Endocytosis of flotillin-1 and flotillin-2 is regulated by Fyn kinase

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Summary
Flotillin-1 and flotillin-2 co-assemble into plasma membrane microdomains that are involved in the endocytosis of molecules such as glycosyl phosphatidylinositol (GPI)-linked proteins. Previous studies suggest that budding of flotillin microdomains from the plasma membrane is a tightly regulated process. Here, we demonstrate that endocytosis of flotillins is regulated by the Src family kinase Fyn. The Src kinase inhibitor PP2 prevents EGF-induced flotillin internalisation, and EGF-induced internalisation does not occur in SYF cells lacking Src, Yes and Fyn. Expression of Fyn, but not Src or Yes, restores EGF-induced internalisation in SYF cells. Expression of an active form of Fyn but not other Src kinases is sufficient to induce redistribution of flotillins from the plasma membrane to late endosomes and lysosomes. Using two partial Fyn constructs that form a functional kinase upon addition of rapamycin to cells, we show that flotillin internalisation from the plasma membrane occurs shortly after Fyn activation. Tyr160 in flotillin-1 and Tyr163 in flotillin-2 are directly phosphorylated by Fyn, and mutation of these residues to phenylalanine prevents Fyn-induced flotillin internalisation. Uptake of the GPI-linked protein CD59 is reduced by expression of the phenylalanine-mutated flotillins. These data establish uptake of flotillin microdomains as a tyrosine-kinase-regulated endocytic process.

Introduction
Flotillin-1 (FLOT1 or reggie-2) and flotillin-2 (FLOT2 or reggie-1) are ubiquitously expressed proteins in mammalian cells (Babuke and Tikkalanen, 2007). They are approximately 50% identical in their amino acid sequence, and belong to a large family of proteins containing a stomatin-prohibitin-flotillin-HflK/C (SPFH) domain, a motif that is suggested to participate in protein oligomerisation (Yokoyama et al., 2008). Flotilins have been reported to have a role in a variety of different cellular processes. These include cell adhesion, endocytosis, phagocytosis and cell signalling events (Bared et al., 2004; Bickel et al., 1997; Hoehne et al., 2005; Langhorst et al., 2007; Rajendran et al., 2003). The proteins localise both to plasma membrane microdomains and to intracellular organelles. Flotillin-1 and flotillin-2 associate to form hetero-oligomeric complexes, which can be visualised by fluorescence microscopy as small puncta, or microdomains, on the plasma membrane (Frick et al., 2007). Flotillin complexes are highly detergent resistant, and therefore flotillins are often used as ‘lipid-raft’ markers (Bickel et al., 1997; Stuermer et al., 2001).

Flotillin microdomains form endocytic structures that are involved in the uptake of plasma membrane components such as glycosyl-phosphatidylinositol (GPI)-linked proteins and glycosphingolipids (Blanchet et al., 2008; Frick et al., 2007; Glebov et al., 2006; Payne et al., 2007; Schneider et al., 2008). Flotillin microdomains are separate from regions of the plasma membrane involved in other alternative endocytic mechanisms, including caveolin-1-positive caveolae (Pelkmans et al., 2004; Stan, 2002), and (on the basis of their differing ultrastructure) the tubular endocytic intermediates of the CLIC-GEEC pathway (Kirkham et al., 2005; Sabharanjak et al., 2002). Flotillin microdomains are abundant, laterally mobile and dynamic structures on the plasma membrane, but they appear to bud into the cell relatively infrequently. We are therefore interested to know how flotillin-dependent endocytosis is regulated.

EGF induces relocation of flotillin-2 from the plasma membrane into intracellular organelles (Neumann-Giesen et al., 2007). Src family tyrosine kinases are activated upon EGF binding to its receptor, and several Src kinases have been shown to bind flotillins (Kato et al., 2006; Liu et al., 2005; Neumann-Giesen et al., 2007; Stuermer et al., 2001). Furthermore, overexpression of Src induces phosphorylation of multiple tyrosines on flotillin-2 (Neumann-Giesen et al., 2007). The aim of this work was to elucidate the role of Src kinases in flotillin-dependent endocytosis.

