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Crystal Structure of the Bovine Lactadherin C2 Domain, a Membrane Binding Motif, Shows Similarity to the C2 Domains of Factor V and Factor VIII

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Abstract

Lactadherin, a glycoprotein secreted by a variety of cell types, contains two EGF domains and two C domains with sequence homology to the C domains of blood coagulation proteins factor V and factor VIII. Like these proteins, lactadherin binds to phosphatidylserine (PS) containing membranes with high affinity. We determined the crystal structure of the bovine lactadherin C2 domain (residues 1 to 158) at 2.4 Å. The lactadherin C2 structure is similar to the C2 domains of factors V and VIII (rmsd of Cα atoms of 0.9 Å and 1.2 Å, and sequence identities of 43% and 38%, respectively). The lactadherin C2 domain has a discoidin-like fold containing two β-sheets of five and three anti-parallel β-strands packed against one another. The N- and C-termini are linked by a disulfide bridge between Cys1 and Cys158. One β-turn and two loops containing solvent exposed hydrophobic residues extend from the C2 domain β-sandwich core. In analogy with the C2 domains of factors V and VIII, some or all of these solvent-exposed hydrophobic residues, Trp26, Leu28, Phe31, and Phe81, likely participate in membrane binding. The C2 domain of lactadherin may serve as a marker of cell surface phosphatidylserine exposure and may have potential as a unique anti-thrombotic agent.

Keywords

Crystal structure; C2 domain; lactadherin

INTRODUCTION

Lactadherin (also known as MFG-E8) is secreted by mammary epithelial cells ¹, epididymal epithelial cells ², vascular cells ³ and activated macrophages ⁴. In mice the protein is present as two splice variants designated MFG-E8L and MFG-E8S ⁴. The shorter protein contains two epidermal growth factor-like (EGF) domains, EGF1–EGF2, followed by two lectin-type C domains, C1–C2, while the longer form has a proline/threonine-enriched domain between the EGF2 and C1 domains. EGF2 contains an Arg-Gly-Asp sequence and binds to the integrins α_vβ₃ and α_vβ₅ while the C2 domain binds to membranes that contain phosphatidylserine ^{5; 6}. The affinity of the lactadherin C2 domain for phosphatidylserine may be modulated by the proline/threonine-enriched domain ⁴. Lactadherin plays an important role as an opsonin bridging apoptotic cells through its C2 domain with phagocytes through its EGF2 domain ⁷. Lactadherin null mice have many unengulfed apoptotic cells in the germinal centers of the

spleen, develop an autoimmune disease similar to lupus and have impaired involution of mammary glands 8; 9

Like lactadherin, blood coagulation proteins factor V and factor VIII have two C domains and also bind to phosphatidylserine-containing membranes through their C2 domains^{10; 11; 12}. Lactadherin competes with factors VIII and V for phospholipid binding sites on phosphatidylserine-containing membranes and inhibits blood coagulation *in vitro*^{13; 14}. Because of its potential as an alternative to annexin V as a marker of phosphatidylserine exposure on cell surfaces and as an anticoagulant, the structure of the lactadherin C2 domain and its relationship to the C2 domains of factors V and VIII is of biological interest. We have now solved the three dimensional structure of the C2 domain of bovine lactadherin expressed in *Pichia pastoris*. The polypeptide backbone shows marked structural similarity to the C2 domains of factor V and factor VIII, and provides a strategy for understanding the nature of the membrane binding properties of this family of proteins.

