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Flotillin-1/Reggie-2 Traffics to Surface Raft Domains via a Novel Golgi-independent Pathway

IDENTIFICATION OF A NOVEL MEMBRANE TARGETING DOMAIN AND A ROLE FOR PALMITOYLATION*

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Flotillins are lipid raft-associated proteins, which have been implicated in neuronal regeneration and insulin signaling. We now show that newly synthesized flotillin-1 reaches the plasma membrane via a Sar1-independent and brefeldin A-resistant targeting pathway. Consistent with post-translational membrane association of flotillin, protease sensitivity experiments suggest that flotillin-1 is not a transmembrane protein but is associated with the cytoplasmic face of the plasma membrane. The N terminus of flotillin contains a prohibitin-like domain (PHB), which shows homology to a number of proteins associated with raft domains including stomatin, podocin, and prohibitin. We show that the PHB domain of flotillin can efficiently target a heterologous protein, green fluorescent protein, to the plasma membrane. Another PHB-containing protein, stomatin, traffics to the plasma membrane via the conventional secretory pathway. Plasma membrane association of both full-length flotillin and the green fluorescent protein-tagged PHB domain of flotillin is dependent on palmitoylation and requires a conserved cysteine residue, Cys-34, in the PHB domain. The results identify a novel targeting mechanism for plasma membrane association of flotillin-1 involving a Golgi-independent trafficking pathway, the PHB domain, and palmitoylation.

The lateral association of lipids in the plasma membrane (PM)[1] of mammalian cells to form lipid microdomains has emerged as a key concept in cell biology. These microdomains, termed lipid rafts, have been implicated in numerous signaling events, including T-cell activation and growth factor signaling (1). Lipid rafts represent organized microdomains of glycosphingolipids, which form a liquid-ordered phase with specific biophysical characteristics (2, 3). Rafts were first defined by their insolubility in non-ionic detergents and low buoyant density on sucrose gradients (4). Such detergent-insoluble lipid-enriched domains have been shown to contain acylated proteins such as Src family kinases (*e.g.* Fyn, Lck), multiple glycosylphosphatidylinositol (GPI)-anchored proteins, caveolins, sphingolipids, and cholesterol (5). Organized microdomains of GPI-anchored proteins have been identified in the PM of living cells (6, 7), and it is now believed that raft microdomains represent a central feature of cellular organization, crucial for membrane trafficking events and for specific signaling cascades. This concept is well illustrated by the demonstration that different Ras isoforms differ in their sensitivity to agents which disrupt raft domains (8). Understanding how membrane proteins associate with lipid rafts, and how they contribute to the formation and function of these domains, is of vital importance in understanding how rafts may act to coordinate cellular functions.

Possibly the best studied raft domain protein is the integral membrane protein, caveolin. Caveolin is associated with a specialized form of raft domain forming a pit termed a caveola (9). These structures perform an important role in signaling, transendothelial transport, and lipid regulation (10–12) but show a more restricted tissue distribution than other, biochemically defined, rafts, which generally appear to be a ubiquitous feature of eukaroytic cells (1). Caveolae and caveolins are abundant in adipocytes, muscle cells, endothelial cells, and fibroblasts, but are rare or absent in lymphocytes and most neuronal cells. More ubiquitous markers of raft domains are the 47-kDa membrane proteins of the Reggie/flotillin family (13, 14). Although both flotillin and caveolin were recently described as novel constituents of raft domains and caveolae (14, 15), flotillin is present in cells that lack morphologically definable caveolae and has been localized to non-caveolar membranes (16, 17). Reggie-1 and -2 were originally identified in regenerating neurons of goldfish optic nerves (13). They were subsequently identified in low density detergent-insoluble complexes of endothelial cells, where they were termed flotillins (14). As well as its postulated role in neuronal regeneration, flotilllin-1 has been implicated in insulin signaling to trigger glucose transporter redistribution in adipocytes (18). Flotillin also associates with phagosomes in macrophages (19).

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[1] The abbreviations used are: PM, plasma membrane; 2BP, 2-bromopalmitate; BFA, brefeldin A; ER, endoplasmic reticulum; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; PHB, prohibitin homology; SPFH domain, stomatin/prohibitin/flotillin/HflK/C domain; BHK, baby hamster kidney; FITC, fluorescein isothiocyanate; HA, hemagglutinin; PBS, phosphate-buffered saline; IP, immunoprecipitation.

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Flotillins contain a recently identified but largely uncharacterized domain, termed the prohibitin homology (PHB) domain or SPFH domain (stomatin/prohibitin/flotillin/HflK/C domain) (20). This domain is evolutionarily conserved and is shared by several eukaryotic and prokaryotic membrane proteins, including stomatin, podocin, and prohibitin, which have apparently diverse functions. Prohibitin has been implicated in regulation of senescence and tumor suppression (21). Mutations in podocin are associated with nephrotic syndrome, which is characterized by disruption of the glomerular filtration barrier (22). Stomatin is absent in genetic disorders associated with hemolytic anemia (23), although stomatin-deficient mice do not show the same phenotype (24). Interestingly, despite PHB domain proteins showing differences in intracellular localization, with prohibitin largely mitochondrial (25) and the other proteins PM localized, several members of the family have been reported to associate with low density detergent-insoluble lipid raft domains (25–27). The role of the PHB domain, which has hydrophobic stretches but no classical transmembrane domain, is unknown. However, a recent study concluded that flotillin is a type I transmembrane protein with a hydrophobic region in the PHB domain of flotillin acting as a transmembrane domain (28). In contrast, both stomatin and podocin have been shown to form a hairpin conformation with both N and C termini cytoplasmic (29, 30). Podocin, stomatin, and prohibitin, although showing homology with flotillins in the PHB domain, differ in having a well defined hydrophobic putative intramembrane domain to the N-terminal side of the PHB domain.

In this study we have investigated the topology of flotillin, the mechanisms by which Reggie-2/flotillin-1 reaches the PM, and examined the possible role of the PHB domain in membrane association. We now show that, although flotillin behaves as an integral membrane protein, it can reach the PM via a Sar1-independent, brefeldin A (BFA)-insensitive pathway. In contrast, stomatin trafficking is through the conventional secretory pathway. The PHB domain of flotillin constitutes a novel lipid recognition motif, which can target a heterologous protein, GFP, to the PM. Association with the PM is dependent on palmitoylation and requires a key conserved cysteine residue close to the N terminus of the protein. These studies reveal a novel pathway for palmitoylation-dependent association of a protein with raft domains.

