 3. Competitive inhibition of C5b-6 and C7 binding to immobilized vitronectin by vitronectin fragments. C5b-6 and either the 43-kD fragment (▲) or the heparin binding peptide (●) were incubated at various concentrations in vitronectin-coated microwells before the addition of C7. Binding of C5b-7 to microwells was quantified using an anti-C7 polyclonal antibody and a peroxidase-conjugated secondary antibody. One hundred per cent binding is expressed as the absorbance of anti-C7 antibody minus that of a non-specific MoAb isotype control.

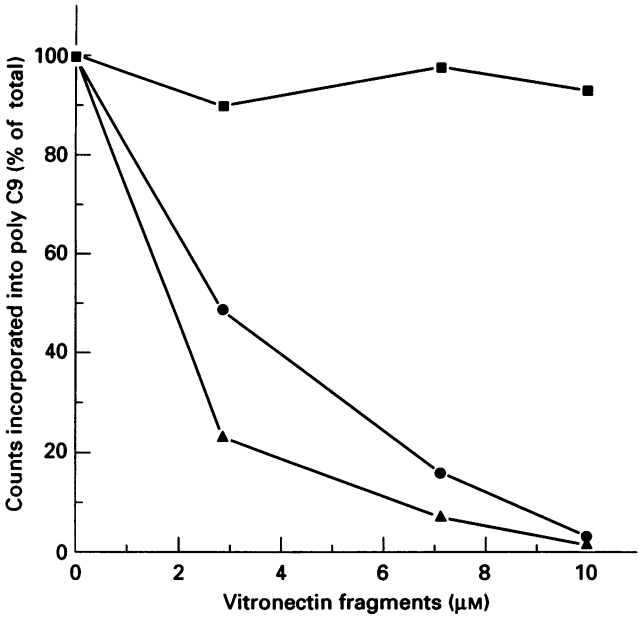

Fig. 4. Inhibition of guinea pig erythrocyte (GPE) C5b-8-induced polymerization of C9 by vitronectin fragments. Amount of ^{125}I-C9 monomer incorporated into polymerized C9 in the presence of increasing concentrations of total cyanogen bromide (CNBr) vitronectin digest (●), 53-kD/43-kD fragment mixture (▲) and heparin binding peptide (■). The heparin binding peptide failed to inhibit C9 polymerization even in high concentration.

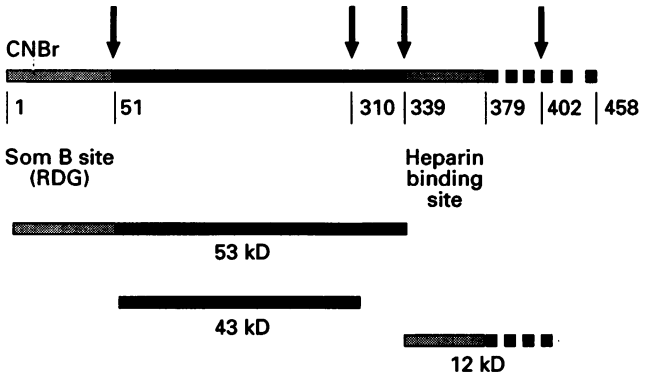

Fig. 5. Schematic representation of cyanogen bromide (CNBr)-generated domains of vitronectin. The CNBr cleavage sites are indicated by arrows. The location of the somatomedin B cell attachment and heparin binding domains are from [8]. The interrupted line represents the sequence after cleavage at Arg 379, which arises from differential sensitivity to proteolysis as a result of the polymorphic site at residue 381 [26]. CNBr-generated fragments of vitronectin are shown.

polymer on SDS–PAGE after induction of C9 polymerization by incubation with the C5b-8 complex. The amount of polymer formed in the presence of various concentrations of vitronectin fragments was measured by counting the radioactivity present in the polymerized C9 band. The 8 h total digest mixture contained effective inhibitors, and the inhibition was concentration-dependent. The heparin binding peptide could not inhibit the reaction in the range 2·85–10 μM, whereas the 43/53-kD fragments caused inhibition (Fig. 4). In fact, inhibition was not observed even at higher concentrations of heparin binding peptide (up to 20 μM, data not shown).