We show that expression of active Fyn kinase, but not the related kinases Src and Lyn, is sufficient to induce redistribution of flotillin-positive microdomains from the plasma membrane to intracellular organelles. Use of a novel system in which recruitment of Fyn kinase domain to the plasma membrane can be induced by use of rapamycin as a heterobivalent crosslinker shows flotillin redistribution to be a rapid and efficient process that occurs in the absence of new protein synthesis. Fyn, moreover, directly phosphorylates both flotillin-1 and flotillin-2 on key tyrosine residues, and mutation of these residues to phenylalanines reduces flotillin-mediated endocytosis of the GPI-linked protein CD59. These data establish endocytosis of flotillin microdomains as a highly regulated process that is likely to be controlled in vivo by activation of receptor signalling upstream of Fyn kinase.

Results
Flotillin-1 and flotillin-2 colocalise on intracellular organelles, including late endosomes/lysosomes and plasma membrane microdomains (Brownman et al., 2007; Frick et al., 2007; Stuermer
et al., 2001). There is some evidence, however, that the two proteins can have different distributions and roles in membrane traffic (Langhorst et al., 2008). As these experiments relied on overexpressed flotillin-GFP constructs we compared the distribution of the endogenous proteins using specific monoclonal and polyclonal antibodies. Flotillin-1 and flotillin-2 showed extensive colocalisation (Fig. 1A). EGF treatment of starved NIH3T3 fibroblasts or of HeLa cells induced redistribution of both flotillins from the plasma membrane pool into intracellular compartments (Fig. 1B,C), as has been shown with flotillin-2-GFP in HeLa cells (Neumann-Giesen et al., 2007). Flotillin-1 and flotillin-2 extensively colocalised both before and after EGF treatment (Fig. 1B). Labelling with antibodies against the late endosomal/lysosomal marker CD63 confirmed that the intracellular organelles in which the flotillins accumulate upon EGF treatment are late endosomes and lysosomes (supplementary material Fig. S1).

To quantify the loss of flotillins from the plasma membrane, we used total internal reflection (TIR) (Merrifield et al., 2002) to illuminate only the bottom ~100 nm of HeLa cells labelled with flotillin antibodies (Fig. 1C,D). As activation of the EGF receptor might cause activation of Src kinases that have been shown to bind to flotillins and increase their phosphorylation, we used the Src family kinase inhibitor PP2 (Kato et al., 2006; Liu et al., 2005; Neumann-Giesen et al., 2007; Stuermer et al., 2001). PP2 treatment of starved NIH3T3 or HeLa cells prior to EGF stimulation prevented flotillin redistribution, implying a role for Src family kinases in regulating the subcellular distribution of flotillin-1 and flotillin-2 (Fig. 1C,D).

To determine which Src family kinase or kinases are capable of inducing redistribution of flotillins, HeLa cells were transfected with fluorescently tagged Src, Fyn or Lyn kinase. The kinases had phenylalanine substitutions on the autoinhibitory photophosphoines in their C-terminal domains, thereby increasing their activity (Resh, 1998). Only FynY531F relocated flotillins into intracellular vesicles (Fig. 2A; supplementary material Fig. S2). Both flotillins redistributed to the same degree, and still showed the same high degree of colocalisation in FynY531F-transfected cells as in untransfected cells (Fig. 2A). In this and all subsequent experiments described in this paper, flotillin-1 and flotillin-2 localised, and redistributed in the same way in response to different perturbations. We therefore present images of one flotillin only in subsequent figures. Projection of multiple z-sections through FynY531F-transfected cells confirmed that FynY531F induces loss of flotillin microdomains from the plasma membrane and accumulation of flotillins in intracellular organelles (Fig. 2B).

TIR was used to quantify the loss of flotillin microdomains from the plasma membrane induced by FynY531F, and to ask whether caveolin-positive caveolae behave in the same way (Fig. 2C,D). There was ~60% less flotillin-2 in the plasma membrane of FynY531F-transfected cells, compared with cells transfected with GFP alone. Src and Lyn did not induce this effect, and none of the Src family kinases had an effect on the amount of caveolin-1 present in the plasma membrane (Fig. 2D). The residual flotillin-2 in transfected cells was still in puncta, rather than being more uniformly distributed in the membrane, so there is no evidence that FynY531F causes disassembly of flotillin microdomains (Fig. 2C). FynY531F had no effect on transferrin uptake, although SrcY527F strongly inhibited this process (supplementary material Fig. S3). We conclude that active Fyn is sufficient to induce a specific redistribution of flotillins from the cell surface to late endosomes and lysosomes.