MATERIALS AND METHODS

Cell Culture and Transfection—Baby hamster kidney (BHK) cells and Vero cells (African green monkey kidney cells) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) serum supreme (BioWhittaker, Walkersville, MD) and 2 mM L-glutamine plus or minus penicillin (100 units/ml) and streptomycin (100 μg/ml). BHK and Vero cells were transiently transfected using LipofectAMINE 2000 or LipofectAMINE Plus reagent (Invitrogen) according to the instructions from the manufacturer.

Antibodies and Other Reagents—Rabbit anti-flotillin-1 antibodies were raised against a synthetic peptide (Mimotopes, Adelaide, Australia) corresponding to the C terminus of mouse flotillin (VNHNKPLRTA) with the addition of a cysteine residue at the N terminus for coupling to a carrier protein or for preparing an affinity purification column (19). Affinity purification was performed exactly as described previously (31). Antibodies were obtained from the following sources: mouse anti-Na^+/K^+ ATPase (Upstate Biotechnology, Inc., Lake Placid, NY); mouse anti-flotillin-1, rabbit anti-caveolin-1, and mouse anti-GM130 (Transduction Laboratories, Lexington, KY); rabbit anti-myc and rabbit anti-hemagglutinin (HA) (Dr. Tommy Nilsson, European Molecular Biology Laboratory, Heidelberg, Germany); and rabbit anti-GFP (32). Brefeldin A, Triton X-100, and 2-bromopalmitate (2-bromo-hexadecanoic acid) (2BP) were purchased from Sigma Aldrich, proteinase K from Roche Diagnostics (Mannheim, Germany), and dextran-FITC from Molecular Probes (Eugene, OR). Media and cell culture reagents were purchased from Invitrogen or BioWhittaker.

Generation of Constructs—The following primers (5′-CGC GGA TCC GCT TCA ACC ATG TTT TTC ACT TGT GGC-3′ and 5′-GG ACA TGC ATG CGG GCT CAT GCT GTC CTT AAA GGC-3′) were used to PCR-amplify nucleotides 151–1453 of the published mouse flotillin cDNA sequence (14) from a 3T3-L1 adipocyte αZAP cDNA library. The resulting product was cloned into the *Bam*HI and *Sph*I sites of pUC19 to form pUC19/flotillin. The authenticity of the cloned sequence was confirmed by sequencing. A *Bam*HI-*Hin*dIII fragment was then excised from pUC19/flotillin and cloned into *Bgl*II-*Hin*dIII sites of pCB6 to generate pCB6/flotillin.

Wild type flotillin was cloned into pBluescript SK (Stratagene, La Jolla, CA) and used as a template to generate an internally HA-tagged flotillin in two parts. The following primers, T3 and 5′-GGC GTA ATC GGG GAC GTC ATA AGG GTA GGA CCC TCC ACT AGA CAC CAG CGT GAT CTT ATT GGC TGA GG-3′, were used in one PCR reaction to produce the left flank of flotillin with an HA tag, and 5′-TAC CCT TAT GAC GTC CCC GAT TAC GCC GGG GGG AGC GGA ACC ATG GGG GCA GCC AAA GTT ACT GGG G-3′ and T7 were used in a second reaction to produce an HA-tagged right flank. The two halves of flotillin were ligated together by utilizing an *Aat*II site naturally occurring within the HA tag and cloned into pC1 (pEGFP C1 (Clontech, Palo Alto, CA) minus GFP). f-PHB-GFP was generated using pBluescript/flotillin as template and employing standard PCR techniques. The PCR product was cloned into the eukaryotic expression vector pEGFP N1 (Clontech). Flot-C34A-HA and f-PHB-C34A-GFP were made by applying a standard site-directed mutagenesis approach employing Flot-HA and f-PHB-GFP as templates, respectively. The authenticity of all constructs was confirmed by sequencing. The following plasmids were kind gifts: GFP-GPI (Chiara Zurzolo, Dipartimento di Biologia e Patologia Cellulare e Molecolare, Naples, Italy), and Sar1(H79G) (Rainer Pepperkok, European Molecular Biology Laboratory, Heidelberg, Germany). GFP-tH and stomatin-myc have been previously described (32, 33).

Light and Electron Microscopy—For immunofluorescence studies, cells were routinely fixed with 4% paraformaldehyde and immunolabeled according to Luetterforst *et al.* (34). For immunoelectron microscopy studies, BHK cells were transfected with the cDNA for flotillin-1. PM sheets were prepared as described previously (32) and labeled with rabbit antibodies to the C terminus of flotillin-1 followed by 10-nm protein A-gold.

Biochemical Procedures—Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis, and Western blotting was performed as described previously (34).

Carbonate Treatment—Crude membrane fractions were prepared from BHK cells as described previously (34) and treated with 150 μl of either 0.1 M $NaCO_3$ (pH 11.5) or HES buffer (pH 7.4) for 15 min on ice.

Protease Sensitivity Experiments—6-cm dishes of transfected BHK cells were washed with PBS and incubated at 37 °C with 1 ml of either 500 μg/ml proteinase K in PBS, 500 μg/ml proteinase K plus 0.01% Triton X-100 (v/v) in PBS, 0.01% Triton X-100 in PBS only, or PBS only for 30min. 100 μl of 100 mM PMSF was added to each dish, and cells were scraped and collected for centrifugation to pellet membranes at 75,000 rpm at 4 °C for 30min in a TLA100.4 rotor. Membranes were resuspended in 150 μl of ice-cold homogenization buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA) and sonicated prior to assaying each sample for protein concentration.

Sucrose Density Gradients—Transfected BHK cells were washed twice with ice-cold PBS and scraped into 250 μl of homogenization buffer plus 1% Triton X-100 and protease inhibitors. Cells were passed 10 times through a 25-gauge needle and centrifuged to pellet nuclei and unbroken cells. 300 μl of the supernatant was mixed with an equal volume of 90% (w/v) sucrose and loaded into a centrifuge tube. The load fraction was overlaid with 228 μl each of 35, 30, 25, 20, 15, 10, and then 5% sucrose and centrifuged at 50,000 rpm in a TLS-55 rotor at 4 °C for 16 h. 11 equal fractions of 150 μl were collected from the top of the gradient, and 1 fraction of 600 μl representing the load fraction. Equal volumes of each fraction were analyzed by Western blotting.

