Epithelial Growth Factor-induced Phosphorylation of Caveolin 1 at Tyrosine 14 Stimulates Caveolae Formation in Epithelial Cells*

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Caveolae are flask-shaped endocytic structures composed primarily of caveolin-1 (Cav1) and caveolin-2 (Cav2) proteins. Interestingly, a cytoplasmic accumulation of Cav1 protein does not always result in a large number of assembled caveola organelles, suggesting a regulatory mechanism that controls caveolae assembly. In this study we report that stimulation of epithelial cells with epithelial growth factor (EGF) results in a profound increase in the number of caveolar structures at the plasma membrane. Human pancreatic tumor cells (PANC-1) and normal rat kidney cells (NRK), as a control, were treated with 30 ng/ml EGF for 0, 5, and 20 min before fixation and viewing by electron microscopy. Cells fixed without EGF treatment exhibited modest numbers of plasma membrane-associated caveolae. Cells treated with EGF for 5 or 20 min showed an 8–10-fold increase in caveolar structures, some forming long, pronounced caveolar “towers” at the cell-cell border. It is known that Cav1 is Src-phosphorylated on tyrosine 14 in response to EGF treatment, although the significance of this modification is unknown. We postulated that phosphorylation could provide the stimulus for caveolae assembly. To this end, we transfected cells with mutant forms of Cav1 that could not be phosphorylated (Cav1Y14F) and tested if this altered protein reduced the number of EGF-induced caveolae. We observed that EGF-stimulated PANC-1 cells expressing the mutant Cav1Y14F protein exhibited a 90–95% reduction in caveolar number compared with cells expressing wild type Cav1. This study provides novel insights into how cells regulate caveolae formation and implicates EGF-based signaling cascades in the phosphorylation of Cav1 as a stimulus for caveolae assembly.

Caveolae are endocytic organelles known to participate in the internalization of specific viruses, toxins, and receptors (1, 2). The major structural components of caveolae are caveolin-1 (Cav1) and caveolin-2 (Cav2) proteins, which share 38% sequence identity (3). Both of the caveolins assemble into higher molecular weight oligomeric complexes (3, 4). Currently little is known about how caveolae are formed in cells and how the assembly process is regulated. Several interesting studies suggest that cells tightly regulate the assembly of caveolae from caveolin monomers/oligomers. For example, Madin-Darby canine kidney (MDCK) cells transport Cav1 and Cav2 complexes to both the apical and basolateral domains, although these proteins are assembled into caveolar vesicles only at the cell base (5). Why few, if any, caveolae are observed at the apical surface in these cells is unclear. Furthermore, selective assembly of caveolae has been observed in migrating endothelial cells in which most of the caveolin protein appears at the leading cell edge, whereas the majority of assembled caveolae are found at the cell posterior (6).

Thus, cells are able to regulate caveolae formation to meet specific physiological needs for membrane trafficking or migration. Several studies have suggested that assembly of caveolae might be regulated by caveolin phosphorylation (5–8). Lisanti and co-workers (9) have demonstrated that Cav1 is phosphorylated at tyrosine 14 after stimulation of epithelial growth factor (EGF) signaling. Phosphorylation of Cav1 also appears to promote profound changes in caveolin localization as assessed by immunofluorescence in cells co-expressing Cav1 and activated c-Src (9). Consistent with these findings, phosphorylation of Cav1 in v-Src-expressing cells was shown to induce aggregation and fusion of caveolae and/or caveolae-derived vesicles (7). Similarly, phosphorylation of Cav1 at tyrosine 14 induced by insulin-like growth factor resulted in translocation of Cav1 in lipid raft membrane microdomains and in the formation of membrane patches on the cell surface (8).