SYF cells lack Src, Yes and Fyn, the most ubiquitous Src-family kinases (Klinghoffner et al., 1999). We used these cells to determine whether these kinases are necessary for EGF-stimulated redistribution of flotillins. EGF had no effect on the distribution of flotillins in serum-starved SYF cells, as determined by quantification of flotillin-2 labelled with a specific monoclonal antibody, in HeLa cells treated as shown. TIR images showing the bottom ~100 nm of cells. EGF was added for 30 minutes. Scale bars: 20 μm. (C) Flotillin-2 detected with a monoclonal antibody, in HeLa cells treated as shown. TIR images showing the bottom ~100 nm of cells. EGF was added for 30 minutes. Scale bars: 20 μm. (D) Quantification of the mean fluorescence intensity of flotillin-2 in TIR images of HeLa and NIH3T3 cells treated as shown. Error bars represent s.e.m.; n=10.
Next, we wanted to verify that the redistribution of the flotillins induced by FynY531F was a result of increased endocytosis from the plasma membrane, and to eliminate the possibility that FynY531F expression diverts the flotillins from a biosynthetic route and thereby causes an accumulation of the newly synthesised proteins on intracellular organelles. This necessitated a system where Fyn activity at the plasma membrane could be induced acutely, rather than gradually accumulating during transient transfection. FynY531F-GFP was expressed as two separate, partial constructs – one comprising the N-terminal 30 amino acids of Fyn, including the acylation sites responsible for membrane targeting, and one comprising the rest of the protein (including the kinase domain) and GFP (Resh, 1998). Heterologous rapamycin-binding domains, which bind to the drug simultaneously (Ho et al., 1996), were fused to both constructs (Fig. 3A). Addition of rapamycin to cells expressing these constructs brought the two Fyn domains together, allowing recruitment of Fyn to the plasma membrane (Fig. 3B). Rapamycin-induced recruitment of Fyn to the plasma membrane causes visible redistribution of flotillin microdomains from the plasma membrane to intracellular organelles, in COS-7 cells pretreated with 20 μg ml⁻¹ cycloheximide (Fig. 3C). Flotillin microdomains redistribute from the plasma membrane to intracellular organelles upon expression of active Fyn. (A) Indirect immunofluorescence indicating that when FynY531F-GFP is expressed in HeLa cells endogenous flotillin-1 and flotillin-2 redistribute from peripheral, membrane-associated puncta to larger perinuclear organelles. A basal confocal section is shown. Merged image is shown on right. Scale bar: 20 μm. (B) Indirect immunofluorescence indicating that when FynY531F-GFP is expressed in HeLa cells, flotillin-1 redistributes from peripheral, membrane-associated puncta to larger perinuclear organelles. The FynY531F-GFP transfected cells are indicated with a red asterisk. The image is a projection of 24 confocal sections, so that all fluorescence in the cells is represented. Scale bar: 20 μm. (C) Total internal reflection (TIR) imaging of flotillin-2 or caveolin-1 labelled by indirect immunofluorescence at the bottom ~100 nm of HeLa cells transfected with FynY531-mCh, and adjacent untransfected cells. Scale bars: 20 μm. (D) Quantification of the amount of flotillin-2 or caveolin-1 labelled by indirect immunofluorescence at the bottom ~100 nm of HeLa cells transfected with the kinases indicated. All kinase constructs had mCherry fused to the C-terminus, and control cells expressed mCh alone. n=10; error bars represent s.e.m. (E) Quantification of the amount of flotillin-2 labelled by indirect immunofluorescence in TIR images of the bottom ~100 nm of SYF cells transfected with the constructs indicated, and treated as shown. All kinase constructs had mCherry fused to the C-terminus. n=15, error bars represent s.e.m.

