Pacsin 2 is recruited to caveolae and functions in caveolar biogenesis

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Summary

The pacsin (also termed syndapin) protein family is well characterised structurally. They contain F-BAR domains associated with the generation or maintenance of membrane curvature. The cell biology of these proteins remains less understood. Here, we initially confirm that EHD2, a protein previously shown biochemically to be present in caveolar fractions and to bind to pacsin, is a caveolar protein. We go on to report that GFP–pacsin 2 can be recruited to caveolae, and that endogenous pacsin 2 partially colocalises with caveolin 1 at the plasma membrane. Analysis of the role of pacsin 2 in caveolar biogenesis using small interfering RNA (siRNA) confirms that EHD2, a protein previously shown biochemically to be present in caveolar fractions and to bind to pacsins, is a caveolar protein. We propose that pacsin 2 has an important role in the formation of plasma membrane caveolae.

Key words: Caveolae, Cavin, Pacsin, F-BAR domain, Syndapin, EHD2

Introduction

Caveolae are flask-shaped invaginations in the plasma membranes of many mammalian cell types, where they have several important functions in diverse processes, including transcytosis, metabolic regulation, clathrin-independent endocytosis and signalling (Doherty and McMahon, 2009; Hansen and Nichols, 2009; Lajoie and Nabi, 2010; Le Lay and Kurzchalia, 2005; Parton and Simons, 2007; Pilch et al., 2007; van Deurs et al., 2003). Caveolae are composed of lipids, caveolin proteins (Fra et al., 1995; Rothberg et al., 1992) and the recently identified cavin proteins (Bastiani et al., 2009; Hansen et al., 2009; Hansen and Nichols, 2010; Hill et al., 2008; Liu and Pilch, 2008; McMahon et al., 2009; Vinten et al., 2005). The GTPase dynamin can also be localised to caveolae and is important during budding of caveolae from the plasma membrane to form caveolar vesicles (Henley et al., 1998; Oh et al., 1998; Pelkmans et al., 2002; Yao et al., 2005). Caveolins 1 and 2 are both multiply acylated (Dietzen et al., 1995; Monier et al., 1996) and have a stretch of hydrophobic amino acids that is likely to be embedded within the lipid bilayer. They hetero-oligomerise in the Golgi complex to form caveolin microdomains (Monier et al., 1995; Parolini et al., 1999) and travel together en route to the plasma membrane (Hayer et al., 2010a; Tagawa et al., 2005), where they associate with the cavin proteins (Bastiani et al., 2009; Hansen et al., 2009; Hansen and Nichols, 2010; Hill et al., 2008; Liu and Pilch, 2008; McMahon et al., 2009; Vinten et al., 2005).

Importantly, the key step in caveolar biogenesis, in which flat caveolin microdomains combine with potentially additional proteins to produce the characteristic caveolar membrane morphology, is still not fully understood.

There are four Eps 15 homology domain (EHD) proteins in mammals (George et al., 2007; Naslavsky and Caplan, 2010; Pohl et al., 2000). EHDs are implicated in endocytosis (Olswang-Kutz et al., 2009; Valdez et al., 2005) and endocytic recycling (Caplan et al., 2002; George et al., 2007; Grant et al., 2001; Naslavsky et al., 2006). EHD proteins have ATPase activity, and both bind to and sculpt cell membranes (Blume et al., 2007; Daumke et al., 2007; Lee et al., 2005). They comprise an N-terminal guanine-nucleotide-binding (G) domain followed by a helical extension and a C-terminal EH domain (Daumke et al., 2007; George et al., 2007; Pohl et al., 2000; Salcini et al., 1997). The structure of murine EHD2 shows that membrane affinity is generated by a polybasic cluster on a concave surface comprising the helical extension region in EHD2 homodimers, and that the binding of ATP takes place in the G-domain (Daumke et al., 2007). Crucially for our study, the biochemical isolation of caveolae carried out by Aboulaich et al., which successfully identified the cavin proteins, also provides strong evidence that EHD2 is associated with caveolae in adipocytes (Aboulaich et al., 2004).