Biosynthetic Labeling and Immunoprecipitation—To measure incorporation of palmitate, transfected BHK cells were incubated for 2 h at 37 °C with 250 μCi/ml [9,10-^{3}H]palmitate (PerkinElmer Life Sciences) in CO_2-independent media containing 20 mM L-glutamine and 3.5 mg/ml fatty-acid free bovine serum albumin ± 100 μM 2-bromopalmitate. Cells were washed three times with IP buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA) then lysed for 30 min at 4 °C in 500 μl of IP buffer containing 1% Nonidet P-40, 0.1% SDS, and protease inhibitors. Cells lysates were cleared by microcentrifugation at 14,000 rpm for 15 min. The volume of cleared lysate was adjusted to 1 ml using IP buffer plus 1% Nonidet P-40. Affinity-purified mouse anti-HA or rabbit anti-GFP antibodies were added overnight at 4 °C followed by protein A/G-agarose for 2 h. Immunoprecipitated proteins were washed twice with IP buffer plus 1% Nonidet P-40, then twice with PBS, and

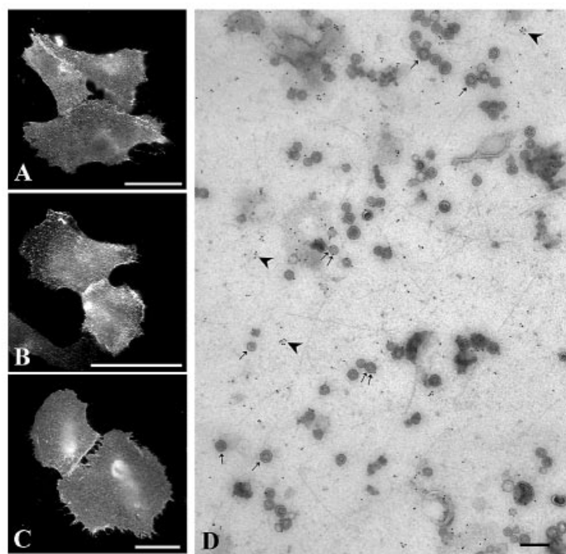

FIG. 1. **Flotillin is targeted to the plasma membrane in cultured cells.** Vero cells (*A* and *C*) and BHK cells (*B* and *D*) were transiently transfected with untagged mouse flotillin-1 (*A*, *B*, and *D*) or flotillin-1 with an internal HA tag proximal to the C terminus (*C*). By immunofluorescence microscopy, flotillin-1 was observed to target efficiently to the PM (*A–C*). *Panel D* is a representative field showing immunoelectron microscopic detection of expressed flotillin-1 on a PM sheet (*D*). Flotillin-1 was detected using anti-flotillin antibodies followed by 10-nm protein A gold. The majority of the gold particles are associated with featureless PM. Some gold labeling is associated with caveolae (*double arrows*), but many caveolae are unlabeled (*single arrows*). Some clusters of flotillin on non-caveolar membranes are apparent (*arrowheads*). Neighboring untransfected cells showed negligible labeling (results not shown). *Bars*, 10 μm (*A–C*) and 200 nm (*D*).

resolved immediately by 12% SDS-PAGE. Gels were incubated in Amplify (Amersham Biosciences) 15–30 min, dried, and analyzed by fluorography. To determine transfection and immunoprecipitation efficiency, equivalent plates of cells were incubated in parallel in the absence of [^{3}H]palmitate and the level of transfected proteins determined by Western blotting. To verify that the radiolabeled fatty acid incorporated into flotillin/PHB was linked by a thioester bond, susceptibility to hydroxylamine was examined as described previously (35).

Microinjection—Vero cells were injected with the indicated cDNAs as described previously (36). In Sar1 experiments, cells were injected with the cDNAs for Sar1(H79G) plus dextran-FITC (to indicate successful injection) and then 4 h later were injected with the Flot-HA or GFP-GPI cDNAs. In BFA and 2-bromopalmitate experiments, Vero cells were microinjected in the presence or absence of either 5 μg/ml BFA or 100 μM 2-bromopalmitate and were cultured for 5 h after injection under the same conditions.

RESULTS

Expressed Flotillin Is Targeted to the Plasma Membrane in Cultured Cells—To investigate the trafficking of flotillin-1, untagged mouse flotillin-1 was expressed in BHK cells or Vero cells and detected by immunofluorescence using affinity-purified antibodies against the C terminus of the protein (19). As shown in Fig. 1 (*A* and *B*), expressed flotillin was predominantly associated with the PM. Although BHK cells have endogenous flotillin-1 (see Fig. 4*E*), untransfected cells show negligible labeling by immunofluorescence under the same conditions. To determine the distribution of flotillin-1 within the PM, we used immunoelectron microscopy on PM sheets as described previously (37). With this method caveolae are clearly evident as uncoated circular profiles associated with the PM (Fig. 1*D*). Specific labeling for flotillin was associated with the inner surface of the PM. Labeling was mainly associated with featureless PM, sometimes in clusters, but low levels of labeling were associated with caveolae. Quantitation showed that ~10% of PM flotillin was associated with caveolae. As caveolae occupy ~3.5% of the PM in BHK cells (38), this suggests a slight concentration of flotillin-1 in caveolae as compared with the bulk PM.

Flotillin Reaches the Plasma Membrane via a BFA and Sar1-independent Trafficking Pathway—We next investigated the use of epitope-tagged forms of flotillin-1 to facilitate the study of flotillin-1 trafficking to the PM. GFP tagging at either the N or C terminus caused intracellular retention of flotillin in large perinuclear aggregates, possibly aggresomes (results not shown). Even small epitope tags such as N-terminal HA and C-terminal myc tags caused similar perturbation of flotillin-1 trafficking (results not shown). We therefore introduced an internal HA tag proximal to the C terminus between Ser-385 and Gly-386 in a region with low sequence conservation in the flotillin/stomatin family of proteins. The HA-tagged protein, here termed Flot-HA, was efficiently transported to the PM in an identical fashion to the wild type untagged protein (Fig. 1*C*). We also examined whether the epitope-tagged protein showed similar behavior to the untagged protein with respect to its association with detergent-resistant low density raft domains. The expressed untagged protein, Flot-HA, and endogenous flotillin all showed similar sedimentation characteristics after detergent treatment and flotation analysis (see Fig. 4*E*).

Most transmembrane proteins are inserted into the endoplasmic reticulum (ER) membrane via a signal peptide. Those destined for the PM first traverse the Golgi en route to their final destination. To determine whether flotillin follows this pathway, we investigated whether flotillin is transported to the PM via the Golgi complex, or by a Golgi-independent pathway such as that followed by K-Ras (39). We employed a number of well characterized agents to specifically disrupt the Golgi trafficking pathway. As a positive control, we utilized a GPI-anchored form of GFP (GFP-GPI), which is transported via vesicular transport through the Golgi complex to the PM (40). We first used BFA, which disassembles the Golgi complex and prevents anterograde transport through the secretory pathway. BFA caused a complete block of GFP-GPI transport to the PM (Fig. 2, *D–F*). Using an identical expression protocol, PM localization of Flot-HA was shown to be completely unaffected by BFA treatment (Fig. 2, *J–L*).