Thus, substantial attention has been given toward testing the effects of activated receptor-tyrosine kinase cascades and downstream Src kinase activation on caveolar dynamics and Cav1 phosphorylation. Whether these activated signaling cascades actually lead to increased numbers of assembled caveolar vesicles and/or their detachment from the plasma membrane, however, remains largely undefined. In this study we report that both neoplastic and normal mammalian epithelial cells respond to EGF stimulation by altering the location of caveolin protein, resulting in a pronounced assembly of caveolae along the cell periphery. In the case of neoplastic pancreatic tumor cells possessing an elevated EGF signaling cascade, excessive caveolae assembly resulted in the formation of long caveolar towers consisting of many connecting caveolae that extended into the central cytoplasm. The vesicular structures in both normal and neoplastic cells were detected using either cholera toxin tagged to HRP or by immunogold electron microscopy (EM) using a Cav1 polyclonal antibody. Corresponding to this assembly process was a shift in the density of Cav1 protein as assessed by sucrose-gradient centrifugation and SDS-PAGE. Because phosphorylation of Cav1 at tyrosine 14 has been observed in cells treated with receptor-tyrosine kinase agonists (8–10), we tested to determine if preventing the phosphorylation at this specific residue might also attenuate caveolar assembly both in vivo and in vitro. Importantly, cells overexpressing a Cav1Y14F-GFP protein, when stimulated with EGF, showed little if any
increase in caveolar structures compared with mock-stimulated cells. To our knowledge this is the first direct demonstration that stimulation of receptor-tyrosine kinase signaling pathways leads to the assembly of caveolar vesicles dependent upon Src-mediated phosphorylation of the Cav1 protein at tyrosine 14.

MATERIALS AND METHODS

Cells and Reagents—Human neoplastic pancreatic ductal epithelial cells PANC-1 (ATCC, Manassas, VA), v-Srcmut-transformed MDCK epithelial cells (a gift from Dr. Fujita, Tokai University School of Medicine), and normal rat kidney (NRK) cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 15 μg/ml penicillin, and 0.05 mg/ml streptomycin. PANC-1 and NRK cells were grown at 37 °C in 5% CO2, and v-Srcmut MDCK cells were maintained at restrictive 40.5 °C temperature under normal conditions and transferred to permissive 33 °C to induce Src activation. Cells were grown in plastic tissue culture dishes for biochemical analyses, on acid-washed coverslips for fluorescence microscopy, and on carbon-coated and glow-discharged grid coverslips (Bellco Glass, Inc., Vineland, NJ) for electron microscopy.

Polyclonal anti-Cav1 antibodies were generated in rabbits and affinity-purified as described previously (11). Monoclonal anti-Cav1 and anti-Cav1 PY14 antibodies were purchased from Transduction Laboratories (Lexington, KY). Secondary goat anti-rabbit and goat anti-mouse IgG antibodies linked to HRP were from Tago, Inc. (Burlingame, CA). ProLong antifade reagent was from Invitrogen, and HRP-cholera toxin B, protein A-Sepharose beads, LYS 294002, lavendustin A, and PP2 were from Sigma.

Generation of Mutant Cav1(Y14F) and Transfection—By using Cav1 in pEGFP vector as a template, a point mutation to change tyrosine 14 to phenylalanine was generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The fidelity of the mutation was confirmed by direct sequencing of the plasmid. The construct and wild type cav1 in pEGFP vector further were used to transfect PANC-1 and NRK cells using Genelamerring agent (Stratagene), according to the manufacturer’s protocol. Twenty-four hours after transfection cells were cultured for 24 h in low-serum Dulbecco’s modified Eagle’s medium containing 0.2% fetal bovine serum. The cells were then treated with 30 ng/ml EGF for microscopy or 100 ng/ml EGF for biochemical assays for the times described in the corresponding figure legends.

Pharmacological Inhibition—for TEM, cells cultured for 24 h in low serum media were pretreated with the drugs PP2 (20 μM), lavendustina A (10 μM), or LY 294002 (100 μM) for 15 min and exposed to HRP-cholera toxin B for 15 min at 4 °C to allow surface binding. Subsequently, cells were washed 3 times in Hanks’-buffered saline solution to remove unbound HRP-cholera toxin, allowed to recover for 15 min at 37 °C, and stimulated with EGF (30 ng/ml) for either 5 or 20 min. Inhibitory drugs were included in all subsequent steps before fixation and HRP treatment as described previously (14).

For biochemical studies, cells cultured on 10-cm dishes were pretreated with drugs for 15 min using the concentrations described above. Drugs were included during EGF-treatment (100 ng/ml) for 5 or 20 min. Cells were then washed three times in Dulbecco’s PBS (D-PBS) and scraped into Eppendorf tubes, and cell lysates were prepared for SDS-PAGE and Western blot analysis using Cav1 phospho-Tyr-14 antibodies.