RESULTS

Model reliability and overall structure of lactadherin C2 domain

The recombinant bovine lactadherin C2 domain (residues 1-158) was expressed in *Pichia pastoris* as a secreted protein. The purified protein yielded a single band upon SDS gel electrophoresis, and its N-terminal amino acid sequence was confirmed by automated Edman degradation. The protein was crystallized and the structure was determined by X-ray crystallography to 2.4 Å. The current model consists of 4 molecules of the bovine lactadherin C2 domain in the asymmetric unit of the crystals, and 119 loosely attached solvent molecules (Table 1). The model was refined against a high quality X-ray diffraction data set (R_{merge} of 0.06) at 2.4 Å with R_{cryst} and R_{free} values of 0.235 and 0.274, respectively (Table 1). Most of the residues are clustered in energetically favorable (81.3%) or allowed (15.9%) regions of the Ramachandran plot. The final Fo-Fc electron density map is flat and featureless, indicating that most of the electron density had been assigned. All four molecules in the asymmetric unit are structurally identical, with rmsd values between 0.19 Å and 0.26 Å. These data suggest that the conformation of the lactadherin C2 domain is relatively rigid and not affected by crystal packing.

The lactadherin C2 domain has a discoidin-like fold with a jelly-roll beta barrel motif (Fig. 1A). This structure has been designated the F5/8 type C domain. The lactadherin C2 domain contains eight major antiparallel strands arranged in two-sheets of five and three strands packed against one another (Fig. 1A). A β -turn and two loops (loop 1 residues 25-32, loop 2: residues 43-47, and loop 3, the β -turn: residues 80-83), all containing solvent-exposed hydrophobic residues, extend from the open end of the barrel (Fig. 1A and B). The factor V and factor VIII C2 domains contain similar loops with loop 3 being a β -turn. The N- and C-terminal regions are linked by a disulfide bridge (Cys 1 and Cys 158). The alignment of the C2 domain amino acid sequences from lactadherin of different species and from factor V and factor VIII (Fig. 2) shows that C2 domain sequences are highly conserved. The overall structure of the lactadherin C2 domain is similar to the C2 domains of factor V and factor VIII, with rmsds for C_{α} atoms of 0.9 Å and 1.2 Å, respectively (Fig. 1A and B)^{15; 16}. The most significant differences in structure occur in three loop regions, loop 1 and the β -turn (loop 3) at the open end of the barrel and loop 4 (residues 106-112) near the closed end of the barrel (Fig. 1A and B).

The recombinant lactadherin C2 domain exists as a monomer in solution as indicated by its gel filtration elution time (data not shown). However, the packing analysis of the lactadherin C2 molecules in the crystal showed that two lactadherin molecules form a dimer with a small contact area. Dimerization occurs through the antiparallel pairing of part of a β -strand (residues 144-146 of β -strand S7, Fig. 1A). In one of two factor V C2 domain structures (PDB code

1czv)¹⁵, the C2 domain also formed a dimer, but through antiparallel pairing of the S6 β -strand. These results suggest the possibility of dimerization of C2 domains under circumstances in which molecules are in close proximity.

Membrane binding mechanisms

The C2 domain of lactadherin binds phosphatidylserine-containing membranes with an affinity similar to full-length lactadherin ($K_d=3.3$ nM), suggesting that the C2 domain is the primary site of lactadherin membrane binding¹⁴. The similarities in the structures of the C2 domains of lactadherin, factor V and factor VIII, the similar phospholipid composition requirements for interaction of these proteins with membranes and the ability of the lactadherin C2 domain to compete with factors V and VIII for membrane binding¹³ suggest similar mechanisms for interaction of these domains with membranes. The lactadherin C2 domain contains a group of solvent-exposed hydrophobic residues: Trp26, Leu28, Phe31 in loop 1 and Phe81 in loop 3 (Fig. 1A). Like the lactadherin C2 domain, factor V and factor VIII C2 domains both exhibit 1 solvent-exposed hydrophobic residues in similar positions as those in lactadherin C2 domain..

Mutational analyses of the factor VIII C2 domain have implicated loop 1 residue Phe2199 and loop 3 residues Leu2251 and Leu2252 in membrane binding (Fig. 1A)^{17; 18}. Similarly, mutational analyses of the factor V C2 domain implicated loop 1 residues Trp2063 and Trp2064 in membrane binding (Fig. 1B)^{19; 20; 21; 22}. It is thus likely that some or all of the loop 1 and loop 3 solvent-exposed hydrophobic residues of lactadherin support membrane binding of the lactadherin C2 domain.

