Mapping of the basic amino-acid residues responsible for tubulation and cellular protrusion by the EFC/F-BAR domain of pacsin2/Syndapin II

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Abstract

The extended Fes-CIP4 homology (EFC)/FCH-BAR (F-BAR) domain tubulates membranes. Overexpression of the pacsin2 EFC/F-BAR domain resulted in tubular localization inside cells and deformed liposomes into tubules in vitro. We found that overexpression of the pacsin2 EFC/F-BAR domain induced cellular microspikes, with the pacsin2 EFC/F-BAR domain concentrated at the neck. The hydrophobic loops and the basic amino-acid residues on the concave surface of the pacsin2 EFC/F-BAR domain are essential for both the microspike formation and tubulation. Since the curvature of the neck of the microspike and that of the tubulation share similar geometry, the pacsin2 EFC/F-BAR domain is considered to facilitate both microspike formation and tubulation.

1. Introduction

BAR domain superfamily proteins deform membranes to a geometry corresponding to the structures of the membrane-binding surface of the BAR-superfamily, and/or bind to the membranes that fit their structures, and thus function to generate specific membrane geometries [1–4]. The BAR domain forms a crescent-shaped dimer, with a positively charged, concave surface [5]. The positively charged surface of the domain binds to the negatively charged inner surface of the plasma membrane to form invaginated tubular membrane structures. Furthermore, the BAR domains from endophilin and amphiphysin have hydrophobic amino-acid residues that are inserted into the membrane on the concave surface or dimer ends [5–7]. The EFC (or F-BAR) domains from CIP4, Toca-1, and FBPI7 form crescent-shaped dimers, and their concave surfaces bind to the membrane [8–10]. The F-BAR domain of FCHo2 also forms a crescent-shaped dimer, but the curvature of its membrane binding, concave surface is larger than those of the EFC/F-BAR domains of CIP4 and Toca-1 [11]. In contrast to the EFC/F-BAR domains of CIP4 and Toca-1, the lateral surface of the F-BAR domain of FCHo2 is curved [11]. The F-BAR domain of srGAP2 and the F-BAR-FX unit of Fes/Fer are involved in the formation of cellular protrusions [12,13], but the underlying mechanisms are unclear because the structures remain unsolved.

Pacsins/Syndapins form one branch of the EFC/F-BAR domain protein family [2,8,14–16]. Pacsins/Syndapins function in the morphogenesis of neurons and in zebrafish notochord development, presumably through endocytosis and/or protrusive structure formation [17,18]. Recently, the structures of the EFC/F-BAR domains of human pacsin1/Syndapin I, pacsin2/Syndapin II, and Drosophila pacsin/Syndapin were reported [17,19], but the formation of cellular structures by these domains was not described. We also solved the structure of the pacsin2 EFC/F-BAR domain, at higher resolution. Here we describe the membrane interactions of the EFC/F-BAR domain of pacsin2/Syndapin II for both tubulation and filopodia-like process formation in cells.
2. Materials and methods

2.1. Liposome assays

The liposome-binding assay was performed as previously described [5, 9, 20]. Liposomes were prepared from total bovine brain lipids (Folch fraction 1, Avanti Polar Lipids) [21]. Dried lipids, under nitrogen gas, were resuspended in XB (10 mM Hepes [pH 7.9], 100 mM KCl, 2 mM MgCl₂, 0.2 mM CaCl₂, and 5 mM EGTA) by mixing with a vortex, followed by hydration at 37 °C for 1 h. No sucrose was added to the XB. This preparation yielded a mixture of liposomes with various diameters (0.1–2 μm), and some of the liposomes were large multi-lamellar vesicles. Liposome co-sedimentation assays were performed as follows. To

