The Stomatin/Prohibitin/Flotillin/HflK/C Domain of Flotillin-1 Contains Distinct Sequences That Direct Plasma Membrane Localization and Protein Interactions in 3T3-L1 Adipocytes*

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Flotillin-1 is a lipid raft-associated protein that has been implicated in various cellular processes. We examined the subcellular distribution of flotillin-1 in different cell types and found that localization is cell type-specific. Flotillin-1 relocates from a cytoplasmic compartment to the plasma membrane upon the differentiation of 3T3-L1 adipocytes. To delineate the structural determinants necessary for its localization, we generated a series of truncation mutants of flotillin-1. Wild type flotillin-1 has two putative hydrophobic domains and is localized to lipid raft microdomains at the plasma membrane. Flotillin-1 fragments lacking the N-terminal hydrophobic stretch are excluded from the lipid raft compartments but remain at the plasma membrane. On the other hand, mutants with the second hydrophobic region deleted fail to traffic to the plasma membrane but are instead found in intracellular granule-like structures. Flotillin-1 specifically interacts with the adaptor protein CAP, the Src family kinase Fyn, and cortical F-actin in lipid raft microdomains in adipocytes. Furthermore, CAP and Fyn associate with different regions in the N-terminal sequences of flotillin-1. These results furthered our understanding for how flotillin-1 can function as a molecular link between lipid rafts of the plasma membrane and a multimeric signaling complex at the actin cytoskeleton.

The plasma membrane of most cell types contains specialized subdomains that are highly enriched in cholesterol and sphingolipids, referred to as lipid raft microdomains (1–4). Lipid rafts are highly organized, dynamic structures connected to the cytoskeleton and are enriched in growth factor receptors, integrins, Src family kinases, glycosylphosphatidylinositol-linked proteins, and adaptor proteins (5–7). The selective enrichment of key signaling molecules in these regions suggests that they could function as organization centers for signaling via the formation of multicomponent complexes.

Lipid raft domains are insoluble in nonionic detergents (Triton X-100) (5). Depending on the tissue and cell type, protein complexes found in these fractions often contain the proteins caveolin and/or flotillin (8–11). The insertion of caveolin-1 into lipid rafts results in the formation of caveolae, flask-shaped invaginations that are abundant in glial, epithelial, endothelial, muscle, and adipose cells (3). The flotillin/reggie proteins are ubiquitously expressed (8, 12). Reggie-1 and -2 were originally identified in developing neurons of goldfish optic nerves (12). The same proteins were subsequently identified from endothelial cells in low density detergent-insoluble complexes that also contained caveolin, where they were named flotillin-2 and -1 (8). Both isoforms were shown to interact and co-localize with the Src family kinase Fyn in lipid rafts derived from neurons and astrocytes, although in Jurkat lymphoma cells, flotillin proteins were found to concentrate in endolysosomal compartments (10). As well as its postulated role in axon regeneration in retinal ganglion cells (12), flotillin-1 has also been implicated in phagosome maturation (13) in macrophages and in NF-κB activation in Jurkat cells (14).

Flotillins belong to a larger family of proteins that share an evolutionarily conserved stomatin/prohibitin/flotillin/HflK/C (SPFH)3 domain (15). The function of SPFH domains remains unclear. Although most SPFH family members are integral membrane proteins with a separate membrane-associated region neighboring the SPFH domain, flotillin-1 has two hydrophobic sequences that are embedded in the SPFH domain (8). It has been suggested previously that flotillin-1/reggie-2 is an integral membrane protein with a short extracellular/luminal domain and a large C-terminal cytoplasmic domain (16). However, a recent study suggested that flotillin-1 was not a transmembrane protein but rather is targeted to the plasma membrane via palmitoylation of cysteine 34 within the SPFH domain (17).

We became interested in flotillin-1 when we identified the protein as a binding partner for CAP/ponsin in 3T3-L1 adipocytes (18). CAP is a multifunctional adaptor protein with three SH3 domains in its C terminus and a region of homology to the gut peptide sorbin in its N terminus. It is highly expressed in insulin-responsive peripheral tissues like skeletal muscle and fat (19). We provided evidence that in adipocytes flotillin-1 was able to anchor the CAP-Cbl complex to lipid rafts, an event that may be important for the insulin-signaling process leading to the stimulation of glucose uptake (20).

