Role of Src-induced Dynamin-2 Phosphorylation in Caveolae-mediated Endocytosis in Endothelial Cells*

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Plasma albumin is transported across the endothelium by transcytosis, a process regulated by the trafficking of vesicles from the luminal-to-abluminal side of the barrier (1–8). The binding of albumin to the 60-kDa endothelial surface albumin-binding protein, gp60, activates albumin transcytosis (1, 4, 6, 9–14). Transendothelial trafficking of plasma membrane-bound steryl pyridinium dyes in endothelial cells also increased following gp601 activation (7, 11). Methyl-β-cyclodextrin, a cholesterol-binding agent that disrupts caveolae (13, 15), inhibited transcellular albumin transport, suggesting that caveolae are the vesicle carriers responsible for transcytosis. Studies in caveolin-1−/− mice showed the absence of caveolae and inhibition of albumin uptake (16), consistent with the essential role of caveolae in albumin transcytosis.

The GTPase dynamin of oligomerization probably plays a crucial role in transcytosis because it triggers fission by constriction of caveolae necks subsequent to the hydrolysis of GTP (17–19). Expression of the GTPase-inactive dynamin mutant (K44A) was shown to prevent the fission of caveolae (18), consistent with this model. Thus, dynamin is an essential component of a “multi-molecular transcytotic complex” in endothelial cells (20) required for the fission of caveolae, the initial step in the migration of caveolae to the basal membrane. However, the upstream signaling events regulating dynamin activation are not known. There is evidence that Src kinase plays an important role in the activation of another component of the endocytic complex, caveolin-1 (11, 21, 22). We have shown that albumin binding to gp60 activated the G-protein-linked Src kinase signaling pathway and induced caveolae-mediated endocytosis (6, 11, 14; for review, see Ref. 23). In the present study, we delineated the regulation of dynamin-2 activation via Src kinase and its role in the mechanism of caveolae-mediated endocytosis. We demonstrate that gp60 activation induced Src-dependent phosphorylation of dynamin-2, the resultant association of dynamin-2 with caveolin-1 and thereby the caveolae-mediated endocytosis and transport of albumin.

EXPERIMENTAL PROCEDURES

Endothelial Cell Cultures—Rat lung microvessel endothelial cells (RLMVEC) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin as described previously (13).

Reagents—All of the reagents were obtained from Sigma unless otherwise stated. PP2 was purchased from Calbiochem. Hanks’ balanced salt solution (HBSS) containing NaHCO3 (4.2 mM) and HEPES (10 mM) was adjusted to pH 7.4. Bovine serum albumin (fraction V, 99% pure, endotoxin-free, cold alcohol-precipitated) was dissolved in HBSS.

Plasmid Transfection—RLMVEC grown to 50–60% confluence in 60-mm-diameter plates were co-transfected with either wild type (WT) or dominant-negative (DN) Src (Y527F,K295M) in vector pS3 (a gift from Dr. Silvio Gutkind, National Institute of Dental Research, National Institutes of Health, Bethesda, MD) and pEGFP (Clontech, Palo Alto, CA) using FuGENE 6 (Roche Applied Science) and were used 24–48 h after transfection.

