ATP-Binding Cassette Transporter B4 Anchors the Cell Adhesion Molecule DdCAD-1 to Cell Membrane in *Dictyostelium discoideum*

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Abstract In *Dictyostelium*, soluble cell adhesion molecule, DdCAD-1, regulates cell–cell interaction through an unknown anchoring protein on the plasma membrane. Far western blot analysis using different probes revealed that the potential DdCAD-1 interacting protein was between 64 and 98 kDa. To isolate and identify the anchoring protein, GST-DdCAD-1 and anchoring protein were cross-linked in vivo by chemical cross-linker and stable protein complex was isolated by co-immunoprecipitation assays. The protein cross-linked to DdCAD-1 was extracted from the gel slice and trypsinized. The peptides were subjected to analysis by mass spectrometry, which showed that the putative anchoring protein belongs to ATP-binding cassette transporter family.

Keywords Anchoring protein · Co-immunoprecipitation · Cross-linking · DdCAD-1 · ABC transporters

Introduction

As a simple social amoeba, one of the essentials of *Dictyostelium* development is the formation of the multicellular structures through the interaction of single cells. In this process, several cell adhesion molecules (CAMs) are expressed to regulate cell–cell contact and cell movement.

As the first expressed CAM, DdCAD-1 is synthesized at the onset of development by *cadA* gene [1]. Although DdCAD-1 has been identified to play its function as a cell adhesion molecule on plasma membrane, the sequence analysis revealed that it was a soluble protein, because N-terminal of DdCAD-1 does not contain a hydrophobic signal peptide or transmembrane domain [2]. Subcellular localization shows that DdCAD-1 was distributed in cytoplasm in the initial of development and is transferred to plasma membrane quickly within 3 h [3]. DdCAD-1 contains two domains, N-terminal domain has homophilic binding function, while C-terminal domain is responsible for tethering DdCAD-1 on cell membrane [4, 5]. DdCAD-1 is transported to cell surface by contractile vacuoles [6], and time-lapse images show that some of DdCAD-1 is imported to contractile vacuoles by membrane invagination [7]. Moreover, DdCAD-1 can form capping structure on the cell surface by antibodies cross-linking experiment, which also demonstrates that DdCAD-1 is anchored on cell surface by certain membrane receptor(s) [8].

To investigate the anchoring protein which tethers DdCAD-1 to cell membrane, in vitro experiments were performed using far western blot to examine the location, and exogenous DdCAD-1 protein was cross-linked to anchoring protein by in vivo cross-linking. Then, the protein complex was isolated by co-immunoprecipitation and the anchoring protein was analyzed by mass spectrometry.

Materials and Methods

Cell Growth and Development

The DdCAD-1 mutant strain *cadA*[−] was derived from the wild-type strain AX4 by homologous recombination as

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described previously [9]. Cells were cultured axenically in HL5 medium [10]. For development, cells were collected and washed with 17 mM phosphate buffer, pH 6.4 at mid-log growth phase, and suspended in same buffer with shaking at 180 rpm, room temperature.

Expression and Purification of Recombinant DdCAD-1 Proteins

The *gst-cadA* cDNA constructs were inserted into the expression vector pGEX. The GST-DdCAD-1 and GST-DdCAD-1-N (fusion between the N-terminal domain of DdCAD-1 and GST) recombinant proteins were expressed in *E. coli* strain BL21(DE3), IPTG with a final concentration of 1 mM was used to induce the expression. After sonication and centrifugation, recombinant proteins were released. GST-DdCAD-1 was adsorbed to glutathione Sepharose beads and then eluted by 50 mM reduced glutathione (BioShop Inc., CA) [2]. His₆-DdCAD-1 was constructed by ligating cDNA of DdCAD-1 sequence into pET32a-derived expression vector. The construct was further transformed into *E. coli* strain BL21 (DE3) and protein expression was induced by adding IPTG to a final concentration of 1 mM. Proteins were purified using Ni-NTA resin (Qiagen Inc., Valencia, CA). His₆-tagged DdCAD-1 was bound onto the Ni-NAT beads through the chelating interaction between histidine residues and the nickel ions immobilized on beads. Elution buffer containing 250 mM imidazole was used to elute the bound His₆-DdCAD-1 from beads [7].