Fluorescence Microscopy—Cells cultured for 24 h on coverslips in low serum media were fixed immediately or first treated with 30 ng/ml EGF and then fixed with 3% formaldehyde in D-PBS for 10 min. After fixation, the cells were washed three times in D-PBS and mounted on slides in ProLong antifade reagent. GFP-tagged Cav1 was visualized by fluorescence microscopy (on Zeiss Axiovert 35 microscope) using a 63X 1.4 NA lens.

Immunoelectron Microscopy and Electron Microscopy—for immunolabeling of ultrathin cryosections, cells were serum-starved and stimulated with 30 ng/ml EGF for the times described in the corresponding figure legends. NRK and PANC-1 cells were fixed with 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After rinsing with phosphate buffer, the cells were scraped and then pelleted by centrifugation. Samples were embedded in 10% gelatin, cooled on ice, and cut into 1-mm3 blocks at 4 °C. The blocks were infused with 2.3 M sucrose at 4 °C overnight and frozen in liquid nitrogen. 50–60-nm-thick cryothin sections were cut at −120 °C using Ultramac FCS (Leica), and sections were picked up in a mixture of 2% methylcellulose and 2.3 M sucrose in 1:1 ratio according to Liou et al. (12).

Immunogold labeling of cryothin sections was according to the protocol described by Slot et al. (13). Briefly, cryothin sections were collected on Formvar-coated nickel grids and incubated with rabbit anti-Cav1 antibody, diluted 1:1000 with 10% fetal calf serum, PBS overnight at 4 °C, then incubated with goat anti-rabbit IgG coupled to 6-nm gold (Sigma) for 2 h at room temperature. After labeling, the sections were treated with 1% glutaraldehyde, counterstained with uranyl acetate, and embedded in methyl cellulose. Cryosections were examined and photographed using a Jeol 1200 electron microscope. HRP-cholera toxin B,
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ruthenium red labeling, and standard EM was as previously described (14).

**Immunoprecipitation, SDS-PAGE Electrophoresis, and Western Blotting**—Immunoprecipitation of Cav1 was performed as previously described (15) with some modifications. Briefly, cells cultured on 10-cm dishes were washed 3 times in D-PBS, scraped into Eppendorf tubes, and lysed in 0.25 ml of ice-cold hypotonic lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.5 ml EGTA, 10 mM NaCl, 1% Triton, and 1% sodium cholate) containing protease (0.5 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (30 mM NaF, 1 mM Na3VO4). Lysates were then preclarified at top speed for 15 min in an Eppendorf microcentrifuge at 4 °C. Cav1 was precipitated using polyclonal Cav1 antibody pre-bound to immobilized protein A-Sepharose beads (Sigma). The immunoprecipitated proteins were washed 3 times in hypotonic lysis buffer containing 150 mM NaCl. Immunoprecipitates were solubilized in an SDS-containing sample buffer and boiled for 5 min. Electrophoresis was performed in 12% acrylamide gels followed by transfer to polyvinylidene difluoride membranes. SDS-PAGE (16), transfer of proteins to polyvinylidene difluoride membranes (17), and Western blotting (18) were as described. For immunoblotting of sucrose gradient fractions, 100 μl of each fraction was solubilized in SDS-containing sample buffer and boiled, and 50 μl of each fraction was separated on 12% SDS-PAGE. Immunodetection of bound antibodies on nitrocellulose membrane was performed using ECL reagents (Amersham Biosciences). All procedures were carried out according to the manufacturer's instructions.