Fig. 2. Flotillin microdomains redistribute from the plasma membrane to intracellular organelles upon expression of active Fyn. (A) Indirect immunofluorescence indicating that when FynY531F-GFP is expressed in HeLa cells endogenous flotillin-1 and flotillin-2 redistribute from peripheral, membrane-associated puncta to larger perinuclear organelles. A basal confocal section is shown. Merged image is shown on right. Scale bar: 20 μm. (B) Indirect immunofluorescence indicating that when FynY531F-GFP is expressed in HeLa cells, flotillin-1 redistributes from peripheral, membrane-associated puncta to larger perinuclear organelles. The FynY531F-GFP transfected cells are indicated with a red asterisk. The image is a projection of 24 confocal sections, so that all fluorescence in the cells is represented. Scale bar: 20 μm. (C) Total internal reflection (TIR) imaging of flotillin-2 or caveolin-1 labelled by indirect immunofluorescence at the bottom ~100 nm of HeLa cells transfected with FynY531-mCh, and adjacent untransfected cells. Scale bars: 20 μm. (D) Quantification of the amount of flotillin-2 or caveolin-1 labelled by indirect immunofluorescence at the bottom ~100 nm of HeLa cells transfected with the kinases indicated. All kinase constructs had mCherry fused to the C-terminus, and control cells expressed mCh alone. n=10; error bars represent s.e.m. (E) Quantification of the amount of flotillin-2 labelled by indirect immunofluorescence in TIR images of the bottom ~100 nm of SYF cells transfected with the constructs indicated, and treated as shown. All kinase constructs had mCherry fused to the C-terminus. n=15, error bars represent s.e.m.

Fig. 3. Use of heterobivalent crosslinking to recruit active Fyn to the plasma membrane. (A) Scheme of experimental design and composition of constructs. FKBP, FK506-binding protein; FRB, FKBP-rapamycin-binding domain. (B) Addition of rapamycin causes rapid recruitment of FKBP-FynY531F31-537-GFP from the cytoplasm to the plasma membrane in cells also expressing Fyn1-30-FRB. Confocal sections approximately 2 μm from the base of the cells are shown. Scale bars: 20 μm. (C) Rapamycin-induced recruitment of FKBP-FynY531F31-537-GFP to the plasma membrane causes visible redistribution of flotillin microdomains from the plasma membrane to intracellular organelles, in COS-7 cells pretreated with 20 μg ml⁻¹ cycloheximide. Basal confocal sections are shown, so the recruitment of FKBP-FynY531F31-537-GFP to the plasma membrane is less readily visualised than in B. Merged images are shown on the right. Scale bars: 20 μm. (D) Quantification of the amount of flotillin-2 labelled by indirect immunofluorescence in TIR images of the bottom ~100 nm of HeLa cells treated with rapamycin. Open circles are untransfected cells, closed circles are cells transfected with FKBP-FynY531F31-537-GFP and Fyn1-30-FRB. n>12; error bars represent s.e.m.
causing a rapid recruitment of the kinase domain from the cytoplasm onto the plasma membrane (Fig. 3B). Even in the presence of cycloheximide (a protein synthesis inhibitor), this was sufficient to induce rapid and efficient redistribution of flotillin microdomains from the plasma membrane into intracellular compartments (Fig. 3C). TIR imaging was used to quantify the amount of flotillin-2 detected in the plasma membrane of rapamycin-treated cells by indirect immunofluorescence (Fig. 3D). Transfection with the Fyn1-30 and FynY531F31-537 constructs resulted in rapid rapamycin-induced removal of flotillin-2 from the plasma membrane, whereas there was no change in untransfected cells.

As FynY531F expression induced endocytosis of flotillin microdomains, we sought to ascertain whether flotillins and Fyn colocalise during endocytosis. In HeLa cells, Fyn-mRFP was present throughout the plasma membrane, and was apparently enriched in focal adhesions (Reddy et al., 2008). TIR microscopy revealed that Fyn-mRFP had a more uniform distribution than flotillin-1-GFP, and was visibly concentrated in less than 5% of flotillin microdomains (Fig. 4A). Apparent budding of flotillin microdomains can be visualised by TIR as the disappearance of these structures from the plane of illumination (Glebov et al., 2006). Such putative budding events occurred both with and without detectable concentration of Fyn (Fig. 4A,B; supplementary material Movie 1), implying that budding of flotillin microdomains might be regulated in different ways or that Fyn activity on the microdomains does not require a visibly detectable concentration of Fyn.