Dynamin-2 Constructs and Stable Endothelial Cell Line Selection—Rat dynamin-2 wild type and K44A mutant were generous gifts from Dr. Mark McNiven (Mayo Clinic, Rochester, MN). Mutants Y231F and Y597F were generated through a two-step PCR as described previously (24). In the first step, two separate reactions with primer pairs Dyn2-Forward R, reverse; ANOVA, analysis of variance; GFP, green fluorescent protein; PH, pleckstrin homology.
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Y231F-R (5'-AGGCGCATGGTGCTCTCAGG-3') or Dyn2-R (5'-GGAGGAGCTTTGCAAGGACGTGCGG-3') and Dyn2-T212F-F (5'-TTGAGAAGAGCTCATCCGCGTTG-3') were used to introduce site-specific mutations in codon 231 while allowing either the region 5' or 3' of the base pair change. Similarly, primer pairs Dyn2-F/Dyn2-Y97F-R (5'-GACGGCTTCTGAAAGTCCTGCT-3') and Dyn2/F-Dyn2-Y97F-F (5'-GAGAACGCATCGAACACGGCGCAG-3') were used to generate site-specific modifications in codon 597. The resulting PCR products were gel-purified and used as overlapping templates in the second step reaction with primer pair Dyn2-F/Dyn2-R to generate full-length Dyn2 mutants. The double mutant was generated as described above for the Y231F mutation using the Y97F mutant as a template. Primer pair Dyn2-F/Dyn2-R was also used to amplify Dyn2 wild type and Dyn2-231F insertion. FLAG-tagged GFP or mCherry fluorescent proteins were also constructed by first cloning into pCMV-Tag4 (Strategene, La Jolla, CA) and then into the EcoRI and PsiI sites of plpXSN/EB vector, a modified pLSXN vector containing an enhanced polylinker site (5'-GAATTCCA-GTTAAGCTGCCCATGCGATCTTATATAGGCGCGC-GCGTCGACTGGGATCC-3' modified towards 5') (12). Dynamin-2-FLAG-tagged constructs were transfected into Phoenix-Eco cells using LipofectAMINE (Invitrogen). Retroviral vector-rich medium was harvested after 48 h post-transfection, clarified by centrifugation, and added to 60–80% confluent RLMEC. Medium was replaced 12 h later with growth medium containing G418 (300 μg/ml) for stable selection. Stable lines were chosen from clones of each group based on Western blot analysis of FLAG expression. The cell lines used herein expressed approximately equal levels of FLAG-tagged dynamin-2 (wild-type or mutant forms) (see Fig. 4A).

Antibodies and Fluorescent Probes—Monoclonal Abs for dynamin-2, caveolin-1, PY20, and Jak-1 and polyclonal Ab against GAPDH were from BD Biosciences. PY416-caveolin-1, PY20, and Jak-1 and polyclonal caveolin Ab were from BD Biosciences. Dynamin-2 monoclonal Ab was from Sigma, pY28–pY28 FLAG-tagged Ab was from Cell Signaling (Beverly, MA). c-Src polyclonal Ab, myc monoclonal Ab, and horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse and anti-rabbit IgG, albumin, and cholera toxin subunit B (CTB) Alexa Fluor conjugates and 4′-6-diamidino-2-phenylindole (DAPC) were obtained from Molecular Probes, Inc. (Eugene, OR). Anti-gp60 Ab was generated as described by us (10).

Immunoprecipitation and Western Blot Analysis—For Western blot analysis, cells were lysed with lysis buffer (30 min at 4 °C in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1.0% Nonidet P-40, 0.1% SDS, 1 mM NaF, 44 μg/ml phenylmethylsulfonyl fluoride, and protease inhibitor mixture), and insoluble materials were removed by centrifugation (14,000 × g for 15 min). For immunoprecipitations, the lysates were incubated with 1–10 μg/ml primary antibodies for 4 h at 4 °C followed by incubation with protein A/G-agarose beads (Santa Cruz Biotechnology) overnight. Most of the dynamin-2 (endogenous or FLAG-tagged) was immunoprecipitated using this protocol as indicated by concentration of dynamin-2 in immunoprecipitates and disappearance of dynamin-2 from lysates after immunoprecipitation. Proteins were resolved by SDS-PAGE as described previously (6).

Phosphorylation of Dynamin-2—Confluent RLMEC were washed with phosphate buffered saline (PBS), pH 7.4, and incubated with serum-free medium for 12–15 h. The cells were then treated for 15 min with 10 μM Na3VO4 (protein tyrosine phosphatase inhibitor), 10 min with 15 μM PP2 (Src family kinase inhibitor), and/or 0–5 min with 1–30 mg/ml albumin, or 20 μM anti-gp60 Ab in HBSS. The cells were then washed with PBS and lysed, and the lysates analyzed for dynamin-2 phosphorylation by immunoprecipitation and Western blot analysis using phosphotyrosine Ab. Relative intensity of phosphotyrosine at the central dynamin band was measured using Scion Image (National Institutes of Health).

