Multifunctional Snake C-Type Lectins Affecting Platelets

Kenneth J. Clemetson Alexei Navdaev Dagmar Dörmann
Xiao-Yan Du Jeannine M. Clemetson

Theodor Kocher Institute, University of Berne, Switzerland

Key Words

Snake venom · C-type lectin · Platelet receptor · Binding sites · Mechanism

Abstract

Snake venoms contain a wide range of components, many of which affect haemostasis by activation or inhibition of platelets or coagulation factors. They can be classified into groups based on structure and mode of action. One group is the snake C-type lectins, so called because of the typical folding which closely resembles that found in classical C-type lectins, such as selectins and mannose-binding proteins. Unlike the classic C-type lectins, those from snakes are generally heterodimeric with two subunits, α and β . Some are multimeric heterodimers. The subunits have homologous sequences and are generally linked by a disulphide bond as well as by swapping loops. One of the first C-type lectins with a defined function was echicetin

which was demonstrated to bind to platelet GPIb and block several functions of this receptor. Since then, many proteins with similar structure have been reported to act on platelet receptors or coagulation factors and several have been crystallized. These proteins were thought to be specific for a single platelet receptor or coagulation factor, i.e. they had only one receptor per heterodimer. Recent studies show that most of these C-type lectins have binding sites for more than one ligand and have complex mechanisms of action.

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Introduction

Venoms produced by snakes have a wide variety of components which kill or weaken their preys. Venoms from some snake families contain mostly neurotoxic proteins, while others such as those of the Viperidae and Cro-

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Dr. K.J. Clemetson
Theodor Kocher Institute, University of Berne
Freiestrasse 1, CH-3012 Berne (Switzerland)
Tel. +41 31 631 41 48, Fax +41 31 921 54 43
E-Mail clemetson@tki.unibe.ch

talidae are mainly haemorrhagic. Snake C-type lectins are an important class among the protein family that has been shown to have haemorrhagic effects. This family is named after the type of folding that occurs in classic C-type lectins, such as mannose-binding protein [1, 2] and the selectins [3]. The 'C' is from calcium-dependent and the 'lectin' from the sugar-binding function but, in fact, most of the snake C-type lectins do not show these properties and the calcium-binding loop is deleted in their structure. We have nevertheless retained this nomenclature since it is that of the primary literature. C-type lectins were first identified as part of metalloprotease/disintegrin/C-type lectin structures without any specific function. A large number of snake C-type lectins have now been characterized with effects on either coagulation factors or platelets. Those affecting platelets either inhibit or activate them by binding to specific receptors like GPIb, $\alpha_2\beta_1$, and GPVI.

Snake C-Type Lectin Targets

C-type lectins that act via GPIb to agglutinate platelets include alboaggregins [4–6], flavocetins A and B [7] and mamushigin [8]. Most of the inhibitory C-type lectins described so far bind to GPIb. These include echicetin [9], jararaca GPIb binding protein [10, 11], tokaricetin [12], CHH-A and B [4], and agkicetin [13]. Echicetin, a heterodimeric snake C-type lectin from *Echis carinatus*, has been shown by several authors to bind specifically to platelet GPIb and to block platelet interactions with von Willebrand factor (vWf) [9] and thrombin [14]. There has been considerable interest in using C-type lectins such as echicetin as antithrombotics, blocking the interaction between vWf and platelets. However, when echicetin or similar snake C-type lectins were injected into small animals to

study their effects in vivo, induction of thrombocytopenia has often been reported [9, 15]. Generally, the platelet count dropped to 20–30% of the control value and then gradually recovered over several hours. This phenomenon has remained unexplained. Other snake C-type lectins bind to different platelet receptors, for example, convulxin, a trimeric heterodimer, has been shown to be specific for GPVI [16]. Several other snake C-type lectins were reported to bind to other platelet receptors, aggrexin (or rhodocytin) [17] and rhodocetin [18], from *Calloselasma rhodostoma* venom and EMS16, a C-lectin type protein from *Echis multisquamatus* venom [19] were shown to interact with $\alpha_2\beta_1$, and, in the case of aggrexin (or rhodocytin) with GPIb and possibly with (an)other receptor(s), which has not yet been identified. Rhodocetin was also the first example described of a snake C-type lectin where the subunits associate noncovalently and there is no disulphide bond between them. This might allow more rapid evolution by enabling subunits to exchange more rapidly. A wide range of other snake C-type lectins has been described that activate coagulation factors or affect other plasma components involved in hemostasis. These include factor IX/factor X binding proteins from *Trimeresurus flavoviridis* [20] and *Echis carinatus leucogaster* [21] venom and coagulation factor X binding protein from *Deinagkistrodon acutus* venom [22].