**Sucrose Gradient Fractionation**—Caveolae membrane microdomains were purified from cultured cells using a modified carbonate method (19). Briefly, serum-starved or EGF-stimulated cells were washed with D-PBS, scraped into 0.2 ml of 0.5 M Na2CO3, pH 11, containing protease and phosphatase inhibitors, and homogenized in a Dounce homogenizer. After that, cells were incubated on ice for 30 min, and membrane fractions were separated from nuclei by centrifugation of the cell homogenates at 1000 × g for 10 min. Subsequently, cell membranes were lysed with three 6-s bursts of a 100-watt Ultrasonicator (Kontes). Equal amounts of cell membranes were adjusted to 40% sucrose by mixing with 0.2 ml of 80% sucrose prepared in MBS buffer (25 mM Mes, pH 6.5, and 150 mM NaCl). This suspension was placed at the bottom of an ultracentrifuge tube and overlaid with 0.4 ml of 30% sucrose-density centrifugation assay was implemented. PANC-1 and NRK cells, either resting or EGF-stimulated, were homogenized, and total membranes were isolated and exposed to sucrose gradient centrifugation. After a 16-h centrifugation at 100,000 × g, 100-μl fractions were removed from the top of the tube and processed by SDS-PAGE and Western blot analysis with Cav1 and phospho-caveolin antibodies (Fig. 1B). Consistent with the findings of others using different agonists (8), we observed a modest but consistent shift of Cav1 protein from a dense to a lighter fraction in both PANC-1 and NRK cells treated with EGF (a total of three distinct experiments). Additionally, in the stimulated cells, Cav1 became present in a larger number of sucrose gradient fractions with slightly different buoyant densities, suggesting that EGF stimulation induced redistribution of Cav1 in lipid raft membrane microdomains in both PANC-1 and NRK cells. The presence of Cav1 in the more buoyant density fractions after treatment with EGF also pointed to an increase in the total amount of Cav1 protein associated with the membranes. Interestingly, co-blotted with the phosphocaveolin antibody showed that the upward shifted (lighter) Cav1 band from the stimulated cells stained more intensely than the lower, less buoyant Cav1 band from the resting cells, suggesting that Cav1 phosphorylation may have played a role in a translocation of Cav1 in membrane microdomains.

**EGF Stimulation Induces Caveolae-like Plasma Membrane Invagination in Normal and Neoplastic Epithelial Cells**—To monitor caveolar-based membrane dynamics in EGF-stimulated epithelial cells, endogenous Cav1 in PANC-1 cells was first monitored by fluorescence microscopy and viewed under resting and serum-starved conditions or after stimulation with 30 ng/ml EGF. In resting cells, Cav1 was localized largely to the periphery along the plasma membrane at cell-cell contacts (Fig. 2, a and a’, arrow) with an additional vesicle-like distribution throughout the cytoplasm. However, after 20 min of EGF stimulation, the Cav1 appeared to move away from the cell borders to a more intracellular location concomitant with the loss of cell-cell interactions (Fig. 2, b and b’). In addition, a substantial increase in Cav1 cytoplasmic puncta was observed, suggesting an active assembly and/or budding of nascent caveolae. Similar alterations in caveolae distribution have been observed in mouse fibroblasts in response to treatment with insulin-like growth factor (8) and in v-Src-transformed NRK cells (7).

To more closely observe the effects of EGF-treatment on caveolae and membrane dynamics in epithelial cells, resting or stimulated PANC-1 cells were fixed and prepared for EM. Consistent with the fluorescence images in Fig. 2, a and b, resting cells displayed largely intact cell borders with relatively modest numbers of associated caveolae-like structures (Fig. 2c). In marked contrast to resting cells, EGF-stimulated cells displayed a dramatic increase in the number of plasma membrane invaginations and formation of caveolae-like vesicles attached to the plasma membrane (formation of caveolae-like vesicles) (Fig. 2d and e). Often these vesicles appeared to fuse together, creating large membrane invaginations that were amply decorated by caveolae-like vesicles (Fig. 2d and e). These
caveolae-like towers generally formed near the sites of disrupted cell-cell borders. Coating cells with ruthenium red dye after fixation helped to identify invaginations continuous with the plasma membrane (Fig. 2, e and e'). Because PANC-1 cells are neoplastic and known to have altered signaling properties or caveolin levels, we conducted identical morphological studies on NRK cells as a control. Consistent with our observations in PANC-1 cells, endogenous Cav1 localized largely to the cell peripheries in resting NRK cells (Fig. 3, a and a') but appeared to move inward and form larger patches after stimulation with EGF (Fig. 3, a and b). EM using ruthenium red dye again showed resting NRK cells with few caveolae-like structures (Fig. 3c), whereas 20 min of EGF treatment induced a pronounced increase in the number of caveolae-like vesicles formed (Fig. 3, d and e). Most of these vesicles appeared as discrete membrane-associated structures and did not appear to coalesce into the membrane towers seen in the neoplastic PANC-1 cells. Both cell types exhibited a marked increase in caveolae-like structures in response to acute EGF treatment. EM-based morphometry of multiple cells was performed to quantitate the number of caveolae-like structures present within 1 μm of the plasma membrane in both cell types under resting or stimulated conditions (Fig. 4). PANC-1 cells responded quickly with a 3-fold increase in the number of plasma membrane-associated vesicles by 5 min and a 4–5-fold increase by 20 min post-EGF treatment. NRK cells displayed only a modest increase in vesicle numbers at early time points but exhibited a remarkable 10–12-fold increase by 20 min after EGF addition. No noticeable changes in clathrin pits were observed in the microscopic fields examined in either cell type.