Pacsins (also known as syndapins) bind to EHD proteins through NPF motifs (Braun et al., 2005; Xu et al., 2004). In addition, pacsins are thought to function in a dynamin-dependent type of endocytosis but do not colocalise with clathrin (Modregger et al., 2000). These observations raise the possibility that pacsins can be targeted to caveolae. There are three mammalian pacsins: pacsin 1 is neuron specific (Plomann et al., 1998; Qualmann et al., 1999), pacsin 2 is nearly ubiquitously expressed and pacsin 3 is predominantly expressed in muscle tissues (Modregger et al., 2000; Ritter et al., 1999). Pacsins are peripheral membrane proteins and contain an N-terminal F-BAR (for Bin–Amphiphysin–Rvs, also termed extended EFC) domain and a C-terminal Src homology 3 (SH3) domain (Suetsugu, 2010; Suetsugu et al., 2010). Three structures of the F-BAR domains of pacsins are available (Edeling et al., 2010). These structures reveal the molecular details of the membrane constriction and tubulation activity of pacsins. The structures disclose that the pacsin F-BAR module comprises a crescent-shaped antiparallel homodimer that gives rise to a central
six-helix bundle. The concave surface of this α-helical coiled-coil region is markedly enriched in positively charged residues, conferring membrane affinity (Edeling et al., 2009; Rao et al., 2010; Shimada et al., 2009; Wang et al., 2009). The propensity of the F-BAR domain in pacsins to bend membranes is regulated by autoinhibition, as the SH3 domain can act as an intramolecular clamp (Edeling et al., 2009; Rao et al., 2010; Shimada et al., 2009; Wang et al., 2009). This clamp is released when the SH3 domain binds to the proline-rich region of dynamin, thereby potentially coupling the membrane-bending function of pacsins to dynamin activity (Kessels et al., 2006; Rao et al., 2010). The SH3 domain might also interact with N-WASP and therefore link pacsin function to the nucleation of F-actin (Kessels and Qualmann, 2002; Kessels and Qualmann, 2004). Pacsins have been implicated in a variety of cellular roles, including endocytosis (Anggono et al., 2006; Kessels and Qualmann, 2002; Modregger et al., 2000), notochord development (Edeling et al., 2009), neuromorphogenesis (Dharmalingam et al., 2009) and gastrulation (Cousin et al., 2008).

The data outlined above make EHD2 a good candidate to be a caveolar protein and raise the possibility that pacsin 2 might also be associated with caveolae. Here, we set out to test these hypotheses and to characterise the potential function of pacsin 2 in caveolar biogenesis.

Results

EHD2 and Pacsin 2 can be recruited to caveolae

In order to confirm that EHD2 can be recruited to caveolae, we expressed GFP–EHD2 in HeLa cells and labelled caveolin 1 using indirect immunofluorescence (supplementary material Fig. S1a). GFP–EHD2 was distributed in membrane puncta and in long tubes (Blume et al., 2007; Daumke et al., 2007). The membrane puncta colocalised with caveolin 1, with over 75% of GFP–EHD2 puncta also containing caveolin 1 (supplementary material Fig. S1a). We used pre-embedding immunolabelling and electron microscopy to ask whether GFP–EHD2 is found in caveolae. Anti-GFP antibodies labelled GFP–EHD2 specifically, and clear labelling of morphologically defined caveolae could be observed (supplementary material Fig. S1b). That these structures are indeed caveolae was confirmed by colabelling with antibodies against caveolin 1 (supplementary material Fig. S1b). These structures are likely to be a caveolar protein.

We have carried out small interfering RNA (siRNA) experiments to address the function of EHD2 in caveolar biogenesis and trafficking, but these experiments do not yield clear phenotypes. The absence of a phenotype in this type of experiment is not necessarily informative as there was residual EHD2 expression in cells transfected with EHD2-specific siRNAs (data not shown). For this reason, and because pacsin 2 knockdown does not generate clear and informative phenotypes, the rest of this paper focuses on the role of pacsin 2 within caveolae.