As an independent test of Golgi involvement in flotillin trafficking to the PM, we used a dominant negative GTP-restricted mutant of Sar1 (Sar1(H79G)). Sar1 GTPase regulates Cop II assembly, and expression of Sar1(H79G) has been shown to inhibit ER to Golgi transport (45). The cDNA encoding Sar1(H79G) was injected into Vero cells together with dextran-FITC to mark expressing cells as in previous studies (36). The same cells were then injected with either the cDNA for GFP-GPI or with the cDNA encoding Flot-HA. Whereas GFP-GPI transport to the PM was completely blocked in cells co-expressing Sar1(H79G) (Fig. 2, *M–O*), Flot-HA transport to the PM was completely unaffected (Fig. 2, *P–R*). These results show that flotillin and GPI-anchored proteins reach the PM using different mechanisms and that flotillin transport to the PM does not require exocytic transport from the ER to the Golgi complex.

Flotillin Topology; Flotillin-1 Adopts a Cytoplasmic Orientation—Flotillin has been shown to associate with the Golgi complex and was proposed to be a transmembrane protein with an N-terminal extracellular/luminal and a transmembrane domain between amino acids 134 and 151 (28). In that study a 14-kDa fragment of flotillin was protected from digestion of Golgi membranes with proteinase K. We have previously shown that the C terminus of the protein is cytoplasmically exposed (19) but were unable to detect any part of the protein exposed extracellularly by labeling the surface of non-perme-

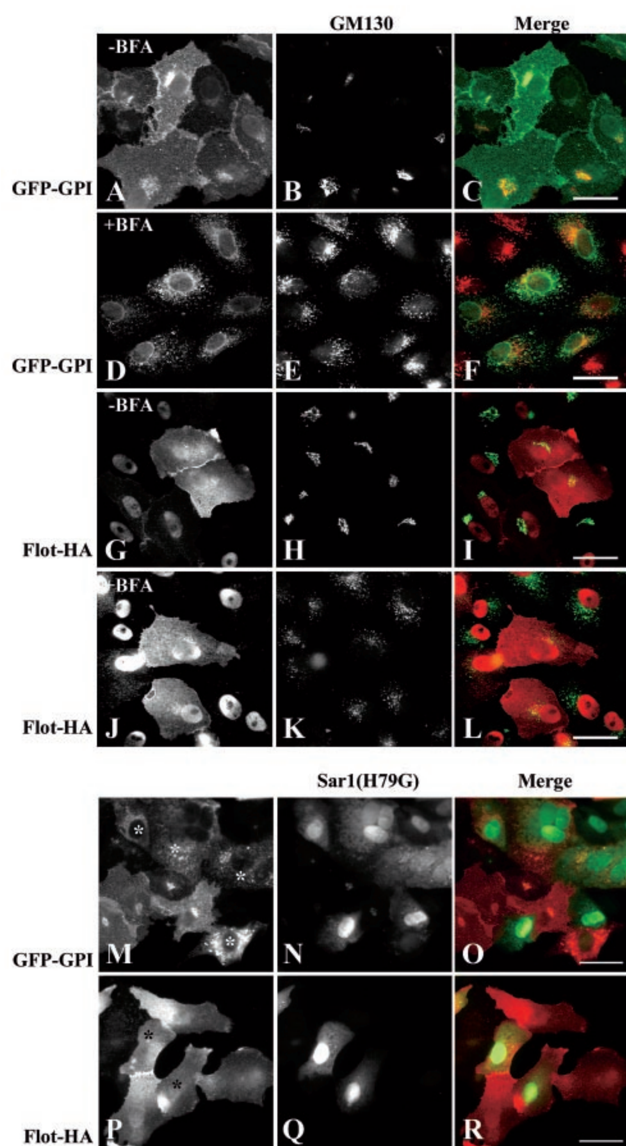

FIG. 2. **Trafficking of nascent Flot-HA to the plasma membrane is insensitive to disruption of ER/Golgi transport.** Vero cells were microinjected with GFP-GPI (*A–F*) or Flot-HA (*G–L*), in the presence (*D–F* and *J–L*) or absence (*A–C* and *G–I*) of 5 μg/ml BFA and incubated for 5 h with or without BFA prior to fixation. Treatment with BFA disrupted the Golgi complex, as revealed by immunolabeling for the Golgi marker GM130 (*E* and *K*). GFP-GPI localization at the PM was disrupted by treatment with BFA (*D*). In contrast, Flot-HA localized to the PM in both absence (*G*) and presence (*J*) of BFA. To confirm that trafficking of Flot-HA to the PM was insensitive to Golgi disruption, Vero cells were injected with Sar1(H79G) plus dextran-FITC and incubated for 4 h at 37 °C to allow protein expression. The same cells were subsequently injected with either GFP-GPI or Flot-HA and incubated for another 5 h at 37 °C. GFP-GPI was detected by labeling for the GFP tag with anti-GFP, followed by goat anti-rabbit-cy3 (to allow detection of both dextran-FITC and GFP-GPI in different channels). GFP-GPI was absent from the PM of cells co-injected with Sar1(H79G) (*asterisks*; *M*), detected by the presence of dextran-FITC (*N*). However, GFP-GPI-injected cells without Sar1(H79G) injections show a typical PM localization. In contrast, cells injected with Flot-HA (P) show cell surface labeling regardless of the presence (*asterisks*), or absence, of Sar1(H79G) expression. *Bars*, 10 μm.

abilized cells at 4 °C with available antibodies (results not shown). We therefore tested whether the flotillin-1 N-terminal domain is exposed extracellularly by expressing flotillin (data not shown), or Flot-HA, in BHK cells and then treating cells with proteinase K in the presence or absence of detergent. Caveolin, which in BHK cells has no detectable extracellular domain (38), was used as a control for cell permeability. As shown in Fig. 3, neither Flot-HA nor caveolin showed a detectable change in mobility upon treatment with extracellular proteinase K. In contrast, a combined treatment with proteinase K and detergent caused a total loss of flotillin immunoreactivity and loss of full-length caveolin. Thus, flotillin-1, like caveolin, has no proteinase K-sensitive extracellular domain.

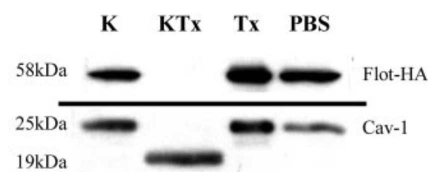

FIG. 3. **Flotillin-HA is insensitive to proteinase K degradation in non-permeabilized cells.** BHK cells were transiently transfected with Flot-HA for 24 h and then incubated with 500 μg/ml proteinase K (*lane 1*), 500 μg/ml proteinase K plus 0.1% Triton X-100 (*lane 2*), 0.1% Triton X-100 (*lane 3*), or PBS (*lane 4*) for 30 min. Both Flot-HA and caveolin were unaffected by proteinase K digestion in intact cells (*lane 1*). In contrast, no Flot-HA labeling is evident and caveolin-1 has been partially degraded after proteinase K treatment of permeabilized cells (*lane 2*). These results suggest that no significant portion of flotillin is accessible to extracellular proteinase K.