The factor V C2 domain was crystallized in two different forms¹⁵. In the “open” conformation, the two consecutive tryptophan residues (Trp2063 and Trp2064) are fully exposed to solvent. In the “closed” conformation loop 1 is displaced significantly closing the groove between loops 1 and 3 leading to a continuous hydrophobic patch formed by the side chains of Trp2063, Trp2064 and Leu2116 (although mutagenesis studies indicated that mutation of the Leu2116 to Ala had no effect on factor V C2 membrane binding^{15; 23}, Fig. 1B). It has been suggested that the “closed” form of the factor V C2 domain represents the circulating form of factor V while the “open form” represents the membrane-bound form of the domain¹⁵. However a recent molecular dynamics study concludes that energetically either conformation is suitable for membrane binding²⁴. The backbone conformations of loop 3 in lactadherin, factor V (open or closed conformation) and factor VIII are quite similar. In contrast, there is greater variability in the conformations of loop 1 in the C2 domains of lactadherin, the open and closed form of the factor V C2 and factor VIII (Fig. 1A and B). Compared with the factor V C2 domain, the lactadherin C2 domain has a longer loop 1 due to a two residue insertion (Fig. 2). As a consequence of this insertion, hydrophobic residues from lactadherin loops 1 and 3 form a continuous hydrophobic patch (Fig. 3A and B)

The solvent accessible area of the four solvent-exposed hydrophobic residues in the lactadherin C2 domain of 491 Å² is similar to the area formed by the solvent exposed hydrophobic residues in loops 1 and 3 of the factor VIII C2 domain (449 Å², Fig. 3B and D) but the solvent exposed hydrophobic residues in factor VIII loops 1 and 3 form independent hydrophobic surfaces (Fig. 3C and D). In the factor V C2 domain Trp2063 and Trp2064 in loop 1 provide an exposed hydrophobic area of 376 Å²²³. Phospholipid binding studies with lactadherin and its C1C2 and C2 domains suggest that the lactadherin C2 domain encompasses the complete membrane binding site of this protein⁶. In contrast, in factor V three domains, A3, C1 and C2, have been implicated in membrane binding^{10; 20; 21; 23; 25; 26; 27}. However, the crystal structure of activated protein C-inactivated factor Va, factor Vai, suggests that the A3 domain is not in a favorable position for interacting with the membrane²⁸. This model does indicate that the factor V C1 domain is likely to be in proximity to the membrane surface and may contribute to membrane binding. Consistent with its position in the structure of factor Vai, C1 domain

mutagenesis experiments indicate that Tyr72 and Leu73 in spike 3 of this domain participate in membrane binding, generating a solvent exposed hydrophobic patch of 344 Å². In contrast, a model of the factor VIII heterodimer, based on a 3-dimensional electron density map of membrane-bound factor VIII derived from electron crystallography, suggests that only the C2 domain of the protein is in contact with the membrane^{29; 30}. Studies of factor VIII membrane binding have yielded mixed results with some indicating that the C2 domain encompasses the complete membrane binding site³¹ while others suggest a role for the C1 and/or A3 domains^{32; 33}. Direct mutagenesis experiments have implicated Met2199 and Phe2200 in loop 1 and Leu2251 and Leu2252 in loop 3 of the factor VIII C2 domain in membrane binding^{17; 18}. While naturally occurring mutations in the C1 and C2 domains of factor VIII have been identified, none have been definitively associated with impairment of membrane binding^{23; 34}. Differences in expression of solvent-exposed hydrophobic surfaces on the C2 domains may contribute to the differences in membrane binding affinity among these proteins. In addition, contributions to binding from C2 domain:phosphatidylserine headgroup interactions as well as differences in participation of other domains in membrane binding likely play a role.

While lactadherin membrane binding, like that of factor V and factor VIII, is Ca²⁺-independent, requires phosphatidylserine with a preference for the L over the D headgroup, and prefers membranes of high curvature, there are significant differences in membrane binding among these proteins. Like factor V but in contrast to factor VIII, lactadherin binds to many binding sites on membranes with a phosphatidylserine content of less than 4%.