We expressed GFP–pacin 2 in HeLa cells. After fixation, limited colocalisation was observed with endogenous caveolin 1 using confocal microscopy (data not shown), and the bulk of the GFP–pacin 2 was clearly cytosolic. It seemed probable that the prominent cytosolic pool of GFP–pacin 2 could mask recruitment of GFP–pacin 2 to caveolae. Consistent with this, when cells were transfected with both GFP–pacin 2 and the caveolar marker cavum 2 (also known as serum deprivation-response protein, SDPR) fused to mCh and analysed using total internal reflection (TIR) imaging, colocalisation in punctate structures could be detected (in addition to the cytosolic pool of GFP–pacin 2; Fig. 1a). As cavum 2–mCh colocalises with endogenous caveolin 1 (Hansen et al., 2009), colocalisation between cavum 2–mCh and GFP–pacin 2 suggests that GFP–pacin 2 is recruited to caveolae.

Although GFP–pacin 2 colocalised with cavum 2–mCh, both the high density of structures containing these markers and the presence of additional GFP–pacin 2 made higher resolution analysis desirable. GFP–pacin 2 could be detected on caveolar membrane profiles when HeLa cells were labelled with anti-GFP antibodies before embedding and processing for immunolabel-electron microscopy. In addition, dual labelling with anti-GFP and anti-caveolin 1 antibodies showed that GFP–pacin 2 is present in caveolin-1-positive caveolae (Fig. 1b). We carried out quantification of the number of gold particles representing GFP–pacin 2 and caveolin 1 staining in morphologically defined caveolae as opposed to the rest of the plasma membrane (Fig. 1c). This analysis again showed that GFP–pacin 2 is localised...
to caveolae, and confirmed that in HeLa cells caveolin 1 is itself found in caveolae as opposed to other regions of the plasma membrane (Rothberg et al., 1992).

**Endogenous pacsin 2 colocalises with caveolin 1**

The distribution of endogenous pacsin 2 in HeLa cells was ascertained using indirect immunofluorescent labelling. This revealed puncta, brighter linear structures and, apparently, cytosolic staining. Co-staining of pacsin 2 and caveolin 1 revealed that many, but not all, caveolin 1 puncta also contain pacsin 2 (Fig. 2a; see supplementary material Fig. S2a for larger images). Approximately 35% of caveolin 1 puncta were positive for pacsin 2. This implies that pacsin 2 is present in caveolae. The colocalisation between endogenous pacsin 2 and caveolin 1 was more distinct and readily visualised than was observed in the case of the fluorescent fusion proteins imaged in Fig. 1a. It is possible that overexpressed GFP–pacsin 2 has a different distribution to the endogenous protein or that it functions differently. For these reasons, we decided to use the antibody against endogenous pacsin 2 rather than overexpressed pacsin 2 chimeric constructs in further experiments. The distribution of pacsin 2 antibody staining was compared with that of the membrane microdomain marker flotillin 1 (Glebov et al., 2006) and clathrin heavy chain. Significantly less overlap was observed between pacsin 2 and flotillin 1 or clathrin heavy chain than between pacsin 2 and caveolin 1 (compare supplementary material Fig. S2a,b and c).

The linear structures labelled with anti-pacsin 2 antibodies were observed with a frequency of ~1 to ~3 per cell. The fact that they could themselves be labelled with antibodies against the GPI-linked protein CD59 (Fig. 2b), and with the glycosphingolipid-binding B subunit of Cholera toxin (supplementary material Fig. S1c) demonstrated that they are likely to be membrane tubes. In order to ascertain the relationship between pacsin 2 in caveolae and in membrane tubes, we turned to experiments in mouse embryonic fibroblasts (MEFs) from caveolin-1-null mice (Razani et al., 2001). Initially, we confirmed that in wild-type MEFs pacsin 2 and caveolin 1 colocalise as in HeLa cells (Fig. 3a). Side-by-side stainings of wild-type and caveolin-1-null MEFs with anti-pacsin 2 antibodies did not reveal a detectable difference in the distribution of pacsin 2, and linear structures likely to correspond to membrane tubes were detected in the knockout cells (Fig. 3b). Caveolin 1 is, therefore, not required for formation of pacsin 2-positive membrane tubes.