The overall sorbin homology (SoHo)/SH3 organization of CAP is also found in other proteins, including vinexin-α and

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1 The abbreviations used are: SPFH, stomatin/prohibitin/flotillin/HflK/C; PM, plasma membrane; CHO, Chinese hamster ovary; CAP, Cbl-associated protein; HDM, high density microsome; LDM, low density microsome; DMEM, Dulbecco’s modified Eagle’s medium; MDCK, Madin-Darby canine kidney cells; TES, 2-(2-hydroxy-1,1-bis(hydra-}

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Differentiation of 3T3-L1 cells to adipocytes was transfected by electroporation as described. COS-1 cells in 60-mm dishes were transfected by using FuGENE 6 reagent as described previously (29).

Construction of Recombinant Lentinus—We used a lentivirus vector (pHRTC5-CMV-WPRE) provided by Dr. M. Zhang (University of Michigan) to introduce flotillin-1 into different cell lines. The flotillin-1 cDNA fused at the C terminus with FLAG epitope was inserted into the BamHI and XhoI sites of the lentivirus vector. The vector (10 μg) was transfected into 293T cells cultured in a 15-cm dish together with the packaging mix (22 μg) by using Lipofectamine2000 from Invitrogen. The culture medium was collected at 60-h point post-transfection and was used directly to infect target cells. The target cells were usually seeded in a 6-cm dish overnight and were cultured subsequently in infectious media containing 8 μg/ml Polybrene for 12 h.

Immunoprecipitation and Immunoblotting—COS-1 cells in 60-mm dishes or 3T3-L1 adipocytes in 150-mm dishes were washed twice with ice-cold phosphate-buffered saline and were lysed for 30 min at 4 °C with buffer containing 50 mM Tris-HCl (pH 8.0), 135 mM NaCl, 1% Triton X-100, 1.0 mM EDTA, 1.0 mM sodium pyrophosphate, 1.0 mM sodium orthovanadate, 10 mM NaF, and protease inhibitors (1 mini tablet per 7 ml of buffer). The clarified lysates were incubated with the indicated antibodies for 2 h at 4 °C. For anti-CAP immunoprecipitation, 1 or 4 μg of anti-CAP antibodies (LY-1) from Santa Cruz Biotechnology, 1 or 4 μg of monoclonal antibodies against Pyk2-Cbl complex by ArgBP2 to flotillin-containing lipid rafts was implied to be critical for actin reorganization in growing neurites of differentiating PC12 cells (26).

In this study we characterize domains in flotillin-1 that contribute to its distinct subcellular localization and protein interactions in 3T3-L1 adipocytes.

MATERIALS AND METHODS

Antibodies and Reagents—The APS (V-19), PTG (N-19), CAP (P-17), Fyn (FY3N), and Myc (9E10) antibodies were purchased from Santa Cruz Biotechnology. The FLAG polyclonal antibody was obtained from Upstate Biotechnology, Inc. The cavelolin polyclonal antibody and the monoclonal antibodies against LAMP-1 and GM130 were purchased from BD Biosciences. Horseradish peroxidase-linked secondary antibodies were from Pierce. The Alexa Fluor secondary antibodies and phallolidin were from Molecular Probes. The anti-phosphotyrosine monoclonal antibody (4G10) and anti-CAP polyclonal antibody were from Upstate Biotechnology, Inc. The anti-phosphotyrosine monoclonal antibody (4G10) and anti-CAP polyclonal antibody were from Upstate Biotechnology, Inc. The anti-phosphotyrosine monoclonal antibody (4G10) and anti-CAP polyclonal antibody were from Upstate Biotechnology, Inc.

Plasmids and Mutagenesis—Myc-tagged cDNA constructs for different CAP isoforms and cavelolin-1 were prepared as described previously (27, 28). Mouse flotillin-1 full-length cDNA was derived from glutathione S-transferase-flotillin plasmid by PCR (20). FLAG-tagged flotillin-1 was constructed by placing flotillin-1 cDNA with FLAG tag fused at the C terminus in-frame in the BamHI and EcoRI sites of pBKH vector. Truncation mutants of flotillin-1 were generated by using a PCR-based cloning method. Point mutations of Cys-34 and internal deletion of the second hydrophobic domain were made by using the Stratagene QuikChange mutagenesis kit, according to the manufacturer’s protocol. The mutations and cloning products were confirmed by automated DNA sequencing. A diagram of different flotillin-1 constructs used in the present study is shown in Fig. 1.