Having demonstrated that Fyn activity is sufficient to induce endocytosis of flotillins, we asked whether Src kinases phosphorylate flotillins directly, and sought to map relevant phosphorylation sites. An in vitro kinase assay using recombinant proteins revealed that Src can directly phosphorylate both flotillins, and that Fyn directly phosphorylates flotillin 2 (Fig. 5A). These experiments did not reveal whether Fyn phosphorylates flotillin-1, because autophosphorylated Fyn has approximately the same molecular mass as the recombinant flotillin-1. As a control, bovine serum albumin was not phosphorylated. Mutation of Y163 has been shown to alter endocytosis of flotillin-2 (Neumann-Giesen et al., 2007), although direct evidence that this tyrosine is phosphorylated is lacking. We generated phospho-specific antibodies, that recognise phosphorylated Y160 of flotillin-1 or Y163 of flotillin-2 (Y160 in flotillin-1 being the equivalent of Y163 in flotillin-2). These antibodies bound to recombinant flotillin proteins only after in vitro phosphorylation by Fyn (Fig. 5A, immunoblots). By contrast, Src did not detectably phosphorylate Y160 of flotillin-1 or Y163 of flotillin-2, implying that this kinase targets other tyrosine residues (Fig. 5A, immunoblots).

The phosho-specific antibodies were used for immunoprecipitation of endogenous flotillins from EGF-stimulated NIH3T3 fibroblasts. Although the anti-flotillin-2 Y163-P antibody proved unsuitable for immunoprecipitation experiments, the anti-flotillin-1 Y160-P antibody was sensitive enough to pull down endogenous flotillin-1 (Fig. 5B). The highest level of flotillin-1 phosphorylation was detected after 5 minutes of EGF-stimulation of cells. Thus, Fyn can specifically and directly phosphorylate flotillin-1 on Y160 and flotillin-2 on Y163 in vitro, and phosphorylation of at least flotillin-1 Y160 correlates with EGF-stimulated redistribution of flotillin microdomains.

Y160 of flotillin-1 and Y163 of flotillin-2 were mutated to phenylalanine, and we produced C-terminal GFP fusions to carry out experiments designed to confirm the role of flotillin phosphorylation in endocytosis. Coexpression of both wild-type flotillins results in formation of flotillin microdomains de novo in the plasma membrane (Frick et al., 2007). When flotillin-1 Y160F and flotillin-2 Y163F were coexpressed, they formed microdomains in the same way as the wild-type proteins (see below), and immunoprecipitation experiments confirmed that flotillin heterooligomerisation is not altered by mutation of these tyrosine residues (Fig. 6A). FynY531F induced accumulation of flotillin-1-GFP and flotillin-2-GFP in intracellular organelles, as observed with endogenous flotillins. However, flotillin-1 Y160F-GFP and flotillin-2-GFP Y163F did not redistribute into intracellular organelles in response to FynY531F expression, but rather remained at the plasma membrane (Fig. 6B). Thus Y160 and Y163 are required for the Fyn-induced internalisation of flotillin-1 and flotillin-2 respectively.

Loss of flotillin-1 expression reduces the rate of internalisation of the GPI-linked protein CD59 (Glebov et al., 2006). Since coexpression of flotillin-1 Y160F and flotillin-2 Y163F results in formation of microdomains that are restricted to the plasma membrane, and these mutants also bind to endogenous flotillins, we tested whether coexpression of flotillin-1 Y160F and flotillin-2 Y163F has a dominant-negative effect on the uptake of antibodies against CD59. In cells expressing both mutants at high levels there was a clear and statistically significant reduction in the amount of internalised CD59 after 45 minutes of uptake (Fig. 6C,D). The magnitude of this effect, however, was relatively small: uptake in flotillin-1 Y160F and flotillin-2 Y163F transfectants was ~70% of that observed in untransfected cells or cells expressing wild-type flotillins (Fig. 6D). This might reflect the fact that endocytosis of CD59 can take place via additional endocytic pathways (Mayor and Pagano, 2007).

