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Snake C-Type Lectin-Like Proteins and Platelet Receptors

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Platelets · C-type lectin-like proteins · Glycoprotein Ib · Glycoprotein VI · Collagen

Abstract
Snake venoms are complex mixtures of biologically active proteins and peptides. Many affect haemostasis by activating or inhibiting coagulant factors or platelets, or by disrupting endothelium. Snake venom components are classified into various families, such as serine proteases, metalloproteinases, C-type lectin-like proteins, disintegrins and phospholipases. Snake venom C-type lectin-like proteins have a typical fold resembling that in classic C-type lectins such as the selectins and mannose-binding proteins. Many snake venom C-type lectin-like proteins have now been characterized, as heterodimeric structures with α and β subunits that often form large molecules by multimerization. They activate platelets by binding to VWF or specific receptors such as GPIb, $\alpha_2\beta_1$ and GPVI. Simple heterodimeric GPIb-binding molecules mainly inhibit platelet functions, whereas multimeric ones activate platelets. A series of tetrameric snake venom C-type lectin-like proteins activates platelets by binding to GPVI while another series affects platelet function via integrin $\alpha_2\beta_1$. Some act by inducing VWF to bind to GPIb. Many structures of these proteins, often complexed with their ligands, have been determined. Structure-activity studies show that these proteins are quite complex despite similar backbone folding. Snake C-type lectin-like proteins often interact with more than one platelet receptor and have complex mechanisms of action.

Introduction

C-type lectin-like proteins are an important group among the haemorrhagic components in snake venom. These proteins are named after the folding in classic C-type lectins, such as mannose-binding protein and the selectins [1]. The 'C' is from calcium-dependent and the 'lectin' from the sugar-binding function. Most of the snake venom C-type lectin-like proteins do not, in fact, have these properties and the calcium-binding loop is deleted. Based on their structures and biological functions, snake venom C-type lectins can be classified into: the true C-type lectins, with one canonical C-type lectin carbohydrate recognition domain (CRD) [2], that binds sugars and agglutinate erythrocytes; and C-type lectin-like proteins with CRD-related non-carbohydrate binding C-type lectin-like domains (CTLDs) [3] that do not bind sugars.

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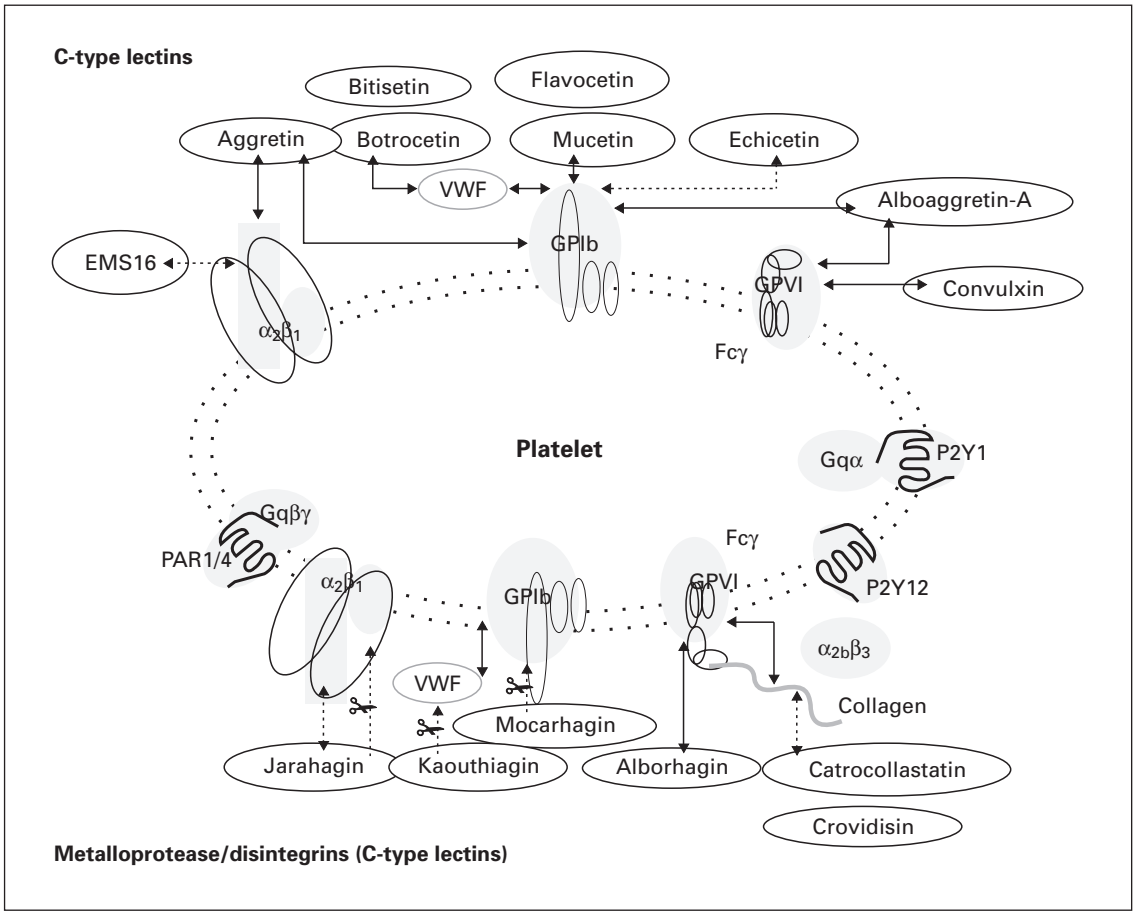