Phosphatidylethanolamine enhances binding of factor V and factor VIII to membranes with lower phosphatidylserine content but has no effect on lactadherin membrane binding^{14; 35; 36; 37; 38; 39; 40}. Whether based on differences in size and distribution of surface exposed hydrophobic patches or other aspects of protein:membrane interaction such as the nature of the phosphatidylserine head group binding sites, it is clear that although they share significant homology in their membrane binding domains lactadherin, factor V and factor VIII recognize somewhat different binding sites on phosphatidylserine bearing membrane surfaces. Direct competition studies indicate that lactadherin competes more effectively for factor VIII binding than for factor V binding to surfaces coated with an optimal ratio of phosphatidylserine:phosphatidylethanolamine:phosphatidylcholine¹³.

DISCUSSION

Factor Va and factor VIIIa serve as activated cofactors in the prothrombinase and tenase enzyme complexes of blood coagulation respectively. In these complexes factor Va binds with factor Xa and factor VIIIa binds with factor IXa on phospholipid membranes in a process dependent on phosphatidylserine and Ca²⁺ ions. Factor X and factor IX are members of the vitamin K-dependent family of blood coagulation proteins that contain another important membrane binding motif, the γ-carboxyglutamic acid-rich (Gla) domain. These domains, found at the N-termini of vitamin K-dependent blood coagulation and regulatory proteins, contain ten to twelve γ-carboxyglutamic acid residues, fold into their membrane binding conformations in a Ca²⁺-dependent manner and require phosphatidylserine for membrane binding⁴¹. The structure of the Gla domain of a vitamin K-dependent protein, prothrombin, has been elucidated in complex with lysophosphatidylserine⁴². In this structure the phosphatidylserine head group is anchored by its interaction with protein-bound Ca²⁺, whereas calcium is not involved in C2 domain interaction with phospholipids. However, other aspects of phosphatidylserine-Gla domain interaction bear similarity to proposed modes of binding of C2 domains with phosphatidylserine-containing membranes. In the presence of lysophosphatidylserine the ω-loop of the prothrombin Gla domain assumes a conformation in which hydrophobic residues, Phe5, Leu6 and Val9, are solvent-exposed. Several lines of evidence indicate that the ω-loop region of the Gla domain inserts into the interfacial membrane region⁴³. In addition the glycerol backbone and phosphate moiety of the lipid are stabilized by interaction with

positively charged residues close to the ω -loop. While neither factor V nor factor VIII compete with Gla domain-containing proteins for binding to phospholipids membranes, lactadherin does compete for membrane binding of the vitamin K-dependent proteins¹³. Thus lactadherin membrane binding sites appear to have less stringent requirements than either the C2 domain or Gla domain-containing blood coagulation proteins.

Annexin V is a member of a family of membrane binding proteins and is widely used to identify cells bearing exposed phosphatidylserine. However the membrane binding properties of the lactadherin C2 domain and annexin V are quite different. In contrast to lactadherin, annexin V binds better to membranes of low surface curvature, is Ca^{2+} -dependent and binds to membranes containing acidic phospholipids other than phosphatidylserine. Recent studies define more precisely the difference in exposed phosphatidylserine concentration required for lactadherin versus annexin V binding^{44; 45}. Lactadherin binds well to membranes with as little as 0.5% phosphatidylserine independent of phosphatidylethanolamine concentration, while annexin V at physiologic Ca^{2+} concentrations binds to membranes with a threshold phosphatidylserine concentration of about 8% in the presence of 2% phosphatidylethanolamine and at about 2.5% phosphatidylserine at 10% phosphatidylethanolamine⁴⁴. For both lactadherin and annexin V binding appears to be maximal at about 8% phosphatidylserine regardless of the concentration of phosphatidylethanolamine. Thus, lactadherin may provide a more sensitive probe of cell surface exposure of phosphatidylserine than annexin V.