Cell Culture and Transient Transfection—Chinese hamster ovary (CHO) cells were cultured in α-minimum Eagle’s medium containing 10% fetal bovine serum. Human cervical carcinoma (HeLa), human hepatocellular carcinoma (HePG2), dog kidney (MDCK), and COS-1 cells were grown in DMEM containing 10% fetal bovine serum. Mouse 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% calf serum, 100 units/ml penicillin G, soybean and 100 μg/ml streptomycin sulfate. Differentiation of 3T3-L1 cells to adipocytes was induced with 1 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutyl-1-methylxanthine as described previously. The cells were then cultured in DMEM containing 10% fetal bovine serum. Rat L6 myoblasts were maintained in DMEM with 15% fetal bovine serum. Differentiation of L6 cells to myotubes was induced by replacing the cells in DMEM containing 1% fetal bovine serum for 3 days. 3T3-L1 adipocytes were transfected by electroporation as described. COS-1 cells in 60-mm dishes were transfected by using FuGENE 6 reagent as described previously (29).

RESULTS

Subcellular Localization of Flotillin-1 in Cultured Cells—Given the numerous cellular processes in which flotillin-1 has been implicated, we wanted to examine and compare its localization determinants in flotillin-1 constructs.
Flotillin-1 is localized differently in preadipocytes and adipocytes. Differentiation-dependent re-localization of flotillin-1 appears to be cell type-specific. Relative distribution between these two compartments appears to be cell type-specific.

**The Plasma Membrane Localization of Flotillin-1 in 3T3-L1 Adipocytes Is Differentiation-dependent**—To determine whether flotillin-1 is localized differently in preadipocytes versus adipocytes, we extracted 3T3-L1 preadipocytes and 3T3-L1 adipocytes with Triton X-100 under low pH conditions. Proteins from soluble and insoluble fractions were analyzed by immunoblotting with different antibodies (Fig. 4). In preadipocytes, ~75% of flotillin-1 protein was present in the Triton-insoluble fraction, whereas ~25% of the protein remained in the soluble fraction. Upon differentiation into mature adipocytes, flotillin-1 protein was exclusively found in the Triton-insoluble fraction. Most interestingly, Fyn, a Src family tyrosine kinase that was shown to interact with flotillin-1 in rat brain and PC12 cells, also exhibited a similar differentiation-dependent lipid raft distribution. As a control, caveolin was found to be localized exclusively to lipid rafts regardless of the differentiation status of the cells.

Previous studies have shown that lipid raft subdomains in the plasma membrane can serve as anchors for unique actin cytoskeleton structures (3, 6). To explore the role of the actin cytoskeleton in flotillin localization, we treated cells with or without the actin filament disrupting drug cytochalasin D prior to the Triton extraction. As shown in Fig. 4, disruption of actin cytoskeleton had no effect on the lipid raft localization of either flotillin-1 or caveolin in both pre-adipocytes and adipocytes.

**The N-terminal Hydrophobic Stretch Is Required for the Lipid Raft Localization of Flotillin-1 in Adipocytes**—To identify the structural determinants responsible for the localization of flotillin-1 to the lipid raft domains of adipocytes, we generated a series of truncation mutants of flotillin-1 tagged at the C terminus with a FLAG epitope. We first examined the effects of these truncations on the flotillin-1 localization in intact 3T3-L1 adipocytes (Fig. 5A). As expected, transient expression of full-length flotillin-1 resulted in a predominant PM localization. Deletion of either the N-terminal 36 residues that contain the first hydrophobic stretch or the whole C-terminal portion after the second hydrophobic stretch had no significant effect on this PM distribution. In contrast, expression of a fragment (flotillin-1(1–134)) that lacked the N-terminal region, including both hydrophobic domains, resulted in predominantly a cytoplasmic localization with only a small amount of protein present at the PM. Moreover, an N-terminal fragment (flotillin-1(1–134)) that ended immediately before the second hydrophobic domain was retained exclusively in intracellular granular compartments. The results demonstrate that the second but not the first hydrophobic domain is required for the trafficking of flotillin-1 to the plasma membrane of 3T3-L1 adipocytes.