Fig. 1. Snake venom C-type lectins interacting with platelet receptors (top section). Metalloproteases/disintegrins where C-type lectins may have a role are shown in the lower section.

Structures of Venom C-Type Lectins

C-type lectins are widely distributed and have roles in many biologically important processes. Both CRD- and CTLD-containing lectins are found in snake venoms. More than 50 snake venom C-type lectins have been completely sequenced. A phylogenetic analysis of these sequences classifies these proteins roughly into three main branches: CRD-containing proteins, factor IX/X-binding proteins and those interacting with platelet receptors. However, sequence alignment data does not readily indicate the binding specificities of the latter group.

CRD-containing snake venom C-type lectins are basically disulfide bond-linked homodimers and often form larger multimers. CTLD-containing snake venom C-type lectins are disulphide-bonded heterodimers. Different members of the C-type lectin-like family are assembled from this basic unit. The first determination of an X-ray crystal structure of a snake venom C-type lectin-like protein indicated that the two subunits associate tightly to form a concave surface by loop-swapping between the two subunits [4]. Higher molecular mass snake venom C-type lectin-like proteins have a complicated subunit structure. Flavocetin-A [5], mucrocetin and convulxin are cyclic tetramers $(\alpha\beta)_4$ made up of four αβ-heterodimers arranged 'head-to-tail' via inter-chain disulfide bridges. The $(\alpha\beta)_4$ structure is one way of forming larger multimeric structures. However, the tetrameric structures of some snake venom C-type lectin-like proteins like aggretin [6] contain two non-covalently linked αβ-heterodimers, while some tetrameric structures such as alboaggregin A have 4 different subunits, α_1, α_2, β_1 and β_2. The concave surface formed between the two subunits is presumed to be the main binding site for the ligand. This was supported by

recent studies on structures of snake venom C-type lectin-like proteins complexed with their ligands [7–9]. However, in heterotrimeric complexes, such as GPIb-VWF A1 domain-botrocetin, other surfaces also participate in complex formation.