The co-stainings with pacsin 2 and caveolin 1 antibodies shown in Fig. 2a, Fig. 3a and supplementary material Fig. S2a demonstrate colocalisation in a substantial fraction of caveolin-1-positive puncta. In order to confirm that the antibody against pacsin 2 does indeed label the pacsin 2 protein specifically, and to provide a tool for functional studies, we used siRNA transfection to knockdown pacsin 2 expression. Four pooled siRNAs, and two out of four single siRNAs, effectively eliminated specific staining of pacsin 2 in over 70% of cells analyzed by indirect immunofluorescence and reduced the total amount of pacsin 2 in the transfected cell population to ~15% of the control levels as determined by western blotting (Fig. 4a,b). Significantly, both the punctate and membrane tube staining was eliminated, so both of these are likely to represent bona fide endogenous pacsin 2.
Reduced pacsin 2 expression reduces the abundance of caveola but increases that of caveolins in the plasma membrane

siRNAs against pacsin 2 were used to further investigate the function of pacsin 2 in caveolar biogenesis and trafficking. We counted the number of morphologically defined caveolar membrane profiles (Fig. 5a) in electron micrographs representing complete reconstructions of single sections through 15 cells from control and pacsin siRNA-transfected cells (Hansen et al., 2009). This was repeated three times in two separate cell lines (HeLa and NIH3T3), using two separate sets of siRNAs. Reduction in expression of pacsin 2 consistently resulted in a reduction in caveolar membrane invaginations, without affecting the abundance of clathrin-coated pits (Fig. 5b). Therefore, not only does pacsin 2 localise to caveolae, but it is also involved in the generation or maintenance of these structures.

Given that in vitro experiments clearly demonstrate a role for pacsin 2 in generating membrane curvature (Edeling et al., 2009; Shimada et al., 2009; Wang et al., 2009), and caveolae are highly curved regions of the plasma membrane, one plausible explanation for the loss of caveola in pacsin-2-siRNA-treated cells is that pacsin 2 plays a direct role in deforming membranes during biogenesis of caveolae. Alternatively, pacsin 2 could be involved in the trafficking, oligomerisation or stabilisation of caveolins or other caveolar components, such as the cavins (Bastiani et al., 2009; Hansen et al., 2009; Hansen and Nichols, 2010; Hill et al., 2008). In order to investigate these possibilities further we asked whether pacsin 2 siRNA affects the distribution or abundance of caveolin 1 or cavin proteins. The abundance of caveolin 1 and cavin 1 (also known as polymerase I and transcript release factor, PTRF) on western blots was not detectably altered by reduction of the expression of pacsin 2 in HeLa cells (Fig. 4a). However, there was a marked increase in the amount of caveolin 1 detected using TIR imaging of the plasma membrane of cells transfected with siRNAs against pacsin 2 (Fig. 6a). Quantification of caveolin 1, caveolin 2 and clathrin heavy chain staining in control and pacsin-2-siRNA-treated cells revealed that depletion of pacsin 2 effectively doubles the amount of caveolin 1 and caveolin 2 detected in the plasma membrane, without altering the distribution of clathrin (Fig. 6b,c). This is surprising given that there are actually less morphologically defined caveolae in pacsin-2-depleted cells (Fig. 5). We used indirect immunofluorescence and epifluorescence imaging to confirm that there is no change in total cellular caveolin 1 in pacsin-2-depleted cells (Fig. 6d,e), as predicted by the western blots shown in Fig. 4a. Caveolin 1 and 2 still colocalised with each other in pacsin-2-depleted cells (data not shown). We conclude that pacsin 2 is not required to stabilise caveolin 1 expression and does not control the oligomerisation of caveolins into membrane microdomains (Parolini et al., 1999; Pelkmans and Zerial, 2005). Rather, pacsin 2 appears to be specifically required for these microdomains to adopt the characteristic curved caveolar membrane profile.