We next examined the localization of full-length and truncated forms of flotillin-1 to the lipid raft microdomains of the PM. We co-expressed caveolin-EGFP protein with various FLAG-tagged flotillin proteins in 3T3-L1 adipocytes. Cells were then extracted with ice-cold Triton X-100 at low pH conditions, and the resultant membrane sheets were visualized by fluorescence microscopy. Although it is likely that not all proteins present in the Triton-insoluble PM sheets are lipid raft-associated, the complete removal of flotillin-1 and caveolin from these structures by cyclodextrin-induced cholesterol depletion argues that this is a valid assay for studying lipid raft localization of flotillin-1 (data not shown). As shown in Fig. 5A, the caveolin-EGFP protein remained attached to the coverslips in a punctate pattern in the Triton-extracted PM sheets. Consistent with its compartmentalization in lipid rafts, the full-length flotillin...
was resistant to cold Triton extraction and remained in the membrane sheets. In contrast, the PM-localized flotillin-(36–428) was completely extracted with Triton and thus absent from the membrane sheets. Because flotillin-(1–134) and flotillin-(151–428) were largely cytosolic, they also were completely removed by Triton treatment. Taken together, these data indicate that the first hydrophobic domain does not contribute to the PM association of flotillin-1 but, instead, plays an important role in targeting the protein to the lipid raft microdomains.

To confirm the results obtained from the histochemical studies, transfected cells were also subject to subcellular fractionation into Triton-soluble and -insoluble fractions. The distribution of various flotillin mutants between these two fractions was examined by anti-FLAG immunoblotting. As shown in Fig. 5B, although over 60% of the full-length protein was found in the Triton-insoluble fraction, less than 30% of flotillin-(36–428) and less than 20% of flotillin-(151–428) were localized in the same fraction. This is consistent with the results obtained from immunostaining as described above. Most surprisingly, flotillin-(1–134), the truncated form that existed in the intracellular granular compartments as revealed by immunostaining, was found to be predominantly present in the Triton-insoluble fraction. Therefore, the first hydrophobic domain is necessary for targeting flotillin-1 to lipid raft microdomains in both PM and intracellular membrane structures.

Previous studies have reported that palmitoylation of Cys-34 is involved in flotillin-1 association with the PM in Vero cells (17). To determine whether palmitoylation of Cys-34 also is required for the PM localization of flotillin in adipocytes, we transfected 3T3-L1 adipocytes with constructs encoding wild type or C34A mutant forms of flotillin-1 together with caveolin-EGFP. As observed previously, the full-length wild type flotillin was associated with the PM in intact cells and localized with caveolin-EGFP in Triton-extracted PM sheets. In contrast, flotillin-(1–134), the truncated form that existed in the intracellular granular compartments as revealed by immunostaining, was found to be predominantly present in the Triton-insoluble fraction. Therefore, the first hydrophobic domain is necessary for targeting flotillin-1 to lipid raft microdomains in both PM and intracellular membrane structures.

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Identification of the Second Hydrophobic Domain as the PM Localization Signal of Flotillin-1—The loss of discrete localization of flotillin-(1–134) and flotillin-(151–428) at the cell surface of adipocytes prompted us to hypothesize that the second hydrophobic domain, between residues 134 and 151, targets flotillin-1 to the plasma membrane. To delineate the sequence requirements for PM localization, we fused various N-terminal portions of flotillin to the FLAG tag and transiently expressed each fusion protein in 3T3-L1 adipocytes. As described above, full-length flotillin and flotillin-(1–134) were localized to PM and intracellular granules, respectively (Fig. 6). Flotillin-(1–142), generated by adding back half of the second hydrophobic stretch to flotillin-(1–134), was still retained in intracellular compartments. In addition, an internal deletion of this sequence from the full-length protein had precisely the same effect on the localization as partial and complete truncations of this region (Fig. 6). On the other hand, C-terminal truncations (flotillin-(1–161) and -(1–151)) that possess the intact second hydrophobic domain exhibited well defined PM staining. Consistent with the finding obtained from the full-length protein (Fig. 5A), deletion of the first hydrophobic sequence from flotillin-(1–161) did not affect its PM targeting. Together, these data indicate that the second but not the first hydrophobic domain of flotillin-1 is needed for the PM localization and for the exclusion of the protein from intracellular granular structures.