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**Fig. 1.** Three-dimensional structure of the pacsin2 EFC/F-BAR domain. (A) Ribbon diagram of the structure of EFCSpacin2 (residues 1–305). N and C indicate the amino and carboxyl termini of the molecule, respectively. The secondary structure elements are colored differently. (B) Side view of the EFCSpacin2 dimer. One molecule is depicted as in (A), while the other molecule is colored magenta. The box indicates the region shown as a larger image in (D). (C) Top view of the EFCSpacin2 dimer. The dimer is rotated by 90° relative to (B). (D) Residues in the insertion loop between helices α2a and α2b are shown as stick representations. (E–G) Electrostatic potential surfaces of the EFCSpacin2 dimer, indicated with blue as positive and red as negative. (E) Bottom view (concave side); (F) side view; (G) top view (convex side). (H, I) Superpositions of EFCSpacin2 (cyan), EFCLpacsin2 (magenta), and the previously determined pacsin2 EFC domain (green). Secondary structures are indicated. (H) The stereo view of the Cα traces around the tips of one coiled-coil region is shown. (I) The ribbon models around the loop region connecting helices α3a and α3b are shown. The prime symbol (') denotes secondary structures in the dimer-related molecule. (J) A stereo view of a 2Fo−Fc omit electron density map, contoured at 1.5σ, of a representative dimer interface of EFCSpacin2. The first and second molecules in the dimer interface are shown as stick models and are colored cyan and magenta, respectively. The hydrogen bonding network in the dimer interface, involving several water molecules, is shown. Hydrogen bonds are shown as dashed lines. Residues involved in the dimer interface are labeled. The prime symbol (') denotes residues in the second molecule of the dimer.
remove the aggregated proteins, the purified proteins were subjected to centrifugation at 25,000 × g for 30 min at 25 °C in a TL100 rotor (Beckman). The proteins (4 μM) were incubated with liposomes (1 mg/ml) in 50 μl XB for 20 min at room temperature (RT), and were then centrifuged at 25,000 × g for 30 min at 25 °C in a TL100 rotor to precipitate the larger liposomes. Supernatants and pellets were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The shape of the liposomes (0.1 mg/ml) in the presence of the proteins (4 μM) was examined by negative-staining electron microscopy, as described previously [22,23]. Alternatively, liposomes after the incubation with the protein were fixed with 2.5% glutaraldehyde for 30 min at RT. The liposomes were then centrifuged at 20,000 × g for 30 min, stained with 1% osmium tetroxide in cacodylate buffer, washed, dehydrated in 50%, 60%, 70%, 80%, 90%, 95.5%, and 100% ethanol, placed in propylene oxide, and embedded in the epoxy resin Quetol-812 (Nissin EM). After polymerization of the resin, 80 nm sections were prepared, and were counter-stained for 10 min in uranyl acetate and for 2 min in lead citrate. The dried sections were examined by transmission electron microscopy.

2.2. Cell culture and transfection

Venus-pacsin2 was prepared by subcloning the mouse pacsin2 cDNA into the pVENUS-C1 vector, in which the GFP in pEGFP-C1 was replaced with Venus [24]. Hela cells were maintained in DMEM containing 10% fetal bovine serum. Transfection was performed using the Lipofectamine LTX and PLUS reagents (Invitrogen), according to manufacturer’s protocol. Cells were stained with an anti-GFP antibody (MBL International), followed by an Alexa488-conjugated anti-rabbit IgG antibody and rhodamine-conjugated phalloidin. Fluorescent images were obtained with an extended peptide segment (Figs. 1A and S1). As in the previously reported structure of the pacsin2 EFC domain, EFCpacsin2 and EFCSpacsin2 form a dimer in the asymmetric units of the crystals (Fig. 1B and C), and their concave surfaces are positively charged, while their convex surfaces are more negatively charged (Fig. 1E-G). A detailed comparison revealed several structural differences. First, the conformations of EFCpacsin2 and EFCSpacsin2 slightly differ from those of the previously determined crystal structure of the pacsin2 EFC domain, in regions such as the tip of the coiled-coil (residues 144–220) and the loop between helices α3a and α3b (Fig. 1H and I). The conformations of these regions of EFCpacsin2 and EFCSpacsin2 also differ between the first and second molecules in the asymmetric units, suggesting the rather flexible nature of these regions. Note that the electron densities were not very clear for a portion of the coiled-coil region (residues 157–206), as indicated by the higher average B-factor values in this region (102.1 Å² for EFCSpacsin2) than in the other regions (Table 1), and