We used a cell-fusion-based approach to visualise assembly of flotillin microdomains and resultant endocytosis directly, to confirm that coassembly of flotillin-1 Y160F and flotillin-2 Y163F results in...
in formation of microdomains that are not capable of being internalised. Cells expressing flotillin-1-YFP were plated with cells expressing flotillin-2-GFP. A non-infectious form of Sendai virus (Okada et al., 2004) was used to induce fusion of the plasma membrane of adjacent cells. GFP fluorescence was resolved from YFP using spectral unmixing. Fusion of flotillin-1-expressing cells with cells expressing flotillin-2 resulted in the rapid assembly (within 10 minutes) of flotillin microdomains containing both proteins in a discrete band around the area of cell fusion (Fig. 7A). Fusion of cells expressing the same flotillin did not result in microdomain formation. Labelling of the cells with antibodies against CD59 during fusion resulted in uptake of the antibodies in formation of microdomains that are not capable of being internalised. Cells expressing flotillin-1-YFP were plated with cells expressing flotillin-2-GFP. A non-infectious form of Sendai virus (Okada et al., 2004) was used to induce fusion of the plasma membrane of adjacent cells. GFP fluorescence was resolved from YFP using spectral unmixing. Fusion of flotillin-1-expressing cells with cells expressing flotillin-2 resulted in the rapid assembly (within 10 minutes) of flotillin microdomains containing both proteins in a discrete band around the area of cell fusion (Fig. 7A). Fusion of cells expressing the same flotillin did not result in microdomain formation. Labelling of the cells with antibodies against CD59 during fusion resulted in uptake of the antibodies in a population of flotillin-positive endosomes within 20 minutes, again in the area of cell fusion (Fig. 7B). When the Y160F and Y163F mutants were used in this approach, a band of flotillin-positive microdomains formed just as with wild-type flotillins, there was no significant alteration in the size and brightness of these microdomains; but importantly, no colocalisation between flotillins and CD59 in endosomal structures was now detected (Fig. 7B). Thus coassembly of flotillin-1 and flotillin-2 results in rapid formation of microdomains within the plasma membrane, which can internalise CD59. The Y160F and Y163F mutations block internalisation, but not microdomain formation.

Discussion

We have shown here: (1) that flotillin-1 and flotillin-2 are endocytosed from the plasma membrane to intracellular compartments in response to Fyn activity; (2) that Fyn is required for the EGF-induced endocytosis of the flotillins; (3) that Fyn kinase can directly phosphorylate Y160 in flotillin-1 and Y163 in flotillin-2; and (4) that mutation of these tyrosine residues blocks flotillin-dependent endocytosis.

Flotillin microdomain formation requires both flotillin proteins, and occurs as a result of coassembly and hetero-oligomerisation. We did not find conditions where one flotillin was redistributed or trafficked independently, and both proteins always exhibited a high degree of colocalisation. Fyn activity induced redistribution of flotillins, apparently without disassembly of flotillin microdomains, because we never observed release of either flotillin from puncta to a more uniform distribution within the plasma membrane. Cell fusion experiments highlight the ability of the flotillins to coassemble rapidly into endocytosis-competent microdomains, and suggest that microdomain formation is driven primarily by binding of flotillin-1 and flotillin-2, rather than induced by other factors.
Fig. 7. Coassembly of flotillin-1 Y160F and flotillin-2 Y163F produces microdomains that are not competent for internalisation. (A) Use of cell fusion to study assembly of flotillins into microdomains and consequent endocytosis. A flotillin-1-GFP-expressing cell has fused with a cell expressing flotillin-2-YFP. Assembly of the two proteins into microdomains at the area of plasma-membrane fusion has occurred. Merged image is shown on the right. Image acquired 30 minutes after initiation of fusion reaction. Scale bar: 20 μm. (B) Endocytic structures containing flotillins and CD59 are generated in the region of cell fusion when cells expressing wild-type flotillins fuse, but not when the tyrosine mutants are expressed instead. Basal confocal sections (z=0 μm) demonstrate that microdomain assembly occurs normally in both cases; higher (z=2 μm) sections demonstrate presence or absence of CD59 and flotillin-positive endosomes (arrowed) in the region of cell fusion. Scale bars: 5 μm.

How the size of flotillin microdomains is specified remains unclear (Frick et al., 2007). Functional interdependence between flotillin-1 and flotillin-2 agrees well with the loss of stability of one flotillin when the expression of the other is reduced using siRNA in mammalian systems or gene disruption in flies (Babuke and Tikkanen, 2007; Hoehe et al., 2005).

