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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

Lin Lin, Qing Huai, Mingdong Huang, Bruce Furie and Barbara C. Furie*

Division of Hemostasis and Thrombosis, Beth Israel Deaconess Medical Center and Department of Medicine Harvard Medical School Boston, MA 02215, USA

*Corresponding author

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 C_α 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 antiparallel 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; factor V; factor VIII

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 $\alpha_v\beta_3$ and $\alpha_v\beta_5$ 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.[10–12] 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.

E-mail address of the corresponding author: bfurie@bidmc.harvard.edu

Results

Model reliability and overall structure of lactadherin C2 domain

The recombinant bovine lactadherin C2 domain (residues 1–158) was expressed in *P. 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 four 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 F_o–F_c 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 (Figure 1(a)). 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 (Figure 1(a)). 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 (Figure 1(a) 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 (Cys1 and Cys158). The alignment of the C2 domain amino acid sequences from lactadherin of different species and from factor V and factor VIII (Figure 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 (Figure 1(a) 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 (Figure 1(a) 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, Figure 1(a)). 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.

Table 1. Data collection and refinement statistics for lactadherin C2

X-ray diffraction data	
Size of crystal (μm)	70×70×100
Cell parameters (Å)	108.12, 107.79, 82.75
Space group	$P2_12_12$
Temperature (°C)	−170
Diffraction limit (Å)	2.15
No. of unique reflections	51,324 (3797)[a] (5268)[b]
Data redundancy	4.2 (2.5)[a] (4.4)[b]
Data completeness (%)	95.6 (71.8)[a] (99.7)[b]
R_{merge}	0.087 (0.916)[a] (0.566)[b]
I/sigma	16.0 (0.78)[a] (2.6)[b]
Refinement	
Resolution range (Å)	50–2.4
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 (Å, °)	0.006, 1.46
Average temperature values (all atoms)(Å^2)	44.4

[a] These data are for the highest resolution shell (2.23–2.15 Å).
[b] These data are for the resolution shell (2.52–2.42 Å).

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 binding.[14] 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 binding[13] 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 (Figure 1(a)). Like the lactadherin C2 domain, factor V and factor VIII C2 domains both exhibit loop 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 (Figure 1(a)).[17,18] Similarly, mutational analyses of the factor V C2 domain implicated loop 1 residues Trp2063 and Trp2064 in membrane binding (Figure 1(b)).[19–22] It is thus likely that some or all of the loop 1 and loop 3 solvent-exposed hyrdrophobic residues