The PHB Domain of Flotillin-1 Is Sufficient for Membrane Association of a Heterologous Protein—The above results suggested that flotillin may be produced as a soluble protein, which subsequently associates with the PM. We therefore investigated the means of membrane anchoring of the flotillin protein and investigated the possibility that the N-terminal portion of the protein, containing the PHB domain or SPFH domain, can target an unrelated cytosolic protein, GFP, to the PM. As the exact boundaries of the domain are difficult to define, we generated a series of fusion proteins, PHB1–PHB6, which comprised the N-terminal regions of flotillin fused to the N terminus of GFP (shown schematically in Fig. 4*A*). Upon expression in BHK cells, PHB1-, 3-, 4-, 5-, and 6-GFP showed varying levels of cytosolic or possible aggresomal labeling (results not shown). However, PHB2-GFP, corresponding to amino acids 1–237 (subsequently termed f-PHB-GFP for flotillin-PHB-GFP fusion protein), was efficiently transported to the PM in both Vero and BHK cells (Fig. 4, *B* and *C*, respectively). This was confirmed biochemically; both f-PHB-GFP and flotillin were predominantly present in the membrane fraction after a high speed spin to remove cytosolic proteins (Fig. 4*D*). To remove peripheral membrane proteins, the membranes were treated with sodium carbonate (pH 11.5). Flotillin and f-PHB-GFP were unaffected by carbonate treatment, showing that both proteins behave as integral membrane proteins (Fig. 4*D*). We next tested whether the PHB domain of flotillin was able to confer raft association on GFP as judged by association with low density Triton X-100-insoluble membranes at 4 °C. As shown in Fig. 4*E*, f-PHB-GFP showed significant association with low density fractions. However, this was consistently a smaller fraction of the total protein than with endogenous flotillin-1 or expressed flotillin-1 (tagged or untagged).

To investigate whether the f-PHB domain reaches the PM using a similar pathway to flotillin, we tested whether f-PHB-GFP also reaches the PM in a Golgi-independent manner. As shown in Fig. 5 (*D–F*), f-PHB-GFP targeting to the PM is completely resistant to BFA treatment. Real-time microscopy of living cells expressing f-PHB-GFP showed association of f-PHB-GFP with the PM within 45 min after intranuclear injection of the cDNA (results not shown). No evidence of ER localization was ever seen for the f-PHB-GFP, unlike caveolin-GFP, but some Golgi labeling was observed. The significance of this is presently unknown but could indicate the existence of an alternative pathway for flotillin-1 trafficking.

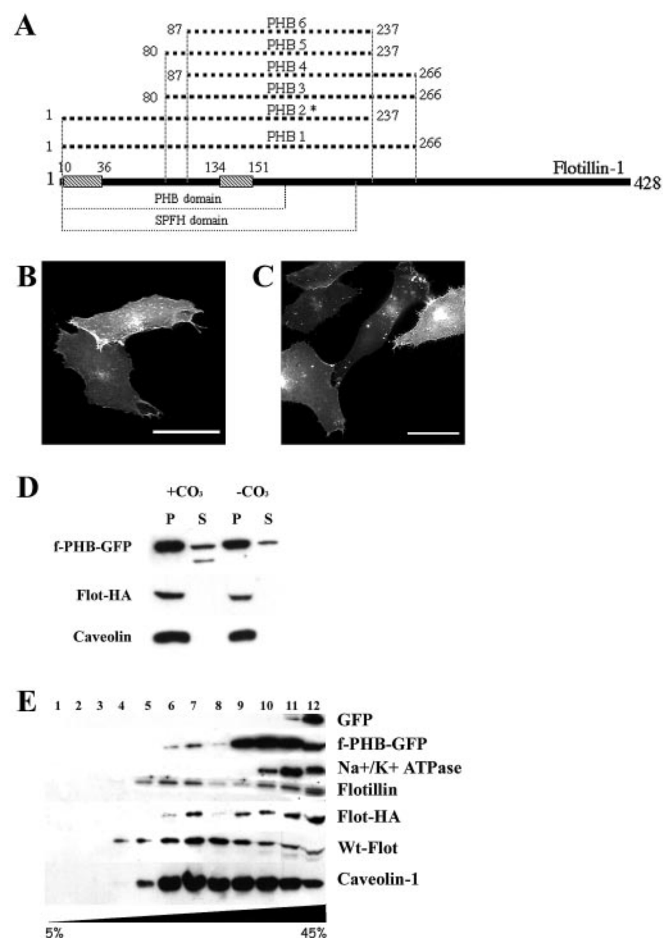

FIG. 4. **The PHB domain of flotillin-1 is sufficient to target a heterologous protein to the plasma membrane.** A series of truncation mutants of flotillin were fused to the N terminus of GFP. Their positioning relative to full-length flotillin-1 is represented in a schematic (*A*), which also shows the two hydrophobic domains (*shaded rectangles*), the putative PHB domain, and SPFH domain. Of these constructs, PHB-2 GFP (*asterisk*), subsequently called f-PHB-GFP, was found to target to the PM in Vero and BHK cells (*B* and *C*, respectively) with some evidence of possible aggresomal localization (puncta). f-PHB-GFP and flotillin are predominantly associated with the membrane pellet after high speed centrifugation of transfected BHK cells in the presence or absence of sodium carbonate (pH 11.5) treatment (*D*). Overexpressed wild type flotillin and Flot-HA show similar sedimentation characteristics to endogenous flotillin and caveolin after Triton X-100 treatment and flotation in a 5–45% sucrose gradient (*E*). f-PHB-GFP shows a greater proportion of protein floating than a soluble protein, GFP, and a non-raft membrane protein, Na^+/K^+ ATPase. However, a smaller percentage of total f-PHB-GFP floats to the lower density sucrose fractions than seen with full-length flotillin. *Bars*, 10 μm.

Finally, we investigated whether trafficking to the surface via a Golgi-independent pathway is a feature of other PHB-domain containing proteins. Stomatin is a related PHB-containing PM protein, which also associates with raft domains. Unlike flotillin-1, PM association of stomatin is strongly affected by BFA treatment (Fig. 5, *J–L*), showing that it is trafficked in a Golgi-dependent manner. Sequence alignments of a number of proteins containing the PHB motif show that stomatins fall within a distinct class of proteins (including stomatin, podocin, Mec2, Unc-1, and Unc-24), all of which contain a putative, N-terminal hairpin-like, intramembrane domain that is not present in the flotillin family (Fig. 6*A*). This domain may confer cotranslational insertion into the ER, and this suggests that the PHB domain may function in the context of both transmembrane proteins and cytoplasmic membrane-associated proteins.