Isolate the Plasma Membrane by Aqueous Two-Phase Polymer System

Cells were collected and broken by tissue homogenizer in cold 17 mM phosphate buffer, pH 6.4, cell homogenate was spun down at 5,900 g for 15 min to pellet the unbroken cells, nuclei and large plasma membrane fragments. Pellets were resuspended in the upper phase solution at 10⁸ cell equivalent/ml, and equal volume of the lower phase solution was added. Pellets and two-phase solution were mixed well by shaking and then spun down at 5,900 g for 10 min at 4 °C. Membrane was collected at the interface of two-phase system and washed twice with cold 17 mM phosphate buffer by 8,000 g for 10 min. Two-phase solution was prepared as described previously [11], 40 g Dextran 500 (lower phase) and 30.9 g polyethylene glycol 6,000 (upper phase) were dissolved in 600 ml H₂O. 100.5 ml of 0.5 M NaH₂PO₄ and 46.2 ml of 0.5 M Na₂HPO₄ were added to the mixture. The mixture was transferred into a 2 l separatory funnel, PEG and Dextran were separated out in the cold.

Far Western Blot Analysis

Plasma membranes were isolated from *cadA*[−] cells at different stages of development. Membrane proteins were solubilized in 2 % SDS and reduced by boiling in 5 % β-mercaptoethanol. Nitrocellulose membrane containing plasma membrane proteins were blocked with 4 % skim milk for 1 h and then incubated with exogenous His₆-DdCAD-1 or GST-DdCAD-1 to probe the anchor protein. After overnight incubation on a rocker at 4 °C, either anti-His₆ monoclonal antibody (Cell Signaling Technology) or anti-DdCAD-1 polyclonal antibody (rabbit anti serum) was incubated with membrane and followed by secondary antibodies.

Alexa 488-Stained Exogenous DdCAD-1 Rescued *cadA*[−] Cells

Recombinant His₆-DdCAD-1 was concentrated with the Eluta tube™ Dialysis Kit (Fermentas, St. Leon-Rot, Deutschland) to a final concentration of 15 mg/ml. To label the protein, 1 mg Alexa Fluor 488 dye (Invitrogen, Eugene, Oregon, USA) was dissolved in 100 μl DMSO and then added to 1 ml His₆-DdCAD-1. The mixture was incubated on a rocker for 1 h at room temperature. Labeled protein and un-reacted labeling reagent were separated by Zeba™ Desalt Spin Columns (Pierce Biotechnology). *CadA*[−] cells were incubated with stained DdCAD-1 at 3 mg/ml with rotation on platform. After 4 h development at 180 rpm, 10⁶ cells were deposited on positively charged coverslips for 30 min, fixed with 4 % paraformaldehyde and prepared for confocal microscopy using the Carl Zeiss LSM510 microscope.

Chemical Cross-Linking

Recombinant GST-DdCAD-1 was added to *cadA*[−] cell at 1 h of development at a final concentration of 10 μg/ml which would be enough even in wide types cells [3]. After another 4 h of development, the cleavable chemical cross-linker DSP (Thermo-Fisher Scientific, Ottawa, ON) was added to a final concentration of 0.1 mM with gentle rotation (60 rpm) at room temperature [12]. After 30 min of incubation, Tris-HCl (pH 7.6) was added at a final concentration of 100 mM to quench the reaction. After 15 min, cells were washed and prepared for membrane isolation and co-immunoprecipitation.