CAP Is an Actin-binding Protein at the Plasma Membrane of 3T3-L1 Adipocytes—CAP/ponsin is a multivalent adaptor pro-
tein highly expressed in fat and muscle (19). Because it was thought to be a vinculin-binding protein at the adherens junctions in fibroblasts (25) and a PM lipid raft component in adipocytes (18), we attempted to determine whether CAP would associate with the cortical actin cytoskeleton at the PM of adipocytes. Immunofluorescence staining was performed on 3T3-L1 adipocytes by using a CAP-specific antibody to detect endogenous CAP. Fluorescein-conjugated phalloidin was used to probe for F-actin (Fig. 7). In the middle section of the cells, confocal microscopy revealed that endogenous CAP was predominantly confined to the cortical F-actin structure with less detectable intracellular staining. At the bottom plasma membrane section, endogenous CAP was found to be co-localized exceptionally well with F-actin at the cell edge and the adhesion-like punctate structures. Most interestingly, CAP concentrated at the ends and the branching points of short actin filaments at the basal plasma membrane level. Moreover, we also found that CAP was capable of binding directly to both G-actin and F-actin in in vitro glutathione S-transferase pull-down and sedimentation experiments.

Identification of a region important for PM localization of flotillin-1 in 3T3-L1 adipocytes. 100 µg of FLAG-tagged constructs encoding for full-length flotillin-1, flotillin-1–134, flotillin-1–142, flotillin-1–161, flotillin-(36–161), flotillin-(1–151), and full-length (FL) flotillin deleted in the second hydrophobic stretch (JHS2) were transiently expressed in differentiating adipocytes by electroporation. The cells were allowed to recover on coverslips for 36 h before fixation and subsequent immunostaining with a polyclonal FLAG antibody to visualize flotillin-1.

Previous studies have shown that flotillin-1 and flotillin-2 are associated with the Src family kinase Fyn in detergent-resistant fractions of T lymphocytes, suggesting that flotillin may play an important role in the formation of signal transduction centers (10). To confirm this interaction, we transfected COS-1 cells with wild type or kinase dead mutants of Fyn with flotillin-1 tagged with FLAG. Western blot analyses of the anti-FLAG immunoprecipitation revealed that both forms of Fyn associated with flotillin-1. No Fyn was detected in the control immunoprecipitates lacking flotillin-1. Anti-phosphotyrosine blotting revealed that the kinase inactive mutant of Fyn had no auto-phosphorylation activity (Fig. 11B). Thus, the Fyn kinase forms a complex with flotillin-1 independently of its kinase activity.

To map the Fyn-interacting region in flotillin-1, we co-expressed Fyn and various truncation mutants of FLAG-tagged flotillin-1. Co-immunoprecipitation assays showed that deletion of the N-terminal 36 amino acids or the large C-terminal portion of the second hydrophobic domain did not eliminate Fyn binding, indicating that these regions are not required for the interaction. However, N-terminal deletion to the end of the second hydrophobic domain in flotillin completely abolished anti-Myc antibody followed by anti-FLAG immunoblotting. As shown in Fig. 8B, flotillin-FLAG was only specifically co-precipitated with CAP4 but not with the other three isoforms. A reciprocal immunoprecipitation was performed using anti-FLAG antibody and revealed the same result (Fig. 8C). Therefore, the interaction of flotillin with CAP is isoform-specific.

Co-localization of CAP4 with Flotillin-1 and F-actin—The finding that CAP4 binds both flotillin-1 and actin led us to test whether the PM localization of CAP4 is independent of the interaction of CAP4 with flotillin-1. We next co-expressed Myc-CAP4 with flotillin-FLAG in differentiated 3T3-L1 adipocytes (Fig. 9B). Both CAP proteins displayed PM rim staining similar to flotillin-FLAG in intact cells. When cells were extracted with cold Triton-100, we observed that the staining of CAP4 overlapped exactly with flotillin-FLAG in the resultant Triton-resistant PM sheets. Pretreatment of cells with cytochalasin D to disrupt cortical F-actin largely removed CAP4 from the PM rim as well as the Triton-insoluble membrane sheets. On the other hand, the loss of cortical actin had no effect on the localization of flotillin in the PM lipid rafts. These results indicate that CAP4 is associated with flotillin-1 in the PM lipid raft microdomain. This co-localization is dependent on the integrity of the cortical F-actin structure.

Identification of CAP4 and Fyn Kinase Interaction Domains in Flotillin-1—We performed deletion analyses to define the region of flotillin-1 required for binding to CAP4. Flotillin-(1–161) and -(1–331) interacted with Myc-CAP4 with the same affinity as the full-length flotillin, whereas no Myc-CAP4 was precipitated when vector alone was co-expressed. Moreover, deletion of the sequence that includes the first hydrophobic domain (1–35) from either full-length or flotillin-(1–161) gave rise to a protein that bound CAP4 to a lesser extent. Immunoblotting showed that a comparable amount of the Myc-CAP4 protein was present in the various lysates (Fig. 10). These observations suggested that the first hydrophobic domain of flotillin-1 is needed for its interaction with CAP4.