Cavin proteins associate with caveolin oligomers soon after their arrival at the plasma membrane and are required for both the stabilisation of caveolin protein expression and for formation of morphologically defined caveolae (Bastiani et al., 2009; Hansen et al., 2009; Hayer et al., 2010a; Hill et al., 2008; Liu and Pilch, 2008; McMahon et al., 2009). We asked whether the association...
between cavin 1 and caveolin 1 is altered when pacsin 2 is depleted. Indirect immunofluorescence and TIR imaging of control and pacsin 2 siRNA transfected cells showed the expected increase in caveolin 1 staining, and revealed that there is less cavin 1 present in caveolin-1-positive puncta when pacsin 2 is depleted (Fig. 7a).

In order to quantify this effect, the ratio between cavin 1 and caveolin 1 staining was calculated on a pixel-by-pixel basis in multiple images, and then the frequency distribution was plotted as a histogram (Fig. 7b). The ratio between cavin 1 and caveolin 1 intensities was clearly reduced when pacsin 2 was depleted. As cavin 1 is thought to be involved in caveolar morphogenesis, this is consistent with the observed accumulation of caveolin 1 but loss of caveolae within the plasma membrane.

Overexpression of the F-BAR domain of pacsin 2 causes loss of caveolae

The F-BAR domain of pacsin 2 is likely to play an important role in functions related to membrane morphogenesis (Edeling et al., 2009; Rao et al., 2010; Shimada et al., 2009; Wang et al., 2009). Overexpression of the F-BAR domain could interfere with such functions. We expressed the F-BAR domain (pacsin 2 N-305) and asked whether this visibly altered the distribution of caveolin 1 detected by indirect immunofluorescence in HeLa cells. At low expression levels, pacsin 2 N-305 colocalised extensively with endogenous caveolin 1 (Fig. 8a). At high expression levels, pacsin 2 N-305 caused a pronounced redistribution of caveolin 1, and colocalised with caveolin 1, seemingly in intracellular aberrant membrane structures (Fig. 8b).
pacsin 2 F-BAR domain to lysine (N-305, D40K) increases membrane affinity and in vitro membrane tubulating activity (Shimada et al., 2009). Expression of this mutant again resulted in colocalisation with caveolin 1 at low levels and redistribution of caveolin 1 at higher expression levels (Fig. 8c,d). Importantly, pacsin 2 N-305 and pacsin 2 N-305, D40K did not colocalise with or cause any apparent redistribution of clathrin heavy chain or flotillin 1 (supplementary material Fig. S3a,b).

In order to demonstrate that association of the F-BAR domain of pacsin 2 with caveolae is specific, we also expressed the related F-BAR domains of CIP4 (CIP4 N-303), FBP17 (FBP17 N-299), and a version of the FBP17 domain containing a potentiating mutation analogous to pacsin 2 N-305, D40K (FBP17 N-299, E39K) (Frost et al., 2008; Shimada et al., 2007; Shimada et al., 2009). None of these constructs colocalised with caveolin 1 (supplementary material Fig. S3a). The redistribution of caveolin 1 in cells expressing different F-BAR domain constructs was quantified using TIR imaging. Pacsin 2 N-305 and N-305, D40K, but not the other F-BAR domain constructs, caused a marked reduction in the abundance of plasma membrane caveolin 1 (Fig. 8e). We also used electron microscopy to quantify the abundance of morphologically defined caveolae in cells transfected with pacsin 2 N-305, D40K. Consistent with the observed loss of caveolin 1 from the plasma membrane, pacsin 2 N-305, D40K caused a significant reduction (P<0.05) in the abundance of caveolae. Negative controls were provided by overexpressing either full length pacsin 2 (which has no effect on the abundance of caveolae or the distribution of caveolin 1) or the pacsin 2 N-305, R50D mutant [which has previously been shown to not bind to membranes (Shimada et al., 2009) and is not recruited to caveolae], (Fig. 8f). There was, however, a slight increase in total caveolin 1 protein levels in these cells (supplementary material Fig. S4).