2.3. Crystallization, data collection, and structure determination

Detailed methods can be found in Supplementary data. Briefly, selenomethionine (SeMet)-substituted fragments of the human pacsin2 EFC/F-BAR domain, EFCpacsin2 (residues 1–343) and EFCSpacsin2 (residues 1–305) were synthesized in a cell-free system, selenomethionine (SeMet)-substituted fragments of the human 2.3. Crystallization, data collection, and structure determination counted, and the number was converted to the relative number ing, the number of cellular tubulations or microspikes was ered to have tubulations or microspikes, respectively. In live imag,ing, the number of cellular tubulations or microspikes was counted, and the number was converted to the relative number that was set to 100%, representing the maximum number of tubulations or microspikes counted during the observation.

2.4. PDB accession number

Coordinates have been deposited in the Protein Data Bank (PDB), with the accession codes 3ABH and 3ACO.

3. Results and discussion

We solved the crystal structures of EFCpacsin2 (residues 1–343; EFC/F-BAR domain + C-terminal extension) and EFCSpacsin2 (residues 1–305; EFC/F-BAR domain) at 2.7 and 2.0 Å resolutions, respectively. The present models contain residues 16–299 for EFCpacsin2 and residues 16–303 for EFCSpacsin2. The structures of EFCpacsin2 and EFCSpacsin2 were very similar to the recently reported crystal structure of a different construct of the same protein [19]. Superposition of either EFCpacsin2 or EFCSpacsin2 with the previously reported pacsin2 EFC domain structure gave an rms deviation between the corresponding Cα atoms of 1.19 Å over 284 residues, whereas that of EFCpacsin2 with EFCSpacsin2 gave an rms deviation between the corresponding Cα atoms of 0.66 Å over 284 residues. This suggests that the presence of the C-terminal extension outside the core EFC domain (residues 1–305) does not significantly affect the overall structure of the core EFC domain. EFCpacsin2 and EFCSpacsin2 are composed of three long α helices (two with insertions) and a short α helix, followed by an extended peptide segment (Figs. 1A and S1). As in the previously reported structure of the pacsin2 EFC domain, EFCpacsin2 and EFCSpacsin2 form a dimer in the asymmetric units of the crystals (Fig. 1B and C), and their concave surfaces are positively charged, while their convex surfaces are more negatively charged (Fig. 1E-G). A detailed comparison revealed several structural differences. First, the conformations of EFCpacsin2 and EFCSpacsin2 slightly differ from those of the previously determined crystal structure of the pacsin2 EFC domain, in regions such as the tip of the coiled-coil (residues 144–220) and the loop between helices α3a and α3b (Fig. 1H and I). The conformations of these regions of EFCpacsin2 and EFCSpacsin2 also differ between the first and second molecules in the asymmetric units, suggesting the rather flexible nature of these regions. Note that the electron densities were not very clear for a portion of the coiled-coil region (residues 157–206), as indicated by the higher average B-factor values in this region (102.1 Å² for EFCSpacsin2) than in the other regions (Table 1), and

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Values in parentheses are for the highest resolution shell.

a Rmerge = ΣiΣj|Fobs| − |Fcalc|/ΣiΣj|Fobs|, where h refers to unique reflection indices and i indicates symmetry-equivalent indices.

b Rwork = ΣiΣj|Fobs| − |Fcalc|/ΣiΣj|Fobs| for the working set reflections (95% of the data) used for the refinement.