Subcellular Fractionation—Confluent cells were treated with 30 mg/ml albumin, washed with PBS, and scraped in a detergent-free buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, and protease inhibitor mixture) on ice. Samples were sonicated on ice four times using 30-s bursts with 30-s intervals and centrifuged at 100,000 × g for 1 h in a TL-45 rotor (Beckman Instruments, Palo Alto, CA) at 4 °C. The 100,000 × g supernatant (cytosolic fraction) was compared with the pellet (membrane fraction). Equal amounts of protein were boiled in loading buffer and subjected to SDS-PAGE and Western blotting.

Immunostaining and Confocal Microscopy—Confluent RLMEC were washed with PBS, incubated for at least 3 h in serum-free medium, and incubated with Alexa 488- or 594-conjugated albumin or CTB in HEPES-buffered HBSS for 30–60 min at 37 °C. Subsequently, the cells were washed with pH 2.5 buffer (0.2 M acetic acid and 0.5 μM NaCl) to remove non-internalized/membrane-associated traces. The cells were then fixed, permeabilized, and stained with anti-FLAG or anti-caveolin polyclonal Ab and the nuclear marker, DAPI (1 μg/ml), as described previously (13). Non-confocal DAPI images were acquired using Hg lamp excitation and UV filter set. Confocal microscopy was performed using a Zeiss LSM 510 microscope with 488- and 543-nm excitation laser lines. Fluorescence emission was detected in optical sections <1 μm in thickness (pinhole set to achieve 1 Airy unit) separately for each fluorophore using a multi-track configuration. Average whole cell fluorescence intensity (per pixel) in the acquired confocal images (n = 6/treatment group) was determined using Zeiss LSM 510 META software. Using the LSM 510 META z-sectioning software, orthogonal views (y–z) were obtained from sequential images acquired in 0.5-μm step increments. The average background fluorescence detected in each experimental condition was subtracted from the total fluorescence to yield specific cellular fluorescence intensity.

RESULTS

Gp60-mediated Src Activation and Endocytosis of Albumin—The activation of the albumin-binding protein, gp60, by the addition of a physiological concentration of albumin (1–30 mg/ml) or gp60-cross-linking using the anti-gp60 Ab (10–20 μg/ml) as described previously (6, 11) resulted in Src kinase activation (Fig. 1A). Treatment of cells with Na3VO4 (tyrosine phosphatase inhibitor) or PP2 (Src kinase inhibitor) augmented or abolished the phosphorylation, respectively. We determined the uptake of fluorescent-albumin in cells treated with Na3VO4 or PP2 (Fig. 1B). We also addressed the role of Src kinase in caveola-mediated endocytosis by co-transfecting RLMEC with WT- or DN-Src eDNA and green fluorescent protein (GFP) cDNA (as a transfection marker). Alexa 594-albumin uptake was measured only in the GFP-positive cells. Cells transfected with DN-Src showed a 45% reduction in Alexa 594-albumin uptake compared with cells expressing WT-Src and 35% less uptake compared with cells transfected with GFP alone (Fig. 1C).

Src Phosphorylation of Dynamin-2—Because dynamin-2 is important in caveola-mediated endocytosis, we next addressed whether dynamin-2 is a target of Src kinase. RLMEC lysates were immunoprecipitated with anti-dynamin-2 Ab followed by Western blotting with phosphotyrosine Ab, PY20. As shown in Fig. 2A, dynamin-2 phosphorylation increased within 1 min of gp60 activation (i.e. within the time course of Src activation). Pretreatment with 10 μM Na3VO4 increased dynamin phosphorylation (Fig. 2B). Activation of gp60 further augmented the phosphorylation that was sensitive to Src kinase inhibition (Fig. 2B). Western blot of pY416-Src showed that activation of Src correlated with phosphorylation of dynamin-2. However, phosphorylation of another non-receptor tyrosine kinase, Jak-1, as determined by pY1022/1023-Jak-1 immunoblot analysis, did not increase upon gp60 activation (Fig. 2B).