These data show that the F-BAR domain of pacsin 2 associates with caveolae and, when over-expressed specifically interferes with caveolar biogenesis or dynamics.

**Discussion**

The data presented here establish a role for pacsin 2 in caveolar biogenesis. We have shown that pacsin 2 colocalises with caveolin 1, that reduction in the expression of pacsin 2 using siRNA causes loss of caveolae but an increase in the amount of caveolin proteins found in the plasma membrane and that overexpression of the F-BAR domain of pacsin 2 causes redistribution of caveolin 1 and loss of caveolae. The number of caveolae in cells treated with pacsin siRNAs was ~50% of control levels – a similar reduction to that observed when cavin 1 or cavin 2 are depleted using siRNA (Hansen et al., 2009; Hill et al., 2008; Liu and Pilch, 2008; McMahon et al., 2009). We have also analysed the subcellular distribution of GFP-EHD2 using immuno-fluorescence and immuno-electron microscopy to confirm that EHD2 is likely to be present in caveolae, as first reported by Aboulaich et al. using proteomic analysis of purified caveolar fractions (Aboulaich et al., 2004). As we did not observe effects on the dynamics or abundance of caveolae using EHD2 siRNAs, the function of EHD2 in caveolae biogenesis remains unclear.

In the case of pacsin 2, however, our data suggest a simple working model. We propose that pacsin 2 is involved at the step in which flat caveolin-positive microdomains within the plasma membrane adopt the distinctive invaginated caveolar morphology (Palade, 1953; Rothberg et al., 1992), and that pacsin 2 is therefore not required to be constitutively associated with caveolae in the way that the cavin proteins are. This would explain why colocalisation between pacsin 2 and caveolin 1 is only partial and why morphological caveolae are lost when pacsin 2 is knocked
down. The increase in caveolin proteins in the plasma membrane when pacsin 2 is depleted could then be explained as a consequence of a block in caveolar biogenesis. The model is also consistent with the decrease in the amount of caveolin 1 relative to caveolin 1 revealed by image ratio analysis in pacin-2-knockdown cells. Our data on the effect on pacin 2 F-BAR domain expression on the distribution of caveolin 1 and caveolae provide strong additional evidence for the role of pacin 2 in caveolar biogenesis or trafficking, but will require further information on the interaction of the domain with other proteins and membranes to interpret in a more detailed manner.

More experiments are required to test the model outlined above. We have carried out immunoprecipitation experiments using a previously established protocol to ascertain whether cavins or caveolins are found associated with pacin 2. No clear co-immunoprecipitation was observed (data not shown). One significant limitation with these experiments is that relatively harsh detergent (e.g. 1% Triton X-100, 1% β-octyl glucoside) (Hansen et al., 2009; Hayer et al., 2010a) is required to fully solubilise caveolar membranes and hence detect genuine cellular protein complexes. Overexpression of GFP-pacin 2, in principle, offers a way of following the dynamics of pacin 2 and caveolae, but we are not confident that overexpressed pacin 2 has the same distribution as the endogenous protein. It is clear that overexpression of caveolar proteins can distort caveolae (Hansen et al., 2009) or result in accumulation of non-functional protein and consequent artifacts (Hayer et al., 2010b), so these experiments would have to be interpreted with caution.

As there is a pool of pacin 2 that does not colocalise with caveolin 1, it is likely that pacin 2 has additional functions outside of caveolae. Dynamin 2 is required for budding of both clathrin-coated pits and caveolae from the plasma membrane, so there is precedent for common components being shared between very different endocytic processes (Henley et al., 1998; Oh et al., 1998; Pelkmans et al., 2002; Yao et al., 2005). Pacin proteins have been reported to interact with dynamin, so it might be the case that pacsins also function during clathrin-mediated endocytosis (Anggono et al., 2006; Qualmann et al., 1999; Qualmann and Kelly, 2000; Taylor et al., 2011). The observation that endogenous pacin 2 is found in membrane tubes, and that membrane tubes have been associated with clathrin-independent endocytic pathways (Howes et al., 2010; Kirkham et al., 2005; Lundmark et al., 2008; Romer et al., 2010), again imply that pacsins function in multiple endocytic pathways.