DISCUSSION

In this study we have localized the major inhibitory activity of vitronectin for the TCC to a 43-kD CNBr fragment (Fig. 5). We have shown previously that vitronectin inhibition occurs with formation of the GPE C5b-7 complex [11], and this result further defines the vitronectin reactive site in the complement lysis sequence. Assays of reactive lysis (i.e. using GPE) showed that vitronectin, purified by heparin–Sepharose chromatography in the presence of urea, was approximately five times less active as an inhibitor than the total CNBr digest, indicating reduced accessibility of the active site. Previous studies of inhibition of bystander lysis by vitronectin have shown that the molecule competes with membranes for sites exposed on C5b-7 [5]. Our results indicate that the apolar region responsible for interaction with the phospholipid binding site on metastable C5b-7 is located within the 43-kD fragment.

Vitronectin participates in several macromolecular interactions of physiological importance [16,17]. The heparin binding peptide of vitronectin has been linked to a number of these interactions, including complement inhibition. In addition, the somatomedin B domain has been shown to interact with type 1 plasminogen activator inhibitor [18], and there is evidence to suggest that collagen binding sites occur in the central region of the molecule [19].

The role of vitronectin in the prevention of C9 tubule formation within the SC5b-9 complex was also examined. C9 polymerization induced in the presence of C5b-8 was inhibited in a concentration-dependent manner by vitronectin fragments, albeit at much higher concentrations than required for SC5b-7 formation. However, neither the results from assays of bystander lysis nor the measurements of C9 polymerization are compatible with a major role for the heparin binding peptide in these activities. The peptide isolated in the present study incorporated the entire heparin binding sequence. It was judged pure by SDS–PAGE and N-terminal sequence analysis and was active in heparin binding. This result complements our previous study [11], which showed that two 15-mer peptides based on the sequence of the vitronectin heparin binding domain (residues 348–379) did not inhibit the lysis of EAC1–7 upon addition of C8 and C9, either separately or in combination.

Undoubtedly, ionic effects are important in complement inhibition, as shown by a number of studies with charged macromolecules. Protamine sulphate has been identified as a potent inhibitor of the terminal complement pathway [11,20]. However, in this work, removal of the heparin binding peptide from the CNBr mixture increased the inhibition of C9 polymerization by the remaining fragments. It is possible that a polyanionic sequence is involved in this inhibition. This cluster of residues containing sulphated tyrosines and acidic amino acids (residues 53–64) at the terminal region of the molecule is known to be masked in the tertiary conformation [16]. Indeed, the secondary modification of these tyrosine residues appears to be unique to vitronectin among the haemopexin family of

molecules [21]. Previous reports have implicated the heparin binding peptide in complement inhibition [9]. However, it should be pointed out that we have measured inhibition during the process of C9 polymerization which involves hydrophilic/hydrophobic transition of C9 leading to binding and unfolding of additional molecules. This is distinct from other work, where the interaction of a heparin binding sequence with actual polymerized C9 was examined [9].

Studies of dissociation of vitronectin from the SC5b-9 complex using the anionic detergent deoxycholate demonstrate the complex nature of the interaction [22]. The structural homology of the late complement components and the general feature of hydrophilic/hydrophobic transition which occurs for the individual proteins suggest a common mechanism for all steps in formation of the terminal complex. It is possible that the self-association of C9 molecules and concomitant exposure of membrane binding sites is analogous to the unmasking of apolar sites on binding of C7 and C8 to the C5b-6 complex. Our results show a similar pattern of inhibition by the vitronectin fragments at the inhibition of C5b-7 binding and the C9 polymerization steps. It is likely that the 43-kD component of vitronectin contains both hydrophobic and hydrophilic sites which act in concert in this inhibition.

The conclusions from this study support a recent study which demonstrated that the heparin binding peptide is exposed in the SC5b-9 complex [23]. This specialized heparin binding sequence is probably involved in binding to glycosaminoglycans at cellular sites. This may be important in a number of functions of vitronectin, including complement inhibition where clearance of many different end-product complexes from the circulation is required. Indeed the vitronectin–thrombin–antithrombin III complex has been shown to interact with human umbilical vein endothelial cells via the vitronectin heparin binding site [24].

Vitronectin therefore combines with terminal complement proteins (i.e. to form SC5b-9) through a site(s) in a 43-kD region, which appears to be readily accessible in purified vitronectin. This interaction appears to lead to exposure of the heparin binding [23] and cell adhesion [25] sites. Both sites may be required for binding to cells and the extracellular matrix for the mediation of biological effects and for the elimination of the complex.

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