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Fyn binding (Fig. 11A). These results indicated that the region between the two hydrophobic domains in flotillin mediates the binding of the protein to Fyn.

To determine whether Fyn affected the binding of CAP4 to flotillin-1, COS-1 cells were also transfected with flotillin-FLAG and Myc-CAP4 constructs in the absence or presence of Fyn. Lysates were precipitated with anti-FLAG antibodies. Anti-Myc immunoblotting showed that the amount of Myc-CAP4 co-precipitated with flotillin-FLAG was not altered significantly when either wild type or kinase dead mutant Fyn was co-expressed (Fig. 12A). Moreover, co-expression of CAP4 had no effect on the interaction between Fyn and flotillin in a similar co-immunoprecipitation experiment (Fig. 12B). These results suggest that the interaction regions in flotillin-1 for CAP4 and Fyn do not overlap with each other. Therefore, these three proteins may be capable of co-existing in a multimeric complex.

DISCUSSION

Cell Type and Differentiation-dependent Localization of Flotillin-1—Flotillin-1 and -2 are ubiquitously expressed lipid raft proteins that have been linked with many cellular events. Previous findings from Stuermer and co-workers (9, 10) suggested that flotillin proteins occurred in clusters at the PM of neuron and glial cells. It was also shown recently that flotillin-1 was localized to the cell surface of the kidney-derived cell lines baby hamster kidney and Vero (17). In Jurkat lymphoma cells, however, both flotillins were co-localized with Thy-1 and Fyn in intracellular organelles that resemble endolysosomes (10). In macrophages, flotillin-1 was found to be present on phagosomes (31). Thus, the subcellular localization of flotillin proteins appears to be cell type-specific. Likewise, we demonstrated that although flotillin-1 resided in the intracellular granule-like compartments in cell lines like MDCK and L6, the protein is localized at the plasma membrane in other cells such as HeLa, CHO, and HepG2. Furthermore, we found that flotillin-1 externalized from intracellular compartments to the PM during the process of cellular differentiation. The intracellular flotillin-positive granules in pre-adipocytes were identified as endolysosomes by anti-Lamp-1 immunostaining. Most interestingly, flotillin-1 was found to localize to the Golgi complex in undifferentiated PC12 cells (16). Upon nerve growth factor-induced differentiation, flotillin-1 was localized to the cell surface (9), although myogenic differentiation of L6 cells did not result in a similar re-distribution of flotillin-1.

It is now well established that lipid rafts exist in internal membranes as well as the PM (16, 32, 33). Our results imply a scenario in which differentiation can induce the incorporation of flotillin-1 and probably other raft-associated proteins, previously sequestered in the membrane of intracellular compartments, into the lipid raft microdomains of the PM. Upon reaching the PM, flotillin-1 can participate in processes as diverse as signal trans-
duction, lipid and/or protein trafficking, and cytoskeletal and lipid raft organization. Consistent with this, we found that flotil-
lin-1 was able to interact with the multivalent adaptor protein
CAP at the PM of adipocytes.

Targeting Domains of Flotillin-1—
Although its membrane
association has been determined by biochemical purification
and microscopic immunostaining, whether flotillin-1 is an-
tegral or a peripheral membrane protein remains controver-
sial. Also uncertain is the membrane topology of the protein.
Despite high evolutionary conservation ranging from flies to
mammals, flotillins do not exhibit any recognizable domains or
motifs aside from the so-called SPFH domain in the N-terminal
region (15). Stomatin, whose function remains obscure, is a
32-kDa integral membrane protein found in the PM of eryth-
rocytes. Prohibitin is an inner mitochondrial membrane pro-
tein that functions as a chaperone in the assembly of subunits
of mitochondrial respiratory chain complexes. Whereas both
stomatin and prohibitin have a separate membrane association
sequence outside of the SPFH domain, computer-aided analyses
predicted two hydrophobic stretches (residues 10–36 and

Fig. 9. Co-localization of flotillin-1, CAP4, and F-actin. A, COS-1 cells were
transfected with 0.5 μg of flotillin-FLAG in the presence or absence of 1 μg of Myc-
CAP4. Cells were fixed and subjected to confocal fluorescent microscopy using
FLAG and Myc antibodies and Alexa 594-labeled phallloidin. Both peripheral and
bottom sections are presented. B, 3T3-L1 adipocytes electroporated with 50 μg of
flotillin-FLAG and 100 μg of Myc-CAP4 were incubated on coverslips for 36 h.
Cells were then pretreated with 2 μM of cytochalasin D (cyto D) for 2 h before sub-
jecting to cold Triton X-100 (TX) extrac-
tion. Flotillin-FLAG and Myc-CAP4 in in-
tact cells and Triton-extracted PM sheets
were visualized by immunofluorescent
microscopy.