Co-immunoprecipitation of GST-DdCAD-1-Anchored Protein Complex

After cross-linking GST-DdCAD-1 or GST-DdCAD-1-N to the cell membrane of *cadA*[−] cells, plasma membrane

was isolated and dissolved in lysis buffer [400 mM Tris, 100 mM NaCl, 0.3 % NP-40 (v/v), 0.5 % Triton X-100 (v/v)] at 4 °C overnight. Samples were centrifuged at 1000 g for 1 min and the supernatant was adjusted to 5 mg/ml. The soluble proteins (500 µl) were incubated with 40 µl Protein A Sepharose (Sigma) at 4 °C for 2 h to remove the non-specific material. The pre-cleaned beads were spun down and kept as a negative control in following analysis step. Supernatant was transferred to a fresh tube, 10 µl DdCAD-1 antibody or GST monoclonal antibody (Cell Signaling Technology) was incubated with the supernatant to a final volume of 200 µl with gentle rocking overnight at 4 °C and then 20 µl protein A beads were added to each tube. After 3 h, the beads were washed and 10 µl of beads were prepared for SDS-PAGE with or without prior reduction by boiling for 3 min in the presence of 5 % β-mercaptoethanol.

Mass Spectrometry

The DdCAD-1 anchor protein brought down in the DdCAD-1 cross-linked complex was separated by SDS-PAGE after cleavage of DSP by β-mercaptoethanol. The gel was stained with Coomassie and the band containing the putative DdCAD-1 anchor protein was excised and prepared for mass spectrometry as described previously [12]. The protein was reduced and alkylated and then digested with trypsin. The tryptic peptides were purified using Sephasil C18 resin and submitted to the Mass Spectrometry Facility of the Advanced Protein Technology Centre, the Hospital for Sick Children, for analysis. The remaining masses were submitted to the ProFound search engine and the Protein Prospector search engine (both available on the World Wide Web) for matches.

Results and Discussion

Identification of the DdCAD-1 Anchor Protein by Far Western Blot Analysis

We have previously shown that exogenous recombinant DdCAD-1 is capable of binding to the plasma membrane of *cadA*[−] cells and restore the wild-type phenotype to these cells [13], indicating the presence of a DdCAD-1 anchor protein on the cell surface despite the loss of endogenous DdCAD-1 expression. To identify the anchor protein, both His-tagged and GST-tagged recombinant DdCAD-1 proteins were expressed in bacteria and purified (Fig. 1). Plasma membrane protein derived from *cadA*[−] cells at 0 and 4 h of development were separated by SDS-PAGE and immobilized on nitrocellulose membranes for far western blot analysis. The membranes were probed with either

His₆-DdCAD-1 or GST-DdCAD-1, followed by immunostaining of the bound protein. Both anti-His₆ mAb and rabbit anti-DdCAD-1 antibody were used in order to rule out non-specific staining by these antibodies (Fig. 2). In all cases, specific bands between 64 kDa and 98 kDa were visualized at 4 h lane but not at 0 h. More protein bands appeared in Fig. 2a, c than that in Fig. 2b which may due to the nonspecific binding of DdCAD-1 polyclonal antibody.

Chemical Cross-linking of Recombinant DdCAD-1 with its Endogenous Anchor Protein

To confirm that DdCAD-1 was capable of binding to a cell surface anchor protein, GST-DdCAD-1 was conjugated with the fluorescent dye Alexa-488. Confocal microscopy showed the binding of the fluorescent protein to the surface of >90 % of *cadA*[−] cells, some of them even formed capping structure (arrow in Fig. 3a), which can be explained by the homophilic binding function of DdCAD-1 [5]. Although DdCAD-1 can bind to *cadA*[−] cells membrane easily, we were unsuccessful in isolating anchoring protein by DdCAD-1 directly, which suggested that the affinity between DdCAD-1 and anchoring protein was low, they might bind to each other by non covalent bond. Therefore, we chose chemical cross-linker DSP to conquer this problem. DSP contains two amine-reactive esters which can react with primary amines in proteins if they are close enough and form stable amide bonds, it also has a disulfide bond and is cleavable in the presence of reducing agents like β-mercaptoethanol (Fig. 3b). After cross-linking and membrane isolation, membrane proteins were detected by western blot and far western blot. In western blot data, GST-DdCAD-1 was present around 50 kDa (molecular weight of DdCAD-1 is around 24 kDa) (arrow in Fig. 3c. lane 1), in far western blot data, the potential interesting band appeared between 64 and 98 kDa (arrow in Fig. 3c lane 2), consistent with the data shown in Fig. 2. These results confirmed that GST-DdCAD-1 and anchoring proteins formed complex and were presented in isolated *cadA*[−] cells membrane.