Rfree = ΣiΣj|Fobs| − |Fcalc|/ΣiΣj|Fobs| for the test set reflections (5% of the data) excluded from the refinement.
the coordinates could not be determined as precisely as in the other regions of the molecule. Second, in EFCSpacin2, two and four additional residues at the C-termini of the first and second molecules in the asymmetric units, respectively, could be modeled. Third, probably due to the improved resolution, a number of ordered water molecules were identified in the dimer-interfaces of EFCSpacin2 (Fig. 1).

We analyzed the deformation of artificial membranes formed from the brain Folch fraction, which is rich in phosphatidylserine, upon an incubation with the EFC domain of pacsin2. As reported previously, the EFC domain fragment of pacsin2 deformed liposomes to tubules (Fig. 2B), with diameters comparable to that of the concave surface in the structure of the pacsin2 EFC domain (data not shown) [19].

The liposome interaction is dependent on phosphatidylserine, and thus may be electrostatic [19]. The in vitro tubulation of liposomes induced by the concave surface mutant, R50D, was hardly observed, while that induced by D40K was enhanced (Fig. 2B). The tubulation induced by the each of the convex surface mutants, K189E and R284E, of the EFC domain of pacsin2 in vitro was also less efficient than that induced by the wild-type EFC domain of pacsin2. The affinities of these mutants to the membrane were analyzed in vitro with purified proteins. As a control, the wild-type IMD/I-BAR domain of IRSp53, but not the BPM mutant defective in

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**Fig. 2.** Membrane deformation by the EFC/F-BAR domain of pacsin2. (A) Mapping of mutated amino-acid residues on the structure of the EFC domain of pacsin2. The mutated basic, acidic, and hydrophobic amino-acid residues are colored blue, red, and yellow, respectively. The two chains in the dimer are differently colored. (left) Bottom view (concave side); (right) side view. (B) Liposome tubulation by pacsin2. Liposomes (0.1 mg/ml) were incubated with the purified protein (4 μM) of wild-type pacsin2 EFC domain (EFCL or residues 1–343), or mutants of the pacsin2 EFC domain. Liposomes alone without proteins are also shown. The liposomes were observed by negative-staining electron micrography. (C) The binding of each EFC/F-BAR domain protein (4 μM) or IMD/I-BAR domain protein (4 μM) to Folch liposomes (1 mg/ml) was examined by a co-sedimentation assay. The supernatant (sup) and the precipitate (ppt) were visualized by Coomassie Brilliant Blue staining after SDS–PAGE. (D) Quantification of liposome binding in (A). The values are the means from three independent experiments with standard deviations. There was no statistical significance between the values.
liposome binding [20], bound to the liposome under these conditions (Fig. 2C and D). The co-sedimentation assay showed no significant reduction in the affinities to the membrane by these single or double amino-acid substitutions (Fig. 2C and D). However, the ability to deform the liposomes appears to correlate with the membrane deformation ability (Fig. 2B–D).

We examined the localization of the overexpressed EFC domain of pacsin2 in Hela cells. The wild-type EFC domain was localized at tubular invaginations from the cell periphery that were not colocalized with actin filaments (Figs. 3 and 4). When expressed in cells, the membrane tubulation ability of the 1–305 fragment was similar to that of the 1–343 fragment (Fig. 3A). Full length pac-
sin2 was not localized at tubular invaginations as frequently as the EFC domain, indicating the auto-inhibition of the full length protein [19].

To confirm that the concave surface is essential for the membrane deformation induced by the EFC domain of pacsin2, we mutated several residues on the surface of the EFC domain of pacsin2 (Fig. 2A). The substitutions of basic amino-acid residues on the concave surface, such as R25, R26, R29, R30, R50, K140, K189, K203, and K207, with acidic amino-acid residues strongly inhibited membrane tubulation when the mutants were expressed...
in Hela cells (Fig. 3). The substitutions of M124 and M125 in the insertion loop with hydrophilic threonines impaired the induction of tubulation when the mutant was overexpressed in cells (Figs. 1D, 2A and 3). All of the mutants were expressed at similar levels when transfected into Hela cells (Fig. S2). Furthermore, the substitutions of acidic amino-acid residues on the concave surface, such as D40 and E110, with basic amino-acid residues enhanced the efficiency of tubulation (Fig. 3). Importantly, the substitutions of the basic amino-acid residues on the convex surface, such as K239, R245, and R284, with acidic amino-acid residues reduced the tubulation efficiency, but to a smaller extent than the substitutions on the concave surface. Therefore, the convex surface also contributes to the membrane tubulation induced by the EFC domain.