The physiological function of flotillin microdomains and of flotillin-dependent endocytosis remains to be fully understood. Although flotillins have been associated with several important processes, including phagocytosis, T-cell signalling, cell adhesion and regulation of the actin cytoskeleton (Babuke and Tikkanen, 2007), their precise molecular contribution to these events is still unclear. The finding that Fyn kinase can trigger endocytosis of flotillin microdomains will facilitate further experiments to elucidate the function of these structures. Gene knockout in mice shows that Fyn is involved in T-cell signalling, cell adhesion and brain function (Lowell and Soriano, 1996), and because these functions overlap with those associated with the flotillins, it will be important to ascertain the specific molecular mechanisms involved.

Both Fyn and Src were identified in an siRNA-based screen for kinases involved in clathrin-independent endocytosis (Pelkmans et al., 2005), and tyrosine-kinase inhibitors have been shown to block clathrin-independent endocytic activity in cultured cells (Sharma et al., 2004). Several lines of evidence link Src activity with regulation of the budding of caveolin-1-positive caveolae (Pelkmans et al., 2002; Sverdlov et al., 2007), but substrates for Fyn that are directly implicated in endocytosis have been lacking. Our experiments suggest that Fyn is considerably more active on flotillins than its relatives Src and Lyn, and the finding that active Fyn causes redistribution of flotillins but not caveolin-1 reinforces the idea that these proteins define separate endocytic mechanisms (Frick et al., 2007). It is not completely clear how Src-family kinases achieve specificity in vivo (Palacios and Weiss, 2004; Resh, 1998), and it might be that in some systems phosphorylation of flotillins is mediated by different kinases. Similarly, our ex vivo experiments show that hyperstimulation of EGF-receptor signalling can lead to flotillin phosphorylation and microdomain internalisation, but the signalling events upstream of flotillin endocytosis in vivo might be different. Clearly, the interplay between endocytic and signalling pathways and the full complexity of kinase regulation of the endocytic machinery is only beginning to be understood (Liberali et al., 2008), and in this context our data showing a direct functional link between Fyn activity and flotillin-mediated endocytosis provides an important step towards the complete characterisation of the diverse endocytic mechanisms of mammalian cells.

Materials and Methods

Antibodies

Rabbit polyclonal antisera were raised against phosphorylated Y160 peptide [HDDQDP(p)YLHS] and Y163 peptide [YDKVD(p)YLSS] by Eurogentec, Belgium. Non-specific antibodies were removed and the phospho-specific antibodies were affinity purified with the corresponding phospho-peptides. Polyclonal anti-flotillin-1 has been described (Frick et al., 2007). Commercial antibodies were obtained: α-flotillin-1 mAb, α-flotillin-2 mAb, and α-caveolin pAb (BD Biosciences), and phycoerythrin-conjugated α-CD59 mAb (Autogen Bioclear).

DNA constructs

Full-length wild-type flotillin constructs have been described (Frick et al., 2007). Flotillin-GFP was mutagenised using the QuikChange system (Stratagene). The nucleotide changes were verified by DNA sequencing (Geneservice, Cambridge, UK). Human Fyn-GFP wt and Y531F constructs were a kind gift from Rosa Puertollano (Puertollano, 2005). These constructs were subcloned into mCherry and mRFP vectors. The coding region of human LynY508F, chicken SrcY527F and mouse YesY535F were cloned into the mCherry-N1 expression vector with C-terminal mCherry-tag, using EcoRI and BamHI restriction enzyme sites for Lyn and Src mutants, and Xmal and Kpnl for Yes.

Rapamycin-binding Fyn constructs

Two partial Fyn constructs linked to rapamycin-binding proteins (FRB and FKBP, which bind rapamycin simultaneously), were cloned separately into pBluescript SK+ vectors. Amino acids 1-30 of Fyn (the membrane-targeting domain) were cloned into HindIII and EcoRI sites N-terminally of FRB, and residues 31-537 (with a Y531F substitution) of Fyn plus GFP were inserted into SsrI and XhoI sites C-terminal of FKBP. COS7 cells were cotransfected with both constructs, and after 24-48 hours, the membrane targeting of FKBP-Fyn(31-537)-GFP (harbouring the catalytic domain of Fyn) was achieved by addition of 200 nM rapamycin (Cell Signalling Technology).