The recent emergence of the cavin proteins as structural components of caveolae has already increased our knowledge of the complexity of the protein–protein interactions required for the biogenesis and function of these structures (Bastiani et al., 2009; Hansen et al., 2009; Hansen and Nichols, 2010; Hill et al., 2008; Liu and Pilch, 2008; McMahon et al., 2009). Our new data demonstrating an additional role for pacin 2 identifies a further key player in these processes and opens the way for further detailed molecular analysis of how caveolae are generated.

Materials and Methods

Cell culture

HeLa, mouse embryonic fibroblast (MEF) and NIH3T3 cells were grown in DMEM (Gibco) supplemented with penicillin and streptomycin and fetal calf serum (FCS). HeLa and NIH3T3 cells were transfected using Fugene (Roche), whereas MEFs were transfected by microinjection using a Labtech 100 electroporator. In both cases the transfection was carried out in accordance with the manufacturer’s instructions. MEFs were isolated from embryos following dissection and cell dispersion with trypsin.

Antibodies

Rabbit polyclonal antibodies against GFP and caveolin 1 were from Abcam. The rabbit polyclonal antibodies against pacsin 2 were from Abgent. Monoclonal antibodies against FLAG (M2) and against α-tubulin (DM1A) were from Sigma. Mouse monoclonal antibodies against caveolin 2 and rabbit polyclonal antibodies against caveolin 1 were from BD Bioscience; monoclonal anti-human-CD59 conjugated to phycoerythrin was from Autoagnostics. Monoclonal antibodies raised against caveolin 1 were generously provided by Jørgen Vinten (The Panum Institute, University of Copenhagen, Denmark) (Vinten et al., 2001). Monoclonal X22 raised against clathrin heavy chain was provided by Leon Lagnado (MRC-LMB, Cambridge, UK).

Plasmids

cDNA for mouse pacsin 2 (clone 5031043) was purchased from Geneservice and was amplified with appropriate primers and inserted into pre-digested vector (EcoRI-SalI sites) in pEGFP-C1 and, likewise, to a modified pmCherry-C1 vector. The pacsin 2 cDNA was inserted into the EcoRI-HindIII sites in the pTAg2B vector resulting in a FLAG tag preceding the pacsin 2 open reading frame. Pacsin 2 truncation constructs were generated in a similar manner to the full-length constructs. Plasmids expressing F-BAR domains of mouse CIP4 and human FBP17 were generated by PCR with appropriate primers and subcloned into Xhol and EcoRI sites of pEGFP-C1. Plasmids expressing F-BAR domains of mouse CIP4 and rabbit Cherry–FBP17 were a kind gift from Marcus Taylor (MRC-LMB, Cambridge, UK). Site-directed mutagenesis was carried out using KOD enzyme for PCR amplification and performed using complementary primers containing the desired mutations; the parental DNA was treated with Dnase to digest parental methylated DNA. cDNA for pacsin 2 and EHD2 was purchased from Geneservice and inserted into the Xhol-EcoRI sites in pEGFP-C3.

siRNA

Human-specific siRNAs against pacsin 2 mRNA were used in HeLa cells and mouse-specific siRNAs targeting pacsin 2 mRNA were used in NIH3T3 cells. The siRNAs were ON-Target plus SMART pools from Thermo Scientific (L-019666 and L-045093) respectively. Individual ON-Target plus siRNAs targeting human pacsin 2 mRNA were also used, the target sequences are: 5′-CAUUUUAAUGUGGGCUAGA-3′ (siRNA 1), 5′-CCCUUAUAGGUGCGAAGC-3′ (siRNA 2), 5′-CCUCACUGUCGAAACCC-3′ (siRNA 3) and 5′-CCUCUUGGUGCUCCCA-3′ (siRNA 4). Oligofectamine (Invitrogen) was used as the transfection reagent for the siRNAs. Cells were transfected twice, 72 and 48 hours before the experiment. The siRNA used as a control was a scrambled siRNA, 5′-GAUUUGGAUCUCUCUCAU-3′ (Mund and Pelham, 2010).