**Vesicular Invaginations Induced by EGF Stimulation Are Dynamic Caveolar Clusters That Extend and Bud from the Plasma Membrane**—Although the EM images of plasma membrane-formed vesicles did not display a clathrin coat and resembled caveolae in size and shape, we pursued two additional techniques to confirm that the nascent vesicular structures were caveolae. First, during stimulation with EGF and before fixation, PANC-1 cells were incubated with cholera toxin subunit B conjugated to HRP. This toxin is known to bind to GM1 lipids and is a widely used marker of caveolae-based endocytosis (14, 22) as it fills caveolae with a dark reaction product when coupled to HRP and viewed by EM. As shown in Supplementary Fig. S1, PANC-1 cells stimulated with EGF for 20 min showed substantial numbers of toxin-filled caveolae along the plasma membrane that were not observed in untreated cells (data not shown). Second, because NRK cells do not label consistently with cholera toxin, presumably due to variations in GM1 levels, we performed immunogold labeling of cells with polyclonal Cav1 antibodies. Although resting and serum-starved NRK cells showed only modest gold labeling of the plasma membrane and associated caveolar structures (Supplemental Fig. S1c), we observed a marked increased labeling...
of caveolae-like membranes in EGF-treated cells (Supplemental Fig. S1, d and e). Thus, from standard morphological criteria, toxin labeling, and immunogold EM, we conclude that the vesicular organelles formed along the cell borders in response to EGF treatment are indeed caveolae.

Although informative, the light (Figs. 2 and 3) and EM (Figs. 2–4 and Supplemental Fig. S1) images of fixed cells could not convey membrane or caveolar dynamics induced by EGF treatment. Therefore, we next viewed live cells expressing Cav1-GFP protein under resting or stimulated conditions to test for any dramatic alterations in the distribution, organization, or motility of caveolae in response to stimulation. PANC-1 cells were plated in imaging culture dishes, transfected with Cav1-GFP, and serum-starved for 24 h. Live imaging of PANC-1 cells under serum-deprived conditions showed few movements, which were predominantly perinuclear Cav1-GFP (Fig. 5a). However, stimulation with EGF induced a dramatic increase in Cav1-GFP mobility along the cell periphery. These dynamics included the detachment of Cav1-GFP vesicles from the plasma membrane and subsequent movement toward the cell center (Fig. 5, a and b, arrows). In addition, large, elongated membrane structures formed at the cell–cell borders (Fig. 5a, arrowheads) accompanied by separation and loosening of the cell–cell junctions. Thus, Cav1-GFP membrane-based dynamics in living EGF-stimulated cells were consistent with the increase in the number of nascent individual caveolae and caveolar towers observed by EM. These findings are also consistent with the biochemical-based observations in Fig. 1, suggesting that EGF treatment is inducing an assembly of caveolae along the plasma membrane, presumably in a phosphorylation-dependent manner.