Many snake venom C-type lectin-like proteins binding to VWF or platelet receptors such as GPIb, $\alpha_2\beta_1$ and GPVI have been characterized. Botrocetin and bitiscetin form trimolecular complexes with VWF and GPIb to activate platelets. Recent results suggest that they interact with both proteins, not simply by inducing conformational changes in VWF-A1 [8, 9]. The C-type lectin-like proteins acting via GPIb fall into two categories, those inhibiting platelet activation by blocking binding of VWF/ristocetin and/or thrombin and those either agglutinating platelets or activating and aggregating platelets. Most inhibitory GPIb C-type lectin-like proteins are simple heterodimers, while most multimeric GPIb-binding venom proteins agglutinate or aggregate platelets. GPIb-binding proteins may behave differently in vitro and in vivo. Echicetin specifically binds platelet GPIb and blocks platelet interactions with VWF and thrombin. It is cross-linked by IgMκ to form multimers that agglutinate platelets in vivo [10]. Convulxin, stejnulxin and ophioluxin activate platelets via GPVI [11–13]. They are multimeric proteins composed of heterodimers. Like alboaggregin-A and alboluxin, convulxin also binds to GPIb. EMS16 binds specifically to the α-I domain of the collagen receptor, integrin $\alpha_2\beta_1$, in a metal ion-independent fashion to inhibit collagen binding [14]. Rhodocetin also binds to and inhibits $\alpha_2\beta_1$ and is unusual in that its subunits are not covalently linked. Aggretin and bilinexin activate platelets via $\alpha_2\beta_1$ and GPIb. However, aggretin may also use other unidentified receptor(s). Thus, venom C-type lectin-like proteins often use more than one platelet receptor.

GPIb-Binding Lectin-Like Proteins

The interaction of the GPIb-IX-V complex with its adhesive ligand, von Willebrand factor (VWF), in the subendothelial matrix initiates platelet adhesion under high shear stress in the arterial circulation. The most important component of GPIb-IX-V complex in terms of mass and functional sites is the 125-kDa GPIbα chain containing the binding site for the VWF-A1 domain.

The snake venom C-type lectin-like proteins acting via GPIb fall into two categories, those inhibiting platelet activation by blocking binding of VWF/ristocetin or thrombin, and those which either agglutinate platelets or activate and aggregate platelets in stirred platelet suspensions. Most of the inhibitory C-type lectin-like proteins described so far bind to GPIb. These include echicetin, jararaca GPIb-binding protein, tokaricetin, CHH-A and B, TSV-GPIb-BP, lebecetin, purpureotin and agkicetin. C-type lectin-like proteins that act via GPIb to agglutinate or aggregate platelets include alboaggregins [15], flavocetins A and B, agglucetin, mamushigin, mucrocetin and mucetin [16]. Although these GPIb-binding proteins have similar sequences, they affect platelets differently. Alboaggregin B agglutinates platelets via GPIb and has a weak activating effect only at high concentrations, while alboaggregin A activates platelets via GPIb and GPVI [17], thus it is not GPIb specific. Most GPIb-binding C-type lectin-like proteins inhibit GPIb-VWF binding, but some also inhibit platelet activation by low doses of thrombin, implicating blockage of the thrombin-binding site on GPIb but, since this was not always tested for, it may be valid for others.

Multimeric GPIb-binding venom proteins could attach to several GPIb molecules on the surface of one platelet to cluster GPIb receptors. On the other hand, they might bind to GPIb on different platelets and provide a mechanism for agglutination of platelets. Flavocetin-A, mucrocetin and mucetin (TMVA) [16] are all multimeric C-type lectin-like proteins. These three C-type lectin-like proteins share a high degree of sequence similarity and all act via GPIb; however, they activate platelets to different extents. Mucetin induces full platelet aggregation; mucrocetin induces platelet agglutination, while flavocetin induces only small platelet agglutinates. This cannot be explained on the basis of binding between these C-type lectin-like proteins and GPIb alone. Minor differences in sequence between these C-type lectin-like proteins, mostly lying outside the concave binding site, could affect interactions between the venom C-type lectin-like proteins themselves and may be induced by GPIb binding. Thus, mucetin and mucrocetin may therefore form larger complexes between GPIb on platelets with proportionately more shear stress activation of platelets in stirred or flow systems. Cross-linking GPIb induces actin polymerization, which in turn leads to release of calcium from internal stores independent of external calcium or Src family tyrosine kinases [10].