Fig. 10. Identification of region in flotillin-1 critical for CAP4
association. COS-1 cells were co-transfected with 0.5 μg of Myc-CAP4
and 1 μg of different flotillin-FLAG constructs (lane 1, vector alone; lane
2, full length; lane 3, 36–428; lane 4, 36–161; lane 5, 1–161; lane 6,
1–331; and lane 7, 151–428). Flotillin-FLAG proteins were immuno-
precipitated (IP) with a FLAG antibody. Flotillin-FLAG and Myc-CAP4 in
immunoprecipitates and lysates were detected by anti-FLAG and
-Myc immunoblotting.
Localization Determinants in Flotillin-1

We demonstrated by deletion analyses the surprising result that the first hydrophobic stretch, which includes the postulated palmitoylation site Cys-34, was not needed for the PM localization of flotillin-1 in adipocytes. Instead, it was found to be involved in targeting of the protein to the lipid raft microdomains, evidenced by the fact that the protein lacking this sequence failed to associate with detergent-resistant fractions in both immunostaining and subcellular fractionation procedures. However, mutation of Cys-34 had no effect on the lipid raft association of flotillin-1. The cause of the discrepancy between our results and the previous finding (17) obtained with fibroblastic cells is not clear. One possibility is that the importance of the palmitoylation of Cys-34 in the PM localization may also be cell type-specific. In adipocytes, an important role for the second hydrophobic domain in the subcellular distribution of flotillin-1 was suggested by the truncation or internal deletion of this hydrophobic stretch, which inhibited the PM localization. Moreover, the mutated proteins were all retained in the intracellular granular compartments. These results implied that the second hydrophobic region might serve as a PM localization signal as well as a signal to exclude flotillin-1 from the internal membrane compartments. However, it remains to be established whether the second hydrophobic stretch of flotillin-1 is anchored in the PM. Most interestingly, a hairpin hydrophobic domain has been proposed to give rise to an atypical membrane topology for most proteins in SPFH family (15).

At this point we cannot rule out the possibility that a separate motif in flotillin-1 may be responsible for membrane association through the tight interaction with a separate transmembrane protein. In fact, a flotillin-1 fragment between the two hydrophobic stretches was still able to associate with the internal membrane compartments, suggesting the presence of such a motif in this region. On the other hand, flotillin-2 does not contain any continuous hydrophobic sequence, but it still shows membrane localization similar to that displayed by flotillin-1 in a variety of cells (9, 10). The sequence in flotillin-2 corresponding to the second hydrophobic stretch of flotillin-1 contains two additional charged residues, making it an unlikely membrane-spanning or -anchoring domain (8). Although a recent study indicated that myristoylation of Gly-2 along with palmitoylation of several unique N-terminal cysteine residues are necessary for the PM localization of flotillin-2 (34), it will be interesting to determine what sequences mediate the membrane association of flotillin-2 when present in intracellular compartments like Golgi and endolysosomes.

Flotillin Interacts and Co-localizes with CAP and Actin—CAP has been shown previously to play important roles in insulin signaling via binding to Cbl (19, 20) and in cytoskeletal organization via binding to vinculin (25). The third and the first two SH3 domains of CAP interact with the respective proline-rich regions of Cbl and vinculin. Earlier studies from our group (18) demonstrated a specific interaction between CAP and flotillin-1 in yeast two-hybrid and glutathione S-transferase pull-down experiments. The SoHo domain in CAP was later identified to be the flotillin-binding motif and was required for CAP to be present in the lipid raft microdomains of PM (35). Over-expression of mutant forms of CAP in which either all three SH3 domains or the SoHo region were deleted blocked the insulin-stimulated tyrosine phosphorylation of Cbl in 3T3-L1 adipocytes (18, 35). These results led us to hypothesize that CAP was involved in an insulin-signaling process that would require the lipid raft localization of CAP. Here we presented additional evidence that endogenous CAP and flotillin-1 proteins interact with each other in adipocytes, where CAP also is co-localized with cortical F-actin and actin-enriched adhesion-like structures at the PM. Among the four CAP isoforms known