Tyrosine Phosphorylation of Caveolin-1 Is Essential for Caveolar Assembly and Dynamics Induced by EGF Stimulation—The Src-based phosphorylation of Cav1 at tyrosine 14 has been observed in a variety of different cell types and is believed to play a role in cell growth (9, 10, 23), neoplasia (10), and potentially caveolar dynamics (6). To test if Src activation is essential and responsible for the pronounced increase in caveolae formation, we utilized a widely used v-Srcα-transformed MDCK cell line (24). At the restrictive temperature (40.5 °C), v-Src kinase is inactive, and cells display a normal polarized and cobblestone phenotype when grown to confluency. Activation of v-Src at the permissive temperature (33 °C) leads to disruption of cellular contacts and loss of cell polarity and induces cell motility. Under these conditions we tested if an activation of v-Src without the use of any externally applied agonists might induce Cav1 phosphorylation. Indeed, as shown in Fig. 6a, Cav1 immunoprecipitated from cells grown at the permissive temperature exhibited a greatly increased level of phosphorylation at tyrosine 14 compared with Cav1 isolated from cells at the restrictive temperature. Thus, the activation of v-Src alone is sufficient to markedly phosphorylate Cav1 at tyrosine 14. To assess if this Src-mediated phosphorylation of Cav1 could lead to a marked increase in the number of caveolae formed at the plasma membrane, v-Srcα-transformed MDCK cells were plated on glass coverslips, incubated at 40.5 °C, then switched to the permissive temperature of 33 °C for 3, 4.5, 9, or 17 h to activate v-Srcα. Afterward, cells were fixed and processed for EM, and caveolae within 1 μm of the plasma membrane were counted. When maintained at the restrictive temperature, v-Srcα MDCK cells showed modest numbers of caveolae (Fig. 6, b and b’). In marked contrast to the cells at 40.5 °C, MDCK cells incubated at the permissive temperature of 33 °C for 9 h to activate v-Src displayed a dramatic increase (4-fold) in the number of plasma membrane-associated caveolae (Fig. 6, c and c’). Interestingly, this increase was reduced in cells that were maintained at the permissive temperature for periods up to 17 h (Fig. 6d). These findings suggest that activation of Src kinase is necessary to induce caveolae formation.

As a further test for the participation of the EGFR-Src pathway in caveolae proliferation, we also utilized pharmacological inhibitors of Src and EGFR kinases. These included lavendustin A, an inhibitor of EGFR, PP2, a broad inhibitor of Src family kinases, and LY 294002, an inhibitor of phosphatidylinositol 3-kinase that is an EGF-activated, non-c-Src mediated pathway. PANC-1 cells were serum-starved overnight, pretreated with the selected inhibitor for 15 min, and stimulated with EGF for either 5 or 20 min in the presence of inhibitor. Cells were then lysed, and the homogenates were subjected to SDS-PAGE and Western blot analysis with the PY14 Cav1 antibody (Supplemental Fig. S2a). Although control cells showed a marked increase in the levels of phosphorylated Cav1 protein after stimulation (Supplemental Fig. S2a), cells treated with Src inhibitors showed little if any increase in the levels of
phospho-Cav1. The phosphatidylinositol 3-kinase inhibitor LY 294002 had no effect on Cav1 phosphorylation as predicted. After this biochemical control experiment demonstrating the ability of the Src inhibitors to attenuate EGF-stimulated Cav1 phosphorylation, we next conducted a morphological experiment to test if the inhibitors might reduce the number of EGF-stimulated caveolar structures formed at the plasma membrane.

Although resting cells displayed few surface caveolae (Supplemental Fig. S2a), EGF-stimulated cells showed a 4–5-fold increase in these structures (Supplemental Fig. S2, c and f). Importantly, EGF-treated cells that were first exposed to the Src inhibitory drugs lavendustin A or PP2 to reduce Cav1 tyrosine14 phosphorylation formed few, if any, caveolae. Lavendustin A treatment reduced caveolae formation 4–5-fold, whereas PP2 treatment resulted in an almost complete elimination of caveolae in both resting and EGF-treated cells (Supplemental Fig. S2, d–f). These observations are in strong support of the experiments described in Fig. 6 using the temperature-sensitive v-Src MDCK cell line, indicating that Cav1 phosphorylation by Src kinase results in pro-liferative caveolae assembly.

To provide a direct test for a role of c-Src phosphorylation of Cav1 at tyrosine 14 as an essential step toward activation of caveolae formation in stimulated cells, we constructed a Cav1 GFP-tagged mutant encoding a protein in which tyrosine 14 was mutated to phenylalanine. PANC-1 cells were transfected with either this Cav1Y14F-GFP construct or a Cav1-GFP wild type construct as a control. These cells were allowed to recover for 24 h after transfection, serum-starved overnight, and stimulated with EGF for either 5 or 20 min. Some manipulated cells were viewed by fluorescence microscopy (Supplemental Fig. S3), whereas others cells were fixed in the presence of HRP-cholera toxin and prepared for EM. Light microscopic viewing of PANC-1 cells expressing wild type Cav1-GFP showed caveolar dynamics in response to EGF treatment as observed in Figs. 2, 3, and 5. In these cells a substantial increase in Cav1-GFP cytoplasmic puncta was observed, as caveolae appeared to move away from the cell borders toward a more intracellular location concomitant with the loss of cell-cell junctions (Supplemental Fig. S3, a and b). In contrast to wild type Cav1-GFP-expressing cells, the PANC-1 cells expressing the phospho-mutant Cav1Y14F-GFP remained tightly opposed to each other, with Cav1Y14F-GFP localizing to the plasma membrane even after 20 min of EGF stimulation (Supplemental Fig. S3, c and d). Thus, the activity and location of caveolae in mutant-expressing cells appears to be altered by the expression of the phospho-mutant caveolin protein.