The requirements for lactadherin membrane binding are more closely related to those of factor V and factor VIII than those of annexin V. Because lactadherin also has the ability to compete with vitamin K-dependent proteins for membrane binding it is not surprising that it is a more effective anticoagulant *in vitro* than annexin V¹³. This difference would likely make lactadherin a better marker of sites of active thrombus formation *in vivo* as well. We have recently observed in an intravital model of thrombus formation in mice that fluorescently labeled prothrombin fragment 1 accumulates at the site of a laser-induced vessel wall injury immediately after the insult to the vessel wall and accumulates into the growing platelet thrombus⁴⁶. In contrast, fluorescently labeled annexin V does not appear to accumulate at the injury site or in the platelet thrombus but slowly accumulates on the injured vessel wall away from the site of injury⁴⁶. We anticipate that the behavior of fluorescently labeled lactadherin or its C2 domain would closely mimic that of prothrombin fragment 1 as a marker of thrombus formation in this mouse model. Recent elucidation of the important role of lactadherin in phagocytosis of apoptotic cells and indications that either the absence or overexpression of this protein can induce autoimmune disease mitigate against efforts to develop lactadherin as an anticoagulant⁴⁷. Use of the isolated lactadherin C2 domain may provide a viable strategy for anticoagulant development as the C2 domain cannot provide the bridging function of the whole protein but the consequences of high concentrations of this protein in the blood circulation *in vivo* have not been studied.

MATERIALS AND METHODS

Construction of the lactadherin C2 domain expression vector and expression in *Pichia pastoris*

The lactadherin C2 domain coding region was amplified by PCR using an EST clone (10 Bov 19 J4), a gift from Tad Sonstegard (Bovine Functional Genomics Laboratory, Animal and Natural Resources Institute). The specific primers, agttgtacgtatgcactgaaccctaggcct (forward, SnaBI restriction site underlined) and tataagcggccgcctaacagcccagcagct (reverse, Not I restriction site underlined), were used to amplify the lactadherin C2 domain, residues 1-158 (or 270-427 in SwissProt numbering). The amplified fragment was digested with *Sna*BI and *Not* I and was ligated to pPIC9K vector (Invitrogen, Inc) to generate the expression vector pPIC9K-lacC2. The expression vector was linearized with *Bgl* II and transformed into *P.*

pastoris by electroporation (1500 V, 25 μ F, 2 for 4.5 ms). For expression screening, transformants were incubated in 5 ml BMGY medium (1% yeast extract, 2% tryptone, 1% glycerol, and 100 mM potassium phosphate, pH 6.0) to an OD₆₀₀ of 5. Cells were pelleted and re-suspended in 10 ml BMMY medium (1% yeast extract, 2% tryptone, 1% methanol, and 100 mM potassium phosphate, pH 6.0) to an OD₆₀₀ of 1 and induced every 24 h with 0.5% (v/v) methanol.

Protein expression and purification

A single colony of the expression strain was inoculated into BMGY medium and grown at 28°C with shaking until the culture reached an OD₆₀₀ greater than 5. The culture was harvested by centrifugation at 4000 $\times$ g for 5 min. To induce expression, the supernatant was decanted from the cell pellet and the pellet was re-suspended in BMMY medium at an OD₆₀₀ of 1. Methanol (100%) was added to a final concentration of 1% every 24 h to maintain induction. The culture supernatant was applied to a SP Sepharose column (Pharmacia) and eluted with 20 mM Bicine, 500 mM NaCl, pH 7.3. The eluted fraction was concentrated and further purified on a Superdex 75 column (Pharmacia). The purified protein was collected and concentrated to 5–8 mg/ml for crystallization experiments. The typical expression yield was 50 mg/L. The N-terminal amino acid sequence was determined using an Applied Biosystems 891 Procise HRT protein sequencer.