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**Fig. 11.** In vivo interaction between flotillin-1 and Fyn. A, COS-1 cells were co-transfected with 0.5 μg of Fyn and 1 μg of different flotillin-FLAG constructs (lane 1, vector alone; lane 2, full length; lane 3, 1–161; lane 4, 1–331; lane 5, 36–428; lane 6, 151–428; lane 7, Δcoil). Flotillin-FLAG proteins were immunoprecipitated (IP) with a FLAG antibody. Co-precipitated Fyn was detected by immunoblotting with a Fyn antibody. B, COS-1 cells were transfected with flotillin-FLAG alone (lane 1), flotillin-FLAG and wild type Fyn (lane 2), or kinase dead Fyn (lane 3), or wild type Fyn alone (lane 4). Anti-FLAG immunoprecipitation was performed, and co-precipitated Fyn was revealed by anti-phosphotyrosine immunoblotting.

**Fig. 12.** CAP4 and Fyn do not compete with each other in binding to flotillin-1. A, COS-1 cells were co-transfected with 0.5 μg of Myc-CAP4 and 0.5 μg of flotillin-FLAG in the presence or absence of 1 μg of Fyn. Flotillin-FLAG proteins were immunoprecipitated (IP) with a FLAG antibody. Co-precipitated CAP4 was detected by immunoblotting with a Myc antibody. B, COS-1 cells were co-transfected with 0.5 μg of Fyn and 0.5 μg of flotillin-FLAG in the presence or absence of 1 μg of Myc-CAP4. Flotillin-FLAG proteins were immunoprecipitated with a FLAG antibody. Co-precipitated Fyn was detected by immunoblotting with a Fyn antibody.

134–151) within the SPFH domain of flotillin-1. Co-fractionation with the PM in the presence of sodium carbonate at pH 11.5 suggested that flotillin-1 was an integral membrane protein (8). Results obtained from proteinase K digestion of isolated Golgi membrane vesicles further indicated that flotillin-1 was a transmembrane protein with the second hydrophobic stretch as its single membrane-spanning domain (16). However, Morrow et al. (17) later showed that the SPFH domain mediated the PM association of flotillin-1 in a palmitoylation-dependent manner. Cysteine 34, located near the end of the first hydrophobic stretch, was identified as the sole palmitoylation site. Moreover, proteinase K digestion of non-permobilized cells revealed that flotillin-1 was not a transmembrane protein at the PM (17). This discrepancy over the membrane topology of flotillin-1 led us to investigate the mechanisms by which flotillin-1 is targeted to the PM lipid rafts of adipocytes, and whether the two hydrophobic stretches of flotillin-1 contribute in any way to its subcellular localization.
in adipocytes, CAP4 was the only one capable of binding to flotillin-1 in a co-immunoprecipitation assay. Most interestingly, CAP4 also is the only isoform possessing a unique proline-rich sequence that is believed to mediate the formation of a homodimeric complex. These results suggest that the overall conformational change brought on by this proline-rich sequence is critically involved in the association of full-length CAP with flotillin-1 in vivo, whereas the SoHo domain may function as a direct binding motif.

It is now becoming clear that both the lipid and protein components of rafts communicate with the actin cytoskeleton directly, thereby regulating cellular responses (36). Recruitment of the Pyk2-Cbl complex by ArgpBP2 to flotillin-containing lipid rafts was suggested to play an important role in signals governing cytoskeletal changes during neurite growth (26). In this study, we show by several independent criteria that CAP4 might function as a linker between the actin cytoskeleton and flotillin-containing lipid rafts at the PM. 1) CAP4 was co-localized with flotillin-1 and F-actin at the PM. Although the actin cytoskeleton was sensitive to cold Triton extraction of the cells, CAP4 was found to be present in Triton-insoluble PM sheets derived from adipocytes together with flotillin-1. 2) CAP proteins co-localized and interacted directly with actin filaments. Ectopic expression of CAP promoted actin polymerization and formation of focal adhesion-like structures. 3) Disruption of F-actin by cytochalasin D treatment of cells greatly diminished the expression of Fyn had no effect on the CAP4-flotillin interaction regions in the SPFH domain of flotillin-1. In addition, co-expression of Fyn in the flotillin-CAP-actin complex presumably may aid in maintaining the correct F-actin structure and also increase signal transduction efficacy in the flotillin-containing lipid rafts of adipocytes.

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