Src-dependent Dynamin-2 Phosphorylation Induces its Association with Caveolin-1—To address whether Src phosphorylation of dynamin-2 promotes its association with caveolin-1 and localizes dynamin to caveola, detergent-soluble RLMEC lysates were immunoprecipitated with anti-dynamin-2 Ab, separated by SDS-PAGE, and immunoblotted with anti-caveolin-1 and anti-Src Abs. Immunoprecipitation of dynamin-2 pulled down caveolin-1 as well as Src (Fig. 3A). Similar results were
obtained using anti-caveolin-1 Ab for immunoprecipitation (Fig. 3A). To address whether the association of dynamin-2 and caveolin-1 was dependent on dynamin-2 phosphorylation, cell lysates prepared after gp60 stimulation were immunoprecipitated with dynamin-2 Ab and immunoblotted for caveolin-1 or dynamin-2. Fig. 3B shows the phosphorylation of dynamin-2 (top panel) and total dynamin-2 pulled down (middle panel). Gp60 activation increased the amount of caveolin-1 immunoprecipitated with dynamin-2 (bottom panel). Thus, phosphorylation of dynamin-2 promoted its association with caveolin-1.

To address the role of Src in regulating the phosphorylation-dependent association of dynamin-2 and caveolin-1, co-immunoprecipitation of caveolin-1 with dynamin was assayed in cells pretreated with PP2. Gp60 stimulation of cells induced the phosphorylation of dynamin-2 as well as caveolin-1 (Fig. 3C). Treatment of cells with PP2 decreased the amount of dynamin-2 recruited to caveolin-1 (Fig. 3C). Also, transfection of RLMVEC with dominant-negative Src decreased the gp60-activated phosphorylation of dynamin-2 and caveolin-1 as compared with phosphorylation of these proteins in empty vector- or WT-Src-transfected cells (Fig. 3D). The overexpression of WT-Src increased dynamin-2 phosphorylation, which enhanced the association between dynamin-2 and caveolin-1 (Fig. 3D). We next addressed the role of dynamin-2 residues Tyr231 and Tyr597, i.e. the tyrosine residues phosphorylated by Src (Fig. 4A) (25, 26) in the phosphorylation-dependent association of dynamin-2 and caveolin-1. We generated stable endothelial cell lines using retroviral vector pLXSN/Dyn-2/FLAG constructs (described under “Experimental Procedures”). Endothelial cells expressed the following constructs: WT dynamin-2; tyrosine phosphorylation-defective mutants (Y231F, Y597F, and Y231F/Y597F); and dominant-negative (GTPase-defective) dynamin-2 mutant (K44A). Phosphorylation of Y597F dynamin was reduced by 70%, and phosphorylation of Y231F was reduced by 50% compared with wild-type dynamin-2 (Fig. 4A), whereas the phosphorylation of double mutant Y231F/Y597F was reduced by 90% (Fig. 4A). Src activation as measured by the phosphorylation of Tyr416 on Src showed that Src activation was unaffected by the expression of WT or mutant forms of dynamin-2. The phosphorylation of Tyr416 on Src showed that Src activation was unaffected by the expression of WT or mutant forms of dynamin-2 (Fig. 4A). Src activation as measured by the phosphorylation of Tyr416 on Src showed that Src activation was unaffected by the expression of WT or mutant forms of dynamin-2 (Fig. 4A). Src activation as measured by the phosphorylation of Tyr416 on Src showed that Src activation was unaffected by the expression of WT or mutant forms of dynamin-2 (Fig. 4A).
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were compared with cells subjected to gp60 activation. To control for the level dynamin-2 mutant expressed and immunoprecipitated, the amount of co-immunoprecipitated caveolin-1 was normalized to the dynamin mutant (Fig. 4C). The association of caveolin-1 with Y231F, Y597F, and Y231F/Y597F dynamin-2 mutants was significantly reduced compared with the amount of caveolin-1 associated with WT or K44A dynamin-2. Both single Tyr point mutations, Y231F and Y597F, as well as the double mutant, Y231F/Y597F, showed reduced association with caveolin-1 following gp60 activation as compared with wild type and K44A dynamin-2 (Fig. 4C). The association of dynamin-2 with caveolin-1 was not altered by the GTPase-defective K44A mutation. These findings indicate that phosphorylation of Tyr231 and Tyr 597 on dynamin-2 is a critical determinant of dynamin-2 association with caveolin-1.