Bothrojaracin, is a 27 kD C-type lectin from *Bothrops jararaca* venom which has been characterized as a potent thrombin inhibitor [23]. Bothrojaracin binds to exosites I and II, with a dissociation constant of 0.7 nM, and influences but does not block the proteinase catalytic site. Bothrojaracin also binds the partially exposed anion-binding exosite (proexosite I) of prothrombin and blocks its activation to thrombin [24].

Botrocetin forms a bimolecular complex with vWF that is capable of binding specifically to GPIb α , a component of the GPIb-IX-V complex on the platelet membrane [25]. The venoms of several snake species in the genus *Bothrops* and at least one species in the genus *Bitis* contain an activity with the properties ascribed to botrocetin, but detailed studies have been conducted mostly with the protein isolated from *B. jararaca*.

Bitiscetin from *Bitis arietans* venom [26] forms a bimolecular complex with vWF, like botrocetin; however the binding site of bitiscetin is within the A3 domain [27]. Binding also involves the GPIb-binding site of vWF located on the A1 domain because it was inhibited by a monoclonal antibody to vWF whose epitopes are within this domain and that block binding of vWF to platelets induced by ristocetin or botrocetin.

Ligand Binding Sites

After the characterization of the first few snake C-type lectins it was generally assumed that the α - and β -subunits had separate functions, and several studies supported this conclusion. It is difficult to compare the positive and negative results from such studies because the individual subunits were prepared under a wide variety of conditions. It nevertheless seems plausible that the subunits prepared, after separation under reducing conditions, can refold if the thiol groups are not blocked, to give at least partially active structures. Under these conditions, several authors were able to show that α - and β -subunits had different binding characteristics [28]. However, the first determination of the X-ray crystal structure of a snake C-type lectin by Mizuno et al. [29] indicated that the two subunits were more tightly associated than had previously been suspected and dem-

onstrated the formation of a concave surface formed by loop-swapping between the two subunits as most likely the binding site for the ligand. This was substantiated by a recent study from the same group showing that the binding site for the Gla domain of factor X does indeed lie in this region between the two subunits of the factor X binding protein [30]. Thus, the consensus of opinion moved to a model where snake C-type lectins have essentially one binding site made up of parts of both subunits.

While this model could be valid for some snake C-type lectin interactions with single ligands, it soon became clear that this is not always the case and, in fact, more complex situations may be commoner. One of the first examples of a C-type lectin interacting with two different ligands was echicetin [31]. As mentioned above, echicetin was one of the first snake C-type lectins interacting with platelet receptors to be identified and characterized. It was shown to interact with GPIb and to inhibit GPIb-related platelet functions [9]. Since then C-type lectins with similar properties were identified in a variety of species of snakes [4, 10, 13, 32]. The first indication that echicetin binds to components other than GPIb came from the observation that echicetin induced platelet agglutination in platelet-rich plasma but not with washed platelets. This suggested that there is a component in plasma that is recognized by echicetin and can cross-link echicetin bound to GPIb to link platelets together. Indeed, this component was identified as a subgroup of IgM κ and can be used to affinity-purify echicetin [31]. The results from earlier studies with isolated subunits suggest that the β -subunit is probably involved in binding to GPIb which might indicate (from steric considerations alone) that the α -subunit is mainly involved in binding IgM κ .