Electron microscopy

Quantification of the abundance of ultrastructurally defined caveolae and coated pits was performed as described previously (Hansen et al., 2009). Conventional electron micrographs at 36,000× magnification were used to reconstruct complete perimeters of 10–20 cells, and the occurrence of caveolae or coated pits in each image was scored without knowledge of the identity of the sample.

For pre-embedding electron microscopy, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 1 hour at room temperature. After washing in 0.1 M phosphate buffer followed by Tris-buffered saline (TBS, 0.05 M Tris, 0.05 M HCl, 0.15 M NaCl), cells were scraped off the Petri dish, pelleted at 1500 g for 10 minutes and then permeabilised in TBS containing 0.05% saponin, 3% BSA and 0.02 M glycine for 30 minutes. Note that saponin was present in all subsequent incubation and washing steps. Cells were then incubated in a mix of rabbit anti-GFP antibodies (1:200, Abcam 6556) and mouse anti-caveolin 1 antibodies (1:100) in TBS plus 1% BSA and 3% normal goat serum for 2 hours at room temperature. After washing, cells were incubated in a mix of gold-conjugated goat anti-rabbit-IgG (15 nm, 1,20) and goat anti-mouse-IgG (5 nm gold, 1,20) antibodies, both from Invitrogen, for 1 hour. After washing, cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer and processed as for conventional electron microscopy.

Distribution of 15 nm and 5 nm gold particles was quantified using micrographs at 36,000× magnification. As pre-embedding labelling can result in variable ultrastructural preservation and efficiency of antibody labelling, quantification was carried out using micrographs where specific staining of caveolar membranes with anti-caveolin 1 antibodies was detected. Membrane profile length was calculated by using manual line tracing in Image J, caveolae were defined by morphology.

Cell imaging

Cells were grown in LabTek chambers, and the medium was supplemented with 10 mM Hepes (Sigma) for live-cell imaging. The cells were rinsed in 37°C PBS and fixed at 37°C in 4% paraformaldehyde (PFA) for immunofluorescence. Afterwards, the cells were rinsed with PBS, blocked in 10% FCS supplemented with 1% saponin for permeabilisation. The cells were subsequently incubated with the appropriate antibodies and studied using a 63×, 1.4 NA objective on a Zeiss LSM510 confocal microscope with appropriate standard filter settings.

TIR images were acquired using an Olympus TIR microscope equipped with 488 nm and 546 nm lasers and fitted with a 100×, 1.45 NA objective. Standard filters sets for FITC- and TRITC-type fluorescence were used.
Image analysis and quantification

The proportion of cells containing pacsin 2 staining was quantified by counting individual puncta in ten separate cell images and scoring colocalisation by eye. Quantification of pacsin 2, caveolin 1, caveolin 2 and clathrin heavy chain staining, specifically within the plasma membrane, was carried out using images acquired by TIR illumination. The mean fluorescence per cell for each of the two fluorescent channels was corrected for bleed-through calculated using ImageJ. The values were then background-subtracted and plotted using Graphpad Prism. Ten images containing approximately ten cells per image were quantified. All images were acquired with the same microscope settings to allow for direct comparison between images. Quantification of total protein levels per cell using indirect immunofluorescence was carried out using the confocal microscope with the pinhole wide open (‘epifluorescence’) and using a 25×, NA 0.80 objective. Mean pixel values per cell were measured in Image J and plotted in Prism as above.

Western blots

Samples were lysed in sample buffer, boiled and run on pre-casted 4–20% Tris-glycine gels (Invitrogen). The gels were blotted and the membrane blocked in a PBS solution containing 4% dried skimmed milk powder and incubated with the appropriate antibodies. The blots were developed using Immobilon (Millipore).

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