Src Phosphorylation of Dynamin-2 Induces Its Translocation to the Membrane—We addressed whether Src-mediated phosphorylation of dynamin-2 stimulates its translocation to caveolin-1-rich membrane fractions. Confluent RLMVEC expressing WT dynamin-2 or Y231F/Y597F were subjected to gp60 activation, and the cytosolic and membrane-enriched fractions were separated by high speed centrifugation. Fig. 5A shows that on gp60 activation, the amount of WT dynamin-2 increased in the membrane fraction, whereas the double tyrosine mutant, Y231F/Y597F, failed to translocate to this fraction. Fig. 5B shows the expression pattern of FLAG-tagged WT dynamin-2 (a and c) and Y231F/Y597F dynamin-2 (b and d) (red) together with caveolin-1 (green) before and after gp60 stimulation. As seen in the high magnification-merged images, gp60 stimulation induced an increase in the co-localized staining of WT dynamin-2 and caveolin-1 (a versus c), whereas the Y231F/Y597F dynamin-2-staining pattern did not change (b versus d). Orthogonal views (asterisk) of the optical sections showed an increase in the number of vesicles in which dynamin-2 and caveolin-1 co-localized following gp60 activation. In contrast, the Y231F/Y597F dynamin-2 mutant failed to co-localize with caveolin-1, indicating that Src phosphorylation of dynamin-2 is required for its association with caveolin-1.

Src Phosphorylation of Dynamin-2 Is Required for Caveolae-mediated Endocytosis and Albumin Transport—We addressed the functional significance of Src phosphorylation of dynamin-2 on the uptake of albumin and CTB in confluent RLMVEC monolayers. Endothelial cells were incubated with Alexa 488-albumin or Alexa 488-CTB, permeabilized, and stained with anti-FLAG Ab followed by Alexa Fluor secondary Ab conjugates (Fig. 6A). Albumin uptake in Y597F- or Y231F/Y597F double mutant-expressing cells was reduced by 45% compared with RLMVEC expressing wild-type dynamin-2 or cells transfected with the empty vector (Fig. 6B). The albumin uptake in the Y231F-expressing cells was not significantly different from empty vector or wild-type dynamin-2-transfected cells (Fig. 6B). In K44A dynamin-2 mutant-expressing cells, albumin uptake was reduced by 55%. We also measured 125I-albumin uptake in the dynamin-2 mutant expressing endothelial cell lines as described previously (13). 125I-Albumin uptake was reduced by 70–80% in the cells expressing Y231F/Y597F and K44A dynamin-2 mutants compared with empty vector-transfected or WT dynamin-2-expressing cells. 125I-Albumin uptake by cells expressing Y231F dynamin-2 was not different from cells transfected with empty vector (data not shown).

We determined whether the non-Src-phosphorylatable dynamin-2 mutants interfere with caveolae-mediated uptake of Alexa 488-CTB. As shown in Fig. 6B, the effect of dynamin-2 mutant expression on CTB uptake was similar to that observed using fluorescent albumin as the tracer. Expression of Y597F and the Y231F/Y597F double mutant reduced CTB uptake by 65%, whereas in Y231F dynamin-2-expressing cells, CTB uptake was not different from empty vector-transfected cells. In the K44A-expressing cells, CTB uptake was reduced by 75% compared with empty vector-transfected cells (Fig. 6B). In cells overexpressing wild-type dynamin-2, we observed a 23% increase in CTB uptake relative to empty vector-transfected cells.