Isolation of DdCAD-1-Anchoring Protein Complex by Co-immunoprecipitation

In order to determine the identity of the anchor protein, the cross-linked complex was subjected to co-immunoprecipitation. When DdCAD-1 polyclonal antibody and protein A beads were used to immunoprecipitate the complex, there was a band between 64 and 98 kDa in the lane of immunoprecipitated beads with β-mercaptoethanol. Same experiments were performed with different marker revealed the band was around 72 kDa (arrows in Fig. 4a), while the

Fig. 1 Protein purification. **a** The coomassie blue stained His₆-DdCAD-1, GST-DdCAD-1 and GST-DdCAD-1-N. **b** Protein blots with GST antibody or DdCAD-1 antibody or His₆-tag antibody

Fig. 2 Far western blot of *cadA*⁻ cells membrane protein. **a** His₆-DdCAD-1 (5 µg/ml) probe and DdCAD-1 antibody. **b** His₆-DdCAD-1 (10 µg/ml) probe and His₆-tag antibody. **c** GST-DdCAD-1 (5 µg/ml) probe and DdCAD-1 antibody

negative control groups did not show any band at the same place.

To eliminate the nonspecific binding site of DdCAD-1 polyclonal antibody, GST monoclonal antibody and protein A beads were adopted. Also, GST-DdCAD-1-N was used as the negative control in this experiment since DdCAD-1 is known to associate with its anchor protein via the C-terminal domain [5]. GST-DdCAD-1-N was performed same process as GST-DdCAD-1. Same band was appeared around 72 kDa in SDS-PAGE gel analysis, while no band was present in GST-DdCAD-1-N negative control (frame in Fig. 4b), confirming the specificity of the interaction. Taken together, we demonstrated that the protein located around 72 kDa should be anchoring protein of DdCAD-1.

Mass Spectrometry Identified the Anchoring Protein

To determine the identity of the anchor protein, the 72 kDa band was excised from the gel and the protein was extracted and digested with trypsin. The peptides were purified and prepared for MALDI-TOF mass spectrometry. Eight peptides were found to match with the protein ATP-binding cassette transporter B4 (ABCB4) in the *Dictyostelium* database, which is a member of the ATP-binding cassette (ABC) transporter family. The primary sequence of ABCB4 (GenBank™/EBI Data Bank with accession number DDB_G0279915) is shown with the matched peptides highlighted (Fig. 4c).

ABC transporters are members of transmembrane proteins that utilize the energy of ATP hydrolysis to carry out biological processes including translocation of various substrates across membranes [14]. In *Dictyostelium*, there exist ABC transporters that were involved in the release of signals from cells [15]. 68 members were recognized in *Dictyostelium* ABC transporter family so far [16], and members of some ABCB families were capable of transporting peptides that lack classical export signals [17]. ABCB4 is a half transporter which contains one transmembrane domain (TMD) and one nucleotide-binding domain (NBD), consisting of six transmembrane segments (Fig. 5a). Gene ontology suggests its molecular function contains ATPase activity, transporter activity and ATP-binding function, the biological process of ABCB4 is transport (data came from *Dictyostelium* database, <http://dictybase.org/>).