GPIb-binding proteins often act differently in vitro and in vivo possibly due to additional interactions with plasma or endothelial proteins.

VWF-Binding C-Type Lectin-Like Proteins

Human VWF in solution and platelet GPIb-IX-V do not interact normally unless exogenous modulators, such as botrocetin and ristocetin, are added or shear stress is applied or VWF contains abnormally large multimers. Two well-known snake venom C-type lectin-like proteins that induce VWF-GPIb binding on platelets leading to agglutination and aggregation are botrocetin and bitiscetin. This makes botrocetin an important tool for studies on VWF/platelet interactions and detection of VWF- and GPIb-related disorders. The specific binding site for botrocetin has been identified in the VWF-A1 domain formed by residues in discontinuous sequence. Bitiscetin is a C-type lectin-like protein with similar properties from *Bitis arientans* venom.

The overall fold of these two proteins is like other snake venom C-type lectin-like proteins. The characteristic target recognition is explained mainly by the differences in the surface potential on the central concave surface. A negatively charged patch on the surface of bitiscetin binds to the positively charged surface of the VWF-A1 domain as with botrocetin. However, bitiscetin binds to a distinct site in the A1 domain of VWF spanning the α4 and α5 helices and the loop between α5 and β6 but close to the botrocetin-binding sites. Comparison between the bitiscetin- and botrocetin-VWF-A1 structures shows that the long-axis of bitiscetin is almost perpendicular to that of botrocetin when the bound A1 domains are superimposed [9].

The crystal structures of the botrocetin-A1 complex [8] and the bitiscetin-A1 complex [9] show no significant structural changes in the GPIb-binding sites of the A1 domain compared with uncomplexed A1. Bitiscetin- or botrocetin-induced binding depends mainly on optimising interactions between the A1 domain and the 45-kDa domain of GPIb with the C-type lectin-like proteins clamping the two together. Electrostatic interactions with the anionic peptide domain of GPIb may enhance this and binding studies support such a role, although crystallographic data are not available. The anionic peptide of GPIb is flexible and could bind to a nearby positively charged site on bitiscetin or on botrocetin complexed with VWF-A1. These studies suggested that, unlike the physiological interaction between GPIb and VWF that requires structural changes, snake venom C-type lectin-like proteins act as 'molecular glue' to stabilize GPIb-VWF interactions. Recently, the structure of the three molecules together (botrocetin, VWF-A1 domain, GPIb 45 kDa domain) was determined [18] and indicated that, after first binding to VWF-A1 domain, botrocetin slid round the complex of VWF-A1 domain/GPIb 45-kDa domain to stabilize interactions slowing the off-rate. GPIb constructs containing the anionic peptide, had 6–8 times higher binding constants in the trimolecular complex than those without it, supporting the additional interactions proposed above.

GPVI-Binding C-Type Lectin-Like Proteins

GPVI/FcγR, a 62/28-kDa complex, is the major signaling receptor for collagen on platelets. GPVI is an Ig receptor superfamily member closely related to human FcαR and natural killer (NK) cell receptors [19]. GPVI has a positively charged arginine in its transmembrane region that is essential for association with the Fcγ-chain. Cross linking of GPVI leads to tyrosine phosphorylation of the Fcγ-chain on its immunoreceptor tyrosine-based activation motif (ITAM) by the Src kinases Fyn and Lyn. This leads to binding and subsequent activation of the tandem SH2 domain-containing tyrosine kinase, Syk, which initiates a downstream signaling cascade that activate a number of effector enzymes including PLCγ2, small G-proteins and phosphoinositide-3 kinase.