We noticed that the cells overexpressing the pacsin2 EFC domain fragment contained more microspikes with actin filaments than the non-overexpressing cells (Fig. 4A). Importantly, the percentage of cells with microspikes was roughly proportional to the percentage of cells with tubulation (Fig. 3). This suggested the contribution of membrane binding at the concave surface of the EFC domain of pacsin2 for protrusion formation. Accordingly, the EFC domain fragment was enriched at the necks of the microspikes (Fig. 4B). Therefore, the EFC domain of pacsin2 may facilitate microspike formation by bending the membrane at the neck. Therefore, we performed thin section electron microscopy of liposomes incubated with the pacsin2 EFC domain, to examine the liposome invagination in vitro, in comparison with the IRSp53 IMD/I-BAR domain. A small population of the liposomes with the pacsin2 EFC domain had invaginations in vitro (Fig. 4C). However, the invaginations were shallower than those induced by the IMD/I-BAR domain (Fig. 4C).

If the bending of the membrane at the neck promotes the microspike formation, then the EFC-domain-induced microspikes could be elongated by another mechanism, such as actin polymerization. When the cells were treated with the actin depolymerizing drug, latrunculin B, the number of microspikes decreased (Fig. 4D and E), suggesting that the microspikes were supported by the actin filaments. The amount of tubulation was increased upon latrunculin B treatment, suggesting that the actin filaments beneath the plasma membrane suppress the tubulation (Fig. 4D and E).

It is quite interesting that the overexpression of the EFC/F-BAR domain induced microspikes as well as tubulation. Since the cytosolic surface of the plasma membrane in the microspike neck has a positive curvature that fits the concave surface of the domain, the membrane geometry is identical to that of the membrane tubulation surface of a cellular invagination (Fig. 5). Therefore, the involvement of the membrane deforming proteins at the necks of the cellular microspikes is a reasonable hypothesis.

The amino-acid residues involved in the formation of invaginations were mainly mapped on the concave surface of the EFC domain, as in the EFC/F-BAR domains of FBP17 and FCHO2. However, the amino-acid residues on the convex surface were also involved in the invagination formation. Interestingly, the convex surface mutations, such as R245 and R284, had greater effects on tubulation, rather than protrusion (Fig. 3). The initial binding of the EFC domain of pacsin2 may occur randomly on the convex and concave surfaces; however, the role of the convex surface in membrane binding should be examined in the future.

The IRSp53-MIM homology domain (IMD)/inverse-BAR (I-BAR) domain binds to the membrane through its convex surface [20,28–30]. The protrusions formed by the concave surface of the pacsin2 EFC/F-BAR domain represent another means of protrusion formation in cells. Therefore, there appear to be three modes for membrane curvature formation: the concave surface of the protein binding to the positive curvature of the membrane at the liposome tubule/cellular invagination (amphiphysin, endophilin BARs and FBP17, CIP4, pacsin, FCHO2 EFC/F-BARs), the convex surface of the protein binding to the negative curvature of the membrane at the liposome invagination/cellular protrusion (IRSp53 and MIM IMD/I-BAR), and the concave surface of the protein binding to the positive curvature of the membrane at the neck of the liposome invagination/cellular protrusion (pacsin EFC/F-BAR) [4].

There may be unknown protein–membrane interactions involved in the negative curvature at the neck of the cellular invaginations.
and at the tip of cellular protrusions, and at the positive curvature of the tip of tubulated cellular invaginations. These membrane-binding proteins may classify microspikes into several novel subtypes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.02.058.

References