Our laboratory has shown previously that DdCAD-1 is transported to the plasma membrane via contractile vacuole pathways. The import of DdCAD-1 into the contractile

Fig. 3 Exogenous DdCAD-1 can bind to *cadA*⁻ cells surface and in vivo chemical cross-linking. **a** Alexa Fluor 488-stained His₆-DdCAD-1 can bind to *cadA*⁻ cells membrane by a putative anchor. Bar 20 μm. **b** Schematic diagram of cross-linking. **c** Western blot

(GST antibody, lane 1) and far western blot (10 μg/ml His₆-DdCAD-1 was used for probe and followed by His₆-tag antibody, lane 2) of *cadA*⁻ cells membrane protein after cross-linking

Fig. 4 Co-immunoprecipitation of DdCAD-1-anchoring complex. **a** DdCAD-1 polyclonal antibody co-immunoprecipitation. Pre-cleaning beads (lane 1); immunoprecipitated supernatant (lane 2); immunoprecipitated beads with or without β-mercaptoethanol (lanes 3, 4); 5 μg GST-DdCAD-1 (lane 5); 5 μl DdCAD-1 antibody (lane 6).

b GST monoclonal antibody co-immunoprecipitation. The beads conjugated to GST-DdCAD-1 complex (lane 1) and GST-DdCAD-1-N complex (lane 2). **c** The bands indicated by the arrows were excised and the protein was digested with trypsin for MALDI-TOF mass spectrometry, eight peptides matched with the *Dictyostelium* protein ABCB4

vacuole lumen is enhanced by exogenous ATP or an ATP regeneration system [6], suggesting the involvement of specific ATP-dependent transporter(s) on contractile

vacuoles membrane. Putative ABC transporters have been identified in a proteomic analysis of contractile vacuole from *Trypanosoma Cruzi* [18]. Although the subcellular

Fig. 5 Model of DdCAD-1 transport. **a** Schematic model of ABCB4. **b** Schematic diagram of DdCAD-1 import and export by ABCB4 through the contractile vacuole and the plasma membrane, respectively

localization of ABC transporters in *Dictyostelium* has not been well defined yet, they remain potential candidates underlying the vacuolar transportation of ATP [19]. As the contractile vacuoles fused with the plasma membrane when DdCAD-1 was transported to cell surface [6], it is conceivable that similar receptors exist on the membrane of contractile vacuoles and cell surface. Therefore, we propose that ABCB4 on contractile vacuoles membrane involve in import DdCAD-1 by an ATP-dependent pathway, while ABCB4 on cell surface is responsible for anchoring DdCAD-1 on plasma membrane. Since ABC transporters have been reported to act as both importers and exporters [20], it is also imaginable that a portion of DdCAD-1 in cytoplasm can be exported by ABCB4 on plasma membrane directly (Fig. 5b). An alternative route for DdCAD-1 import into contractile vacuoles is through the invagination of the vacuolar membrane [7].

Conclusion

We have employed far western blot analysis and chemical cross-linking coupled with mass spectrometry to identify

ABCB4 as the anchor protein of DdCAD-1. In addition to serving the function of presenting DdCAD-1 on the cell surface so it may fulfill its function as a cell–cell adhesion molecule, ABCB4 may be involved in the import and export DdCAD-1 in order to regulate the relative level of DdCAD-1 on the cell surface, which in turn modulates the adhesive strength contributed by the *trans*-homophilic interaction of DdCAD-1 between adjacent cells.