Another example of a snake C-type lectin that binds to more than one ligand is alboaggregin A [4, 33–35]. The situation is slightly more complicated here because this is a dimeric heterodimer or tetramer and probably binding sites are present on both heterodimers. Alboaggregin A was first identified as a GPIb-binding protein from *Trimeresurus albolabris* venom as well as alboaggregins B and C [33]. However, unlike the two others it showed much stronger activating effects on platelets. At first this was ascribed to the tetrameric structure, whereas alboaggregin B is simply heterodimeric, leading to more clustering of receptors and hence a stronger signal [4, 34]. However, the fact that the tyrosine phosphorylation pattern in platelets activated by alboaggregin A was different from that in platelets activated by alboaggregin B pointed to the involvement of other receptors. Investigation of the platelet receptors which alboaggregin A binds to clarified this issue by showing that not only GPIb but also GPVI is involved [35, 36]. Activation of platelets by clustering GPVI is well known as the mechanism used by collagen, collagen-related peptides and convulxin, also a snake C-type lectin. It is intriguing that of the four chains making up alboaggregin A, presumably two α (AlboA chains 1 and 2)- and two β (AlboA chains 3 and 4)-chains, two have sequences that resemble the α - and β -subunits of convulxin (2 and 4) whereas the other two (1 and 3) more closely resemble α - and β -subunits of C-type lectins that interact with GPIb [6].

Another example of a C-type lectin with multiple ligands is aggregin (or rhodocytin) [17, 37]. This was originally reported to be $\alpha_2\beta_1$ -specific and not to interact with GPIb [17, 37]. Later studies indicated that both $\alpha_2\beta_1$ and GPIb but not GPVI were involved [38, 39]. On the other hand, platelets genetically lacking $\alpha_2\beta_1$, where GPIb was also removed proteolytically, still showed the same reactivi-

ty with this C-type lectin, suggesting that yet another receptor played a critical role [40].

There are other examples of C-type lectins which were thought to have a simple monovalent activity, but which now appear more complex. A classic example of these is botrocetin, mentioned above. This was thought to cause vWf to bind to GPIb by binding to the A1 domain and changing its configuration to an 'activated' state. More recent work based upon X-ray crystallographic structure determination [41] indicates that this may not be the whole story and that following initial binding between botrocetin/A1 domain and GPIb, botrocetin also engages a second binding site with GPIb and so may link vWf and GPIb together physically.

In the case of bothrojaracin, it is also not clear whether only one binding site is adequate to inhibit both the exosites on thrombin or, again, if two independent bind sites on the C-type lectin are necessary [42]. In order to elucidate this and other problems of C-type lectin-binding sites it will be necessary to have more structural information based on X-ray crystallographic data.

Structure-Function Relationships

Modelling the structure of snake C-type lectins has been a fairly useful approach to establishing putative binding sites on their surfaces based on the premise that interactions with ligands involve electrostatic as well as shape-matching interactions. Comparison of linear sequence data is not always a very helpful indication of similarities in binding ligands for the simple reason that even where ligands are shared between two C-type lectins, for example, from different snake species, it does not mean that the binding site on the ligand is identical. Species differences between the sequence and structure of the target

ligand also need to be considered. If the two snake species have a different distribution of prey species then evolution will optimize C-type lectin/prey ligand interactions for those sequences which are most encountered or which offer the snake the best prey mass/venom protein relationship. Analysis of this situation is complicated by the fact that it is the whole venom that is optimized by evolution and not just the single molecular species but the principle holds nevertheless. Why does one venom contain a number of components, including C-type lectins which can bind to the same ligand and may indeed compete for binding sites? A plausible answer is that again we are dealing with a kind of combinatorial library that is being screened constantly by another library, namely the molecules present in the various prey species. Venom components interact with a wide variety of other cells, endothelial cells, smooth muscle cells, nerve cells, and not only with plasma proteins or platelets in blood, despite these being prime targets. Even if specific components may appear to compete when tested against platelets, they may have individual advantages against other cells in particular prey species.

There are a variety of C-type lectins which are generally considered to interact with a single platelet ligand. A typical example is convulxin, thought to be specific for GPVI. However, even in this case a number of questions can be asked. One of these is whether convulxin has two different binding sites for GPVI with different affinities [43]. An additional possibility is that convulxin has (a) binding site(s) for other convulxin molecules [44]. Whether these are always present or are exposed after binding to GPVI are additional questions that have not been clearly resolved. It may be optimal in terms of amount of venom to attain maximum platelet activation to deliver (or assemble) a few large multimeric structures to the platelet surface rather than a larger number of smaller multimers. These questions can only be addressed by a physico-chemical approach when recombinant C-type lectin molecules with systematically mutated binding sites become available.

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