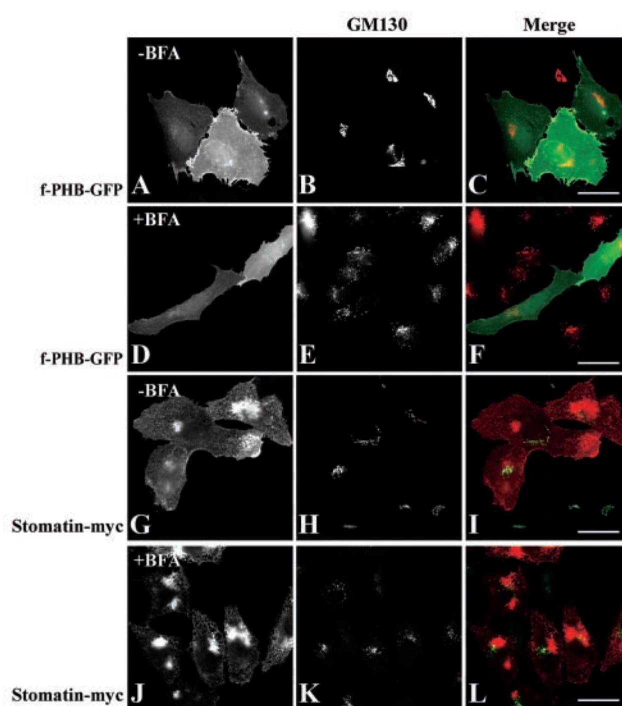

FIG. 5. **Trafficking of nascent f-PHB-GFP to the plasma membrane is insensitive to BFA treatment.** Vero cells were microinjected with f-PHB-GFP (*A–F*) or stomatin-myc (*G–L*) in the presence (*D–F* and *J–L*) or absence (*A–C* and *G–I*) of 5 μg/ml BFA and incubated for 5 h with or without BFA prior to fixation. The PM localization of f-PHB-GFP was not perturbed by BFA treatment (*D–F*), unlike stomatin, which was strongly affected (*J–L*). *Bars*, 10 μm.

Palmitoylation within the PHB Domain Is Involved in Flotillin-1 Association with the Plasma Membrane—The flotillin-related PM protein, stomatin, is palmitoylated at two sites in the N terminus of the protein (30). The region containing the major palmitoylation site in stomatin, Cys-29 (human), is lacking in flotillin-1 (Fig. 6*A*), but a second palmitoylated cysteine residue, Cys-85, is conserved (corresponding to Cys-34 in flotillin-1). Cys-34 is the only cysteine in flotillin-1 that is highly conserved among PHB domain-containing proteins and in flotillin-1 is evolutionarily conserved down to the goldfish, *Carassius auratus*. Hence, we investigated first whether flotillin-1 is palmitoylated, and second whether palmitoylation plays a role in association with the PM.

Cells were transfected with Flot-HA or f-PHB-GFP and then incubated with $[^3H]$palmitate for 2h. Flot-HA or f-PHB-GFP were specifically immunoprecipitated using antibodies to the HA or GFP tag, respectively. Labeling was specifically incorporated into both Flot-HA and PHB-GFP (Fig. 6*B*). Incorporation of label was hydroxylamine-sensitive and was greatly reduced by treatment with the specific inhibitor of palmitoylation, 2BP (41) (Fig. 6*C*). Incorporation of label was completely abolished by mutation of Cys-34 to Ala to create f-PHB-C34A-GFP (Fig. 6*D*), suggesting that Cys-34 is the major palmitoylation site in the PHB domain (which contains all the cysteine residues in flotillin-1). As well as identifying the major palmitoylation site in flotillin-1, these results strengthen our proposed model for the topology of flotillin-1 with both N and C termini cytoplasmically orientated.

We employed two strategies to investigate the role of palmitoylation in flotillin localization. We first treated cells with the palmitoylation inhibitor, 2BP. As a positive control we used GFP-tH, a dually palmitoylated and farnesylated protein that comprises the PM targeting information of H-Ras fused to GFP (39). Treatment with 2BP inhibited association of palmitoy-