We next determined the role of tyrosine phosphorylation of dynamin-2 in the regulation of transendothelial albumin permeability by measuring the luminal-to-abluminal transport of 125I-albumin in RLMVEC monolayers stably expressing the dynamin-2 mutants. All of the cell monolayers were grown to confluence in Transwell chambers. Transendothelial 125I-albumin permeability was determined from tracer accumulation in the lower chamber. In cells expressing the Y597F, Y231F/
Y597F, or K44A dynamin-2 mutants, transendothelial 125I-albumin permeability decreased 30% compared with cells transfected with the empty vector (Fig. 6C). The Y231F dynamin-2 mutant showed no effect compared with empty vector, whereas the overexpression of WT dynamin-2 significantly increased the transport of albumin. The decrease in both albumin endocytosis and transcytosis observed in the Y597F dynamin-2 mutant-expressing cells paralleled the inhibition of caveolin-dynamin co-immunoprecipitation observed in the Y597F mutant-expressing cells (Fig. 4B and C, versus Fig. 6B and C).

**DISCUSSION**

The GTPase dynamin is a key regulator of vesicle fission from the plasma membrane in multiple cell types (18, 19, 23). Dynamin functions by binding to the neck region of vesicles, forming a collar, and by its "pinchase" activity releasing the vesicle from the membrane (27). Expression of the dominant-negative dynamin-1 lacking the GTPase activity in endothelial cells inhibited endocytosis (17). As caveolae are the vesicle carriers mediating albumin transcytosis in endothelial cells (5, 6, 13), the mechanisms mediating the release of caveolae from the plasma membrane are key to understanding the basis of transcytosis. The upstream signals regulating activation of dynamin-2 (the predominant endothelial isoform) (28), its membrane localization, and role in the scission of caveolae remain unclear. c-Src-induced tyrosine phosphorylation of dynamin regulates the endocytosis of β-adrenergic and epidermal growth factor receptors via clathrin-coated vesicles (25, 26). In these studies, Src phosphorylation at Tyr231 and Tyr597 induced dynamin self-assembly and its GTPase activity (26). Thus, in this study, we addressed the role of Src kinase in the activation of dynamin-2 and its role in the mechanism of caveolae-mediated endocytosis and albumin transport.

We assessed the mechanism of albumin endocytosis via caveolae in response to activation of the albumin-binding protein, gp60 (6, 11, 13). Gp60 activation induced both Src phosphorylation and Src-dependent tyrosine phosphorylation of dynamin-2 within 1 min, consistent with the rapid induction of caveolae-mediated endocytosis of albumin (13). This phosphorylation step was required for the uptake of albumin and CTB, a marker of caveolae-mediated endocytosis (29). Previous stud-

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A. N. Shajahan and R. D. Minshall, unpublished observation.
ies showed that the internalized albumin co-localized with CTB and that the uptake of both was blocked by the caveolae-disrupting agent methyl-β-cyclodextrin (13). In this study, we showed that Src phosphorylation of dynamin-2 in endothelial cells signaled caveolae internalization and endocytosis of albumin. Epidermal growth factor–induced tyrosine phosphorylation of dynamin in B82L fibroblasts also occurred via a Src-mediated pathway regulating the internalization of epidermal growth factor receptors (30). In addition, Src and dynamin formed a complex in PC12 cells and regulated membrane trafficking (31). These findings are in accord with our observation demonstrating the important signaling function of Src in activating dynamin-2.

Because Src-induced dynamin-2 phosphorylation was necessary for caveolae release, we next addressed the possibility that Src mediated this effect by regulating the association of dynamin-2 with caveolin-1. We observed that the Src-dependent tyrosine phosphorylation of dynamin-2 following the activation of gp60 promoted the association of dynamin-2 with caveolin-1. It is possible that the association between caveolin-1 and dynamin-2, however, is not the result of a direct interaction per se but rather they are part of a multi-protein complex (20) in which additional factors are required for their interaction. Recently, Predescu et al. (32) showed that the adaptor protein intersectin, which interacts with dynamin, was important in regulating the fission and internalization of caveolae, suggesting that multiple proteins can interact to localize dynamin to caveolae and thus promote caveolae fission.