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References

1. Yang C, Brar SK, Desbarats L, Siu CH (1997) Synthesis of the Ca^{2+} -dependent cell adhesion molecule DdCAD-1 is regulated by multiple factors during *Dictyostelium* development. *Differentiation* 61:275–284
2. Wong EF, Brar SK, Sesaki H, Yang C, Siu CH (1996) Molecular cloning and characterization of DdCAD-1, a Ca^{2+} -dependent cell–cell adhesion molecule, in *Dictyostelium discoideum*. *J Biol Chem* 271:16399–16408

3. Brar SK, Siu C (1993) Characterization of the cell adhesion molecule gp24 in *Dictyostelium discoideum*. Mediation of cell–cell adhesion via a Ca^{2+} -dependent mechanism. *J Biol Chem* 268:24902–24909
4. Lin Z, Sriskanthadevan S, Huang H, Siu CH, Yang D (2006) Solution structures of the adhesion molecule DdCAD-1 reveal new insights into Ca^{2+} -dependent cell–cell adhesion. *Nat Struct Mol Biol* 13:1016–1022
5. Lin Z, Huang H, Siu CH, Yang D (2004) (1)H, (13)C and (15)N resonance assignments of Ca^{2+} -free DdCAD-1: a Ca^{2+} -dependent cell–cell adhesion molecule. *J Biomol NMR* 30:375–376
6. Sesaki H, Wong EF, Siu CH (1997) The cell adhesion molecule DdCAD-1 in *Dictyostelium* is targeted to the cell surface by a nonclassical transport pathway involving contractile vacuoles. *J Cell Biol* 138:939–951
7. Sriskanthadevan S, Lee T, Lin Z, Yang D, Siu CH (2009) Cell adhesion molecule DdCAD-1 is imported into contractile vacuoles by membrane invagination in a Ca^{2+} - and conformation-dependent manner. *J Biol Chem* 284:36377–36386
8. Sesaki H, Siu CH (1996) Novel redistribution of the Ca^{2+} -dependent cell adhesion molecule DdCAD-1 during development of *Dictyostelium discoideum*. *Dev Biol* 177:504–516
9. Wong E, Yang C, Wang J, Fuller D, Loomis WF, Siu CH (2002) Disruption of the gene encoding the cell adhesion molecule DdCAD-1 leads to aberrant cell sorting and cell-type proportioning during *Dictyostelium* development. *Development* 129:3839–3850
10. Sussman M (1987) Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell Biol* 28:9–29
11. Siu CH, Lerner RA, Loomis WF Jr (1977) Rapid accumulation and disappearance of plasma membrane proteins during development of wild-type and mutant strains of *Dictyostelium discoideum*. *J Mol Biol* 116:469–488
12. Harris TJ, Ravandi A, Siu CH (2001) Assembly of glycoprotein-80 adhesion complexes in *Dictyostelium*. Receptor compartmentalization and oligomerization in membrane rafts. *J Biol Chem* 276:48764–48774
13. Sriskanthadevan S, Zhu Y, Manoharan K, Yang C, Siu CH (2011) The cell adhesion molecule DdCAD-1 regulates morphogenesis through differential spatiotemporal expression in *Dictyostelium discoideum*. *Development* 138:2487–2497
14. Jones PM, George AM (2004) The ABC transporter structure and mechanism: perspectives on recent research. *Cell Mol Life Sci* 61:682–699
15. Good JR, Kuspa A (2000) Evidence that a cell-type-specific efflux pump regulates cell differentiation in *Dictyostelium*. *Dev Biol* 220:53–61
16. Anjard C, Loomis WF (2002) Evolutionary analyses of ABC transporters of *Dictyostelium discoideum*. *Eukaryot Cell* 1:643–652
17. Detmers FJ, Lanfermeijer FC, Poolman B (2001) Peptides and ATP binding cassette peptide transporters. *Res Microbiol* 152:245–258
18. Ulrich PN, Jimenez V, Park M et al (2011) Identification of contractile vacuole proteins in *Trypanosoma cruzi*. *PLoS One* 6: e18013
19. Sivaramakrishnan V, Fountain SJ (2012) A mechanism of intracellular P2X receptor activation. *J Biol Chem* 287:28315–28326
20. Davidson AL, Dassa E, Orelle C, Chen J (2008) Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol Mol Biol Rev* 72:317–364