GPVI is an ideal target for snake venom proteins because when clustered it induces massive platelet activation. Several snake venom C-type lectin-like proteins that activate platelets via GPVI have been identified and are powerful tools for studying GPVI-specific platelet activation. Convulxin was the first identified member of this group and was instrumental in the cloning of GPVI [11, 19]. Additional C-type lectin-like proteins from snake venoms shown to activate GPVI include alboaggregin A [17], stejnulxin [12], ophioluxin [13] and alboluxin. All are multimeric and induce platelet activation by clustering the receptor. The binding site of convulxin for GPVI is probably located on the concave surface between the two subunits. It is not clear whether the clustering of 4 GPVI molecules by a convulxin molecule is enough to cause the strong signalling or, like some of the tetrameric GPIb-binding molecules mentioned above, if there may be additional interactions between convulxin molecules, induced by the GPVI binding. Several of these snake toxins bind to a second surface glycoprotein in addition to GPVI. For example, convulxin also binds weakly to GPIb and this could contribute to cross-linking of platelets, although it may only have a minor role in overall signal transduction at the low concentration used to activate platelets [13]. Alboaggregin A or alboluxin binds to GPVI

and GPIb, inducing powerful activation. The interaction with GPIb is not essential for activation, as alboaggregin A activates Bernard-Soulier platelets and cell lines transfected with GPVI. However, signal amplification by cross-linking GPIb and GPVI may play a role in the overall platelet activation. Stejnulxin and ophioluxin are more GPVI specific than convulxin because no residual GPIb binding activity was detected in either of these [12, 13].

$\alpha_2\beta_1$-Binding C-Type Lectin-Like Proteins

The $\alpha_2\beta_1$ integrin ($\alpha_2\beta_1$, GPIa/IIa) is another major collagen receptor on platelets. It mediates adhesive interactions as well as generating intracellular signals that help to stabilize the thrombus. Snake venom C-type lectin-like proteins that interact with $\alpha_2\beta_1$ are aggretin (rhodocytin), rhodocetin, bilinexin and EMS16. Rhodocetin inhibits $\alpha_2\beta_1$ interactions with collagen. The α and β subunits of the heterodimer of rhodocetin are non-covalently associated, in the absence of the disulfide bridge, to maintain activity [20]. Recombinant α_2 I-domain binds to rhodocetin and the binding site was identified as the α3–α4 loop plus adjacent α-helices. Bilinexin is a 110-kDa protein with multiple subunits that agglutinates fixed platelets, washed platelets and platelet rich plasma, without obvious activation, via $\alpha_2\beta_1$ and GPIb. EMS16 from *Echis multisquamatus* venom is a potent and selective inhibitor of $\alpha_2\beta_1$. EMS16 has a unique, positively charged patch on its concave surface that could be a site of interaction with the I-domain of $\alpha_2\beta_1$. The crystal structure of EMS16 complexed with the integrin α_2-I domain revealed that the collagen-binding site of the α_2-I domain does indeed lie on the concave surface in EMS16, and direct binding sites are located at both ends of the surface [7]. The most positively charged residues of the patch in this region bind to residues in the α_2-I domain by water-mediated hydrogen bonds, which may contribute to the stability of the complex and the binding is metal-ion-independent [14]. Unlike the other two proteins above, aggretin strongly activates platelets. Platelets genetically lacking $\alpha_2\beta_1$, where GPIb was also removed by proteolysis, still showed the same reactivity with aggretin, suggesting that yet another receptor plays a critical role.

Role of C-Type Lectin-Like Proteins in P-IV Class Metalloproteases/Disintegrins

C-type lectin-like proteins are also important in the function of this class of protease in providing the binding site that determines specificity. However, in many cases very little is known. Increasingly, the sequence of such proteins is determined from cDNA sequence and since the C-type lectin-like proteins are provided by separate genes they are not detected. Recent research indicates that they are linked to the metalloproteases by a disulfide bridge during post-translational processing. Thus, they will need to be investigated by protein chemistry approaches.

Conclusions

Snake venom C-type lectin-like proteins are useful tools for exploring many facets of platelet function and haemostasis and will undoubtedly continue to do so. The way in which these proteins affect platelets, plasma and vessel wall provides new basic knowledge as well as new possibilities in diagnosis and treatment.

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