Cell treatments

HeLa, NIH3T3, SYF and COS7 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% foetal calf serum (Perbio Science, Cramlington, UK). Cells were transfected with FuGENE HD (Roche Diagnostics) 16-24 hours before imaging. NIH3T3, SYF and HeLa cells were starved of serum for 16 hours, and stimulated with 25 ng/ml recombinant human EGF (R&D Systems). Src kinases were inhibited by adding 10 μM PP2 (Calbiochem) 30 minutes before cell stimulation.

Cell imaging and analysis

For indirect immunofluorescence, cells were fixed with –20°C methanol for 5 minutes to reveal epitopes of endogenous flotillins. Confocal images were acquired using a Zeiss LSM510 equipped with META spectral detector. Standard FITC-TRITC-Cy5 filter sets and laser lines were used. TIR images were acquired with an Olympus TIR illumination system, via a ×100/1.45 NA objective. Illumination was provided with 488 nm and 546 nm laser lines. A dual dichroic mirror and switchable emission filters allowed resolution of GFP and mRFP fluorescence without bleed-through. For live-cell imaging, cells were maintained at 30°C in DMEM, 10% FCS buffered with 25 mM HEPES at pH 7.2. Mean fluorescence intensity of specific cells was quantified using manually defined regions in ImageJ.
Recombinant protein purification

Human flotillin-2 was expressed using the TRX-His-tagged expression vector pET32a (Ding et al., 2005) and rat flotillin-2 was cloned into MBP (maltooligosaccharidebinding protein)-tagged expression vector pNM (A gift from Neil Marshall, MRC-LMB, Cambridge, UK). Recombinant proteins were co-overexpressed in E. coli Rosetta 2 pLysS cells. Briefly, cultures were grown at 37°C and induced with 250 μM IPTG for 20 hours at 16°C. Cells were harvested and lysed in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM EDTA, DTT-free protease inhibitor cocktail (Roche) and DNaseI using an EmulsiFlex-C5 cell lyser. A two-step purification protocol was used to purify flotillin complexes. Proteins were first purified on a column containing amylase resin, and subsequently, eluates were further purified by Ni2+ affinity chromatography. This resulted in a 1:1 mixture of flotillin-1 and flotillin-2 proteins. After purification, the MBP-tag of flotillin-2 was cleaved using PreScission protease (GE Healthcare).

In vitro kinase assay

Cells were labelled continuously with a 1:200 dilution of anti-CD59 monoclonal antibody (Okada et al., 2004). To remove noninternalised antibody, cells were washed briefly three times with CD59 antibodies at the beginning of the incubation with virus particles at 4°C and so were present throughout the fusion process triggered by warming to 37°C. 40 minutes). Cells were harvested and lysed in 50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 0.1 mM EGTA, 30 μM adenosine 5’-triphosphate (ATP) and 0.1 μCi/μl [γ-32P]ATP (Amersham Biosciences) at 30°C for 30 minutes. The proteins were eluted with Laemmli sample buffer, resolved by SDS-PAGE, and protein phosphorylation was analysed by autoradiography of dried gels.

Cell fusion

Cell fusion using inactivated Sendai virus was carried out using the GenomONE-CF HVJ Envelope Cell fusion kit (Ishihara Sangyo Kaisha Ltd, Japan) using the provided reagents and buffers. Cells were transfected with different plasmids in different dishes and scored by FACs 16 hours after transfection to obtain 100% transfected populations. The different populations were mixed and replated together. Cell fusion was carried out 16 hours later according to the manufacturer’s instructions. Cells were labelled with CD59 antibodies at the beginning of the incubation with virus particles at 4°C and so were present throughout the fusion process triggered by warming to 37°C (Okada et al., 2004).

CD59 uptake assay

Cells were labelled continuously with a 1:20 dilution of anti-CD59 monoclonal antibodies conjugated to Alexa Fluor 547 (Serenex) for the time indicated (usually 40 minutes). To remove noninternalised antibody, cells were washed briefly three times with ice-cold 150 mM glucose, pH 3.0 before fixation in 4% formaldehyde in PBS.

Purified recombinant His-Src was a kind gift from Thomas Mund. Deposited in PMC for release after 6 months.

References