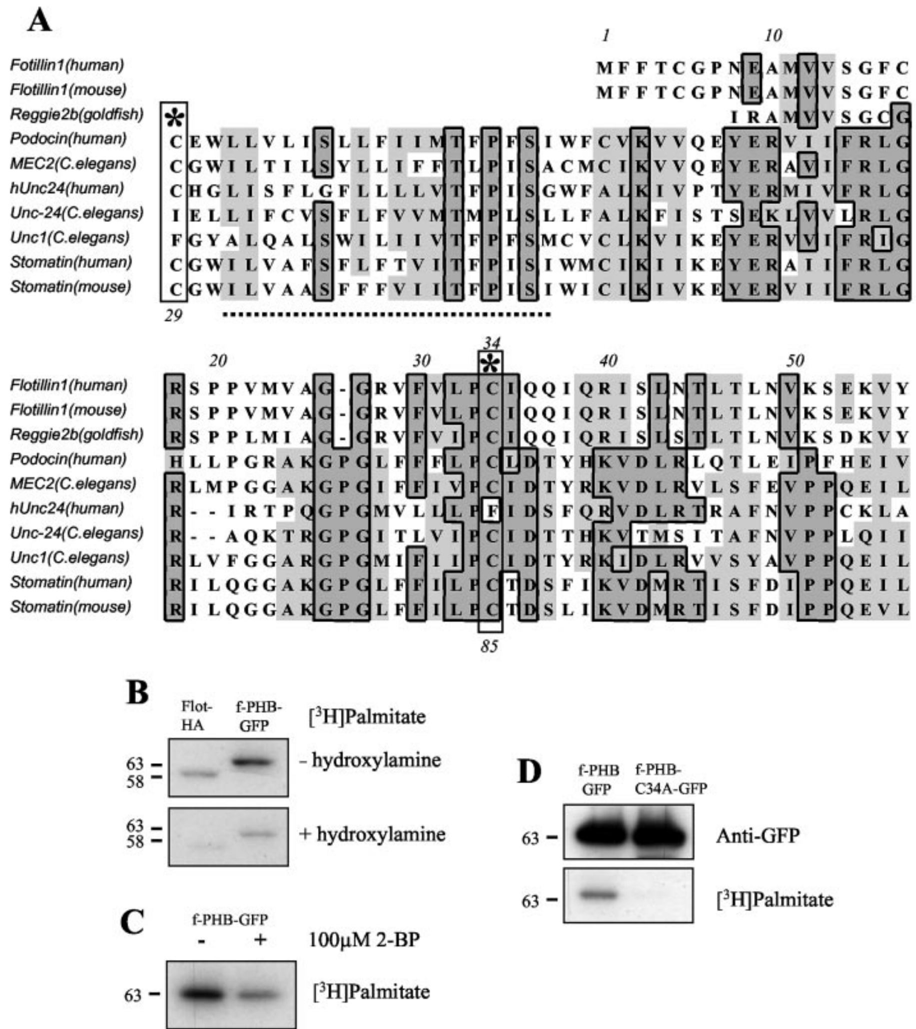

FIG. 6. **Palmitoylation of flotillin.** *A*, Cys-34 is highly conserved within the PHB domain of the stomatin family. A ClustalW alignment was performed with flotillin-1 (human, accession no. 075955), flotillin-1 (mouse, AH04647), reggie2b (goldfish, AK07568), podocin (human, Q9NP85), MEC2 (*Caenorhabditis elegans*, AAA82333), hUNC24 (human, AAD42031), UNC24 (*C. elegans* AAC02604), Unc-1 (*C. elegans*, T34324), stomatin (human, AAA58432), and stomatin (mouse, AAC64173). The two palmitoylation sites in stomatin are indicated (*boxed* regions with *asterisks*). The major palmitoylation site at position 29 in stomatin (30) is absent in flotillin-1; the minor palmitoylation site in stomatin (Cys-85) is conserved in several PHB-containing proteins including flotillin-1 (Cys-34). Flotillin-1 lacks the hydrophobic stretch of amino acids comprising the putative intramembrane domain of stomatin (*broken line*). *Numbering above* the alignment indicates amino acid position in flotillin-1 (human); *numbering below* indicates amino acid position in stomatin (mouse). *B–D*, palmitoylation of flotillin. BHK cells were transfected with either Flot-HA or f-PHB-GFP and then were labeled with [^{3}H]palmitate for 2h. They were subsequently immunoprecipitated with antibodies specific for their epitope tags. [^{3}H]Palmitate was specifically incorporated into both Flot-HA and f-PHB-GFP (which contains all the Cys residues in flotillin). Note that transfection efficiency was lower with Flot-HA giving rise to the lower signal. Samples treated with hydroxylamine to disrupt thioester linkages show a striking decrease in [^{3}H]palmitate signal when compared with untreated samples (*B*). Incorporation of [^{3}H]palmitate was also found to be significantly reduced upon treatment with 2BP in f-PHB-GFP-transfected cells (*C*). No [^{3}H]palmitate was incorporated into f-PHB-C34A-GFP compared with f-PHB-GFP, even though equal loading of protein was evident in the IP (*D*; anti-GFP).

lated GFP-tH protein with the PM (Fig. 7, *A* and *B*) but had no effect on the trafficking of GFP-GPI (Fig. 7, *C* and *D*), confirming the specificity of the agent. Treatment with 2BP caused a significant disruption of f-PHB-GFP association with the PM (Fig. 7*F*). Similar results were observed in cells injected with Flot-HA with greatly reduced PM staining (data not shown). We then investigated the localization of f-PHB-C34A-GFP. The mutant protein showed greatly decreased association with the PM, as judged by immunofluorescence and by membrane fractionation (Fig. 7, *H* and *I*). We tested the effect of the same mutation within the context of the full-length flotillin protein. Flotillin-C34A-HA showed a greatly decreased association with the PM with significant accumulation of the protein intracellularly (Fig. 7, *G* and *I*). Thus, the PHB domain of flotillin-1 can mediate association with the PM, and this requires palmitoylation of Cys-34.

DISCUSSION

In the present study, we have investigated the mechanism by which the raft-associated flotillin-1 protein accesses the PM and associates with specific surface domains. We have shown that association with the PM occurs in a Golgi-independent manner. The results identify a role for the PHB domain and palmitoylation in membrane targeting and have implications for a large family of PHB-containing proteins including stomatin and podocin.

Our results strongly support the hypothesis that flotillin-1 associates with the cytoplasmic face of the PM in an orientation similar to both stomatin and podocin. Previous studies showed protection of a 14-kDa N-terminal fragment of flotillin-1 upon protease treatment of Golgi vesicles, and this was interpreted as indicative of transmembrane orientation of flotillin with the N terminus exoplasmic/luminal (28). We speculate that the protection of a fragment of flotillin may instead reflect insertion of part of the PHB domain into the membrane and that flotillin associates with the inner face of the membrane. First, PM flotillin is unaffected by surface protease treatment. Second, we provide strong evidence for palmitoylation of the N terminus of flotillin and a role for palmitoylation in membrane association. Consistent with this, previous studies described dissociation of flotillin from the membrane upon hydroxylamine treatment of membranes to disrupt palmitoylation (13). Finally, we have shown that both full-length flotillin and an N-terminal domain of flotillin associate with the PM in a Golgi-independent manner, more consistent with post-translational association with membranes than cotranslational insertion of a transmembrane domain into the ER.

Although these studies suggest that PHB-domain-containing proteins such as flotillin and stomatins share a cytoplasmic orientation in the membrane, with both N- and C termini cytoplasmic (29, 33), a striking difference between flotillins and stomatin-like proteins (stomatin, podocin, prohibitin) is the presence of a highly hydrophobic putative intramembrane region in stomatin-like proteins which is absent in flotillins (Fig. 6*A*). This long hydrophobic segment may act as an uncleaved