Protein crystallization and structure determination

The lactadherin C2 domain crystals were grown at room temperature using the dialysis method and a precipitant solution of 15% PEG4K, 20% glycerol, 50 mM HEPES, pH 7.5. Cubic crystals were obtained after 48 hours. X-ray diffraction data were collected on the National Synchrotron Light Sources (NSLS) beam lines X12C and X29 (Table 1). All the diffraction data were indexed and processed with HKL2000⁴⁸. The structure was determined by molecular replacement using AMORE⁴⁹ and a homology model of the lactadherin C2 domain based upon its primary sequence and templates from the C2 domain of factor Vai (1sdd, 1czs) and factor VIII (1d7p) (SwissModel Database)⁵⁰. The model was iteratively refined with CNS and manually adjusted using the graphics program O until convergent^{51; 52}. Four lactadherin C2 molecules exist in the asymmetric unit of the crystal. No non-crystallographic symmetry (NCS) among these four lactadherin molecules was used during the refinement process. The areas of solvent accessible surfaces was calculated using CCP4 AREAIMOL with a probe radius of 1.7 Å⁵³.

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Fig. 1.

Superposition of the bovine lactadherin C2 domain structure with the C2 domains of factor VIII and factor V. A. The lactadherin C2 domain (cyan) shows global similarity with the core of the factor VIII C2 domain (PDB 1CZV, orange). B. Loops 1 and 3 of the factor V C2 domain in its open (PDB 1CZT, green) and closed (PDB 1CZV, orange) conformation are compared with loops 1 and 3 of the lactadherin C2 domain (cyan). The core beta strands are numbered in reference to that of human factor V C2 domain¹⁵. Figures 1 and 3 were made with Pymol 54.

Fig. 2.

Sequence alignment of C2 domains. The sequences of the C2 domains of bovine, pig, rat, mouse and human lactadherin are aligned with the C2 domains of human factor V (FV) and human factor VIII (FVIII). Residues that are homologous or well conserved in all the C2 domains are shown in red. Residues that are homologous or well conserved only in lactadherin C2 domains are shown in green. Loops 1, 2 and 3 of the bovine lactadherin C2 domain are highlighted in gray. Residue numbers are for the isolated C2 domains irrespective of the residue number in the whole protein from which they are derived. Numbering of the C2 domains begins at the amino-terminal cysteine. Residue numbers for the C2 domains of factors V (FV_C2#) and VIII (FVIII_C2#) within the full length proteins are indicated.

Fig. 3. Surface charge distribution of bovine lactaherin C2 domain (A and B) and factor VIII C2 domain (C and D, residues 2171-2330, PDB entry 1D7P) in two orthogonal views. (A and C: oriented as in Fig 1 A; B and D: rotated 90° from A and C; view from cell membrane). CPK coloring code.

Table 1
Data collection and refinement statistics for lactadherin C2

X-ray diffraction data	
Size of crystal, μm	$70 \times 70 \times 100$
Cell parameters, $\text{\AA}$	108.12, 107.79, 82.75
Space group	$P2_12_12$
Temperature	-170°C
Diffraction limit, $\text{\AA}$	2.15
No. of unique reflections	51324 (3797) [#] (5268) [*]
Data redundancy	4.2 (2.5) [#] (4.4) [*]
Data completeness	95.6% (71.8%) [#] (99.7%) [*]
Rmerge	0.087 (0.916) [#] (0.566) [*]
I/sigma	16.0 (0.78) [#] (2.6) [*]
Refinement	
Resolution range, $\text{\AA}$	50–2.4 $\text{\AA}$
R_{cryst}	0.235
R_{free}	0.274
No. of mol. in asymmetric unit	4
No. of protein atoms	5100
Total No. of solvent atoms	119
Ramachandran distribution (% core, allowed, generous, disallowed)	81.3, 15.9, 1.1, 1.6
R.m.s bonds, angles	0.0006 $\text{\AA}$, 1.46°
Average temperature values (all atoms)	44.4 $\text{\AA}^2$

[#] Note: this data is for highest resolution shell (2.23–2.15 $\text{\AA}$);

^{*} this data is for resolution shell (2.52–2.42 $\text{\AA}$).