To determine the functional role of Src-induced association of dynamin-2 with caveolin-1 in mediating membrane trafficking in endothelial cells, we assessed whether the dynamin-caveolin association was required for caveolae internalization and transendothelial albumin permeability. We generated retroviral vector FLAG epitope-tagged rat dynamin-2 mutants: Y231F, Y597F, and the double mutant Y231F/Y597F. These residues were chosen because Tyr231 and Tyr597 are phosphorylated by Src in dynamin-1 (25, 26). Src phosphorylates Tyr231 in the GTPase domain and Tyr597 in the pleckstrin homology (PH) domain, and both regulate GTPase function and self-assembly of dynamin in vitro (20). Expression of these mutants in endothelial cells resulted in significant reductions in the association of dynamin-2 and caveolin-1 as well as caveolae-mediated endocytosis. The amount of caveolin-1 immunoprecipitated by Y231F or Y597F dynamin-2 mutant following gp60 activation was 60–65% less than wild-type dynamin-2, indicating the importance of Src activation in the mechanism of the dynamin-caveolin-1 association. In contrast, caveolin-1 co-immunoprecipitated by the K44A dynamin-2 mutant was similar to wild-type dynamin-2, indicating that the dynamin-2 GTPase activity is not a requirement for the caveolin-1 association with dynamin-2.

We observed markedly less caveolae-mediated uptake of 125I-albumin tracer and CTB in the endothelial cells stably expressing Y597F or Y231F/Y597F dynamin-2 compared with control cells. The albumin and CTB uptake in cells expressing the Y231F dynamin mutant were not reduced relative to control cells, whereas the Y231F dynamin-2 mutant expression showed decreased association with caveolin-1, similar to that seen with Y597F dynamin-2. Thus, it is possible that the Src-regulated association of dynamin-2 with caveolin-1 is dependent on the phosphorylation of both Tyr231 and Tyr597, whereas Tyr597 alone is important for caveolae-mediated endocytosis. Because previous in vitro studies showed that the phosphorylation of Tyr597 regulated GTPase activity of dynamin (26), it is also possible that impairment in dynamin GTPase activity in the Y597F dynamin-2 mutant-expressing cells is responsible for the reduction in the uptake of albumin and CTB. As expected, the endothelial cells expressing K44A dynamin-2 (GTPase-defective mutant) also showed reduced uptake of albumin and CTB, consistent with the requirement of GTPase activity in the mechanism of caveolae fission (17).

Dynamin functions by localizing at the neck of caveolae such as its role in dynamin-2-mediated endocytosis.
that the activation of its GTPase activity leads to vesicle fission (18). Our data show that Src-mediated phosphorylation of dynamin-2 is probably a key signal in directing dynamin to the membrane since dynamin-2 translocated to caveolin-1-rich membrane fractions after gp60 activation. In contrast, the Y231F/Y597F dynamin-2 mutant remained in the cytosolic fraction on gp60 activation. We observed by confocal imaging that dynamin-2, but not the Y231F/Y597F dynamin-2 mutant, co-localized with caveolin-1 in punctate vesicle-like structures at the membrane after gp60 activation. To address the functional significance of Src-regulated association of dynamin-2 with caveolin-1 on endothelial barrier function, we determined the effects of Src phosphorylation of dynamin-2 on transendothelial albumin permeability. Permeability of 125I-albumin in Y597F and K44A dynamin-2-expressing cells was significantly reduced compared with control cells. Because the Src phosphorylation-defective dynamin-2 mutant interfered with caveolea-mediated endocytosis as shown above, the reduction in permeability is evidence that Src phosphorylation of dynamin-2 at Tyr597 is a critical determinant of albumin transcytosis.

The mechanism by which Src phosphorylation of dynamin-2 signals the internalization of caveolae is unclear. Dynamin Tyr597 is located in the PH domain that is involved in protein-protein and protein-lipid interactions (33). Studies showed that the dynamin PH domain is required for activation of endocytosis by clathrin-coated vesicles (34, 35). Thus, a possible mechanism of caveolae internalization as regulated by Src phosphorylation of Tyr597 may involve the binding of the βγ dimer of heterotrimeric G proteins to the PH domain (36, 37). Gβγ binding was reported to regulate dynamin GTPase activity (38). Also, the inactivation of Gβγ was shown to inhibit endocytosis of clathrin-coated pits (39), lending support to Src phosphorylation of Tyr597 of dynamin-2 as being the crucial signal required for caveolea-mediated endocytosis.