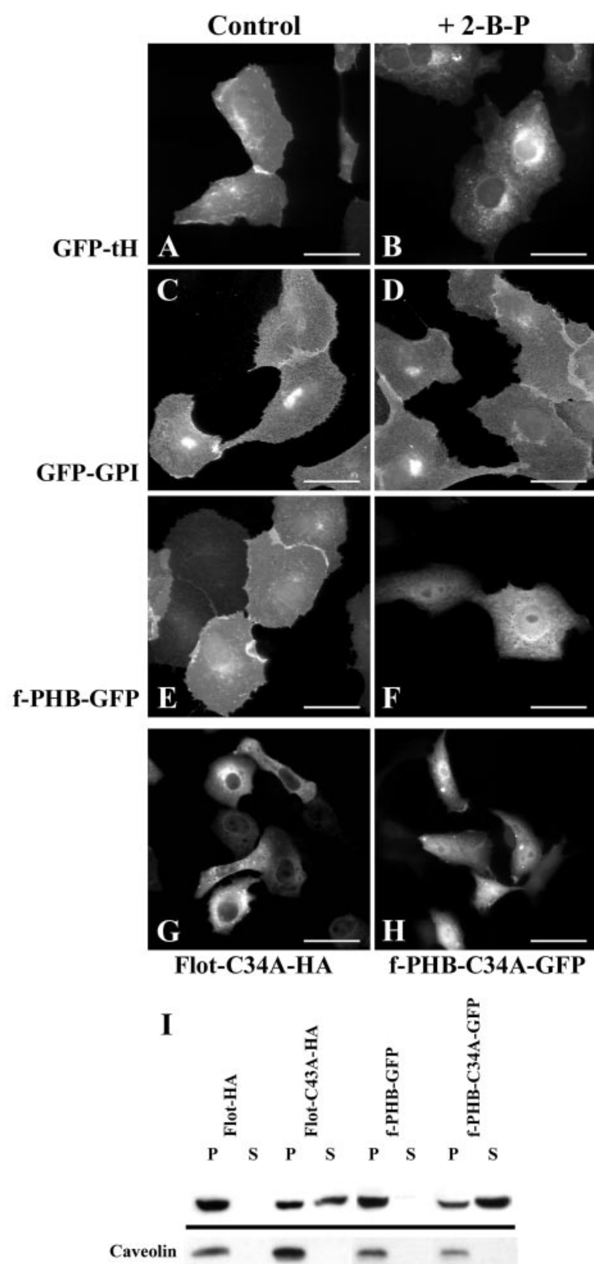

FIG. 7. **Plasma membrane localization of f-PHB-GFP requires palmitoylation of Cys-34.** Vero cells were injected with GFP-tH (*A* and *B*), GFP-GPI (*C* and *D*), or f-PHB-GFP (*E* and *F*) and subsequently incubated at 37 °C for 5 h in the presence (*B*, *D*, and *F*) or absence (*A*, *C*, and *E*) of 100 μM 2BP. GFP-tH and f-PHB-GFP show greatly reduced PM association after 2BP treatment (*B* and *F*, respectively) and an intracellular localization. PM association of the non-palmitoylated protein, GFP-GPI, detected by labeling for the GFP tag with anti-GFP followed by goat anti-rabbit-cy3, was unaffected by 2BP (*C* and *D*). Expression of the flotillin mutants Flot-C34A-HA and f-PHB-C34A-GFP in BHK cells (*G* and *H*, respectively) resulted in an intracellular expression pattern, suggesting that the cysteine at amino acid 34 is important for normal PM association. Western blot indicates the relative distributions of transfected Flot-HA, Flot-C34A-HA, f-PHB-GFP, and f-PHB-C34A-GFP in BHK crude cytosol (*S*) and membrane (*P*) fractions after high speed centrifugation (100,000 $\times$ *g*) (*I*). Flot-HA, f-PHB-GFP, and caveolin are tightly associated with membranes. In contrast, Flot-C34A-HA and f-PHB-C34A-GFP show a significant loss of membrane association and concomitant partitioning into the cytosolic fractions.

signal sequence for insertion into the ER, as suggested for caveolin-1, which also adopts a hairpin-like conformation in the membrane (42). This may give rise to the different trafficking pathways followed by the stomatin-like proteins and flotillin-1. As shown here, stomatin trafficking, unlike flotillin trafficking, is dependent on the Golgi complex.

The flotillin PHB domain possesses information for targeting a heterologous protein, GFP, to the PM and, like the full-length protein, does so via a Golgi-independent trafficking pathway. Thus, flotillin targeting, like that of K-Ras (39), occurs in a BFA- and Sar1-insensitive manner. In contrast, the trafficking of other PM proteins, such as H-Ras and GPI-anchored proteins, are both completely blocked by these agents (this study and Ref. 39). For both K-Ras and flotillin, the mechanisms involved and the role of possible chaperoning proteins remain to be elucidated. Although our studies identify a Golgi-independent trafficking pathway, which can be followed by flotillin-1 upon Golgi disruption, they do not rule out the trafficking of flotillin-1 by more than one pathway. In fact, at early times of expression, we observed significant association of f-PHB-GFP with the Golgi complex as identified by colabeling with the Golgi marker GM130 (results not shown). This raises the possibility that flotillin can use the conventional secretory pathway, at least partially, to reach the PM. This is consistent with a report of Golgi association of flotillin-1 (28).

On reaching the PM, flotillin and the f-PHB-GFP fusion protein are associated with the PM in a sodium carbonate-resistant manner, arguing for tight association with the membrane, presumably via one or both hydrophobic segments in the PHB domain of flotillin, rather than peripheral association with PM proteins or co-association with endogenous flotillin. The PHB domain is present within a diverse family of eukaryotic proteins, which are not all PM-associated, suggesting that differences in this domain, or in targeting pathways, might dictate the cellular location of the proteins. Intriguingly, even though these proteins have distinct destinations in the cell, they have been shown to associate with raft domains as determined biochemically. The archetypal member of the family, prohibitin, associates with mitochondria but was isolated in detergent-insoluble floated fractions from epithelial cells (25). Podocin and stomatin have also been shown to associate with raft domains (26, 27). It is therefore interesting to speculate that the PHB domain itself confers raft association. We find that the flotillin-PHB domain shows affinity for raft domains, as shown by the usual criteria of detergent insolubility and flotation in sucrose density gradients, but does not show the same high level of raft association as flotillin. Other properties of flotillin, such as oligomerization mediated by the coiled coil domain of the protein, outside the PHB domain, may be involved.

Our studies also suggest a role for palmitoylation in association of flotillin, and the PHB domain, with the PM. An inhibitor of palmitoylation reduced association of newly synthesized flotillin with the PM. Mutation of the conserved cysteine residue, Cys-34, in the N terminus of the protein greatly reduced labeled palmitate incorporation into flotillin and also inhibited PM association. The compartment in which palmitoylation takes place remains to be determined. Although the role of palmitoylation in raft association of membrane proteins is well established (43), the best studied pathway by which palmitoylated raft proteins reach the PM involves palmitoylation in the ER and then transport on the cytoplasmic surface of membranes of the secretory pathway to the cell surface. H-Ras, for example, is still palmitoylated when ER to Golgi traffic of newly synthesized H-Ras is inhibited by BFA (39). In contrast, K-Ras accesses the PM using an alternative, Golgi-independent pathway (39, 44).

In conclusion we have shown that the PHB/SPFH domain of flotillin possesses membrane targeting activity, which can confer raft association on a heterologous protein. The PHB/SPFH

domain is conserved even in prokaryotic proteins (20), suggesting an extremely fundamental role for this membrane domain. We have also identified a novel pathway by which flotillin-1 reaches the PM. The role of this trafficking pathway is unclear but may allow highly rapid changes in flotillin-1 localization in response to specific stimuli.

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