In summary, we have demonstrated a novel role for Src kinase in signaling caveolea-mediated endocytosis of albumin in endothelial cells. Caveolin-1 is a scaffolding protein for Src kinase and other components of the endocytic machinery (22). We showed that activation of albumin-binding protein gp60 induced Src activation, which in turn phosphorylated caveolin-1 (11) as well as dynamin-2. Phosphorylation of dynamin-2...
Transendothelial 125I-albumin permeability in Y231F-expressing cells was also less than WT-dynamin-2-expressing cells (Fig. 6). Tyr597 of dynamin-2 is required for endocytosis and albumin transport in endothelial cells. A, confluent monolayers of stably transfected RLMEC were serum-deprived, incubated with Alexa 488-albumin, acid-washed, fixed, permeabilized, and stained with anti-FLAG Ab. Confocal images showed reduced Alexa 488-albumin uptake in the cells expressing Y597F, Y231F/Y597F, and K44A dynamin-2 compared with empty vector (EV), WT, or Y231F-dynamin-2 expressing cells. B, internalized Alexa-488-albumin and Alexa-488-CTB were quantified by measuring the fluorescence intensity per cell (as in A). The graph shows increased CTB uptake in cells expressing WT dynamin-2 compared with EV-transfected cells (p < 0.01; n = 6). Y597F, Y231F/Y597F, and K44A dynamin-2 mutant-expressing cells showed reduced uptake of both albumin and CTB compared with EV-transfected or WT dynamin-2-expressing cells (* p < 0.05; n = 6). Y231F-expressing cells showed no decrease in uptake compared with EV. C, transendothelial 125I-albumin permeability was determined in dynamin-2 mutant-expressing cells by measuring the transcellular flux across endothelial monolayers grown on Transwell filter inserts. Cells expressing WT-dynamin-2 showed a 30% increase in 125I-albumin transport compared with EV-transfected cells (p < 0.01). Transendothelial 125I-albumin permeability of Y231F-transfected cells was similar to EV-transfected cells. In cells transfected with Y597F, Y231F/Y597F, or K44A dynamin-2, 125I-albumin permeability was significantly reduced compared with EV and WT- or Y231F-dynamin-transfected cells (* p < 0.05; n = 6). Transendothelial 125I-albumin permeability in Y231F-expressing cells was also less than WT-dynamin-2-expressing cells (p < 0.05; n = 6).

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Fig. 6. Tyr597 of dynamin-2 is required for endocytosis and albumin transport in endothelial cells. A, confluent monolayers of stably transfected RLMEC were serum-deprived, incubated with Alexa 488-albumin, acid-washed, fixed, permeabilized, and stained with anti-FLAG Ab. Confocal images showed reduced Alexa 488-albumin uptake in the cells expressing Y597F, Y231F/Y597F, and K44A dynamin-2 compared with empty vector (EV), WT, or Y231F-dynamin-2 expressing cells. B, internalized Alexa-488-albumin and Alexa-488-CTB were quantified by measuring the fluorescence intensity per cell. The graph shows increased CTB uptake in cells expressing WT dynamin-2 compared with EV-transfected cells (p < 0.01; n = 6). Y597F, Y231F/Y597F, and K44A dynamin-2 mutant-expressing cells showed reduced uptake of both albumin and CTB compared with EV-transfected or WT dynamin-2-expressing cells (*, p < 0.05; n = 6). Y231F-expressing cells showed no decrease in uptake compared with EV. C, transendothelial 125I-albumin permeability was determined in dynamin-2 mutant-expressing cells by measuring the transcellular flux across endothelial monolayers grown on Transwell filter inserts. Cells expressing WT-dynamin-2 showed a 30% increase in 125I-albumin transport compared with EV-transfected cells (p < 0.01). Transendothelial 125I-albumin permeability of Y231F-transfected cells was similar to EV-transfected cells. In cells transfected with Y597F, Y231F/Y597F, or K44A dynamin-2, 125I-albumin permeability was significantly reduced compared with EV and WT- or Y231F-dynamin-transfected cells (*, p < 0.05; n = 6).
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