To provide an ultrastructural correlation with the fluorescence images described above, wild type and mutant Cav1-expressing cells were also prepared for EM viewing. The experiments were particularly challenging as individual transfected cells had to first be identified from the non-transfected neighbors before thin-sectioning. As shown in Fig. 7a, a’ and c, c’, cells were grown on gridded coverslips, and transfected cells were identified by both phase and fluorescence microscopy. Upon resin embedding and thin-sectioning, low magnification EM images of identified cells (Fig. 7, a’ and c’) were oriented and confirmed with the light images. Electron micrographs of adjacent transfected and non-transfected cells were taken, and the number of caveolae formed along the plasma membrane was counted. Representative images of mutant Cav1-expressing cells and adjacent untransfected cells are shown in Fig. 7, b, b’, d, d’, and d”. Interestingly, the mutant-expressing cells showed markedly fewer caveolae along the plasma membranes than did the non-transfected control cells that exhibited the traditional 4–5-fold increase in caveolae, as observed in Figs. 2–4. Quantitation confirmed that cells expressing the Cav1Y14F mutant protein assembled only 10–20% as many caveolae as did cells expressing wild type Cav1-GFP. These observations are consistent with the prediction that phosphorylation of Cav1 protein at tyrosine 14 is essential for caveolae assembly in EGF-stimulated cells.

DISCUSSION

In this study we have provided evidence that stimulation of epithelial cells by activation of EGF-induced signaling leads to a marked increase in the number of assembled caveolae at the cell surface and that caveolar formation is a result of Src-mediated phosphorylation at Cav1 tyrosine 14. In the two cell types studied, PANC-1, a highly invasive human ductular carcinoma cell line (25), and NRK, a normal rat kidney cell line, we observed a variety of dramatic changes in caveolar dynamics in response to EGF treatment. These include, first, a marked phosphorylation of Cav1 protein at tyrosine 14 within minutes of EGF treatment (Fig. 1), also observed by others in A431 cells (9). Second, we observed a distinct shift of Cav1 protein to a lighter membrane fraction in sucrose density gradients of total cell lysate from stimulated NRK and PANC-1 cells (Fig. 1), also observed by Maggi et al. (8) in IGF-IR mouse fibroblast cells overexpressing insulin-like growth factor receptor. Third, we observed a remarkable 4–12-fold increase in the number of caveolae-like vesicles and long “caveolar towers” from the cell membrane (Figs. 2–4). These vesicles possess the morphological hallmarks of bona fide caveolae in size and shape (Figs. 2 and 3) while having a strong affinity
FIGURE 6. Activation of v-Src kinase in a v-Src\textsuperscript{ts} MDCK cell line induces Cav1 phosphorylation at tyrosine 14 concomitant with caveolae formation. \textbf{a}, Western blot (WB) analysis of immunoprecipitated (IP) Cav1 from MDCK cells expressing a temperature-sensitive v-Src protein. Immunoprecipitates from both inactive cells (40 °C) and active cells (33 °C) were blotted with the phospho-Cav1Y14 antibody and a Cav1 polyclonal to show equal loading. Cav1 was modestly phosphorylated in cells expressing inactive v-Src but highly phosphorylated in cells at the permissive temperature with active v-Src. Inactive v-Src; Active v-Src.

\textbf{b}, low magnification TEM image of v-Src\textsuperscript{ts} MDCK cells grown at the non-permissive temperature of 40 °C. Cells formed polarized monolayers at this temperature. \textbf{b}', higher magnification TEM image of the boxed area in \textbf{b} showing cell-cell interactions with few if any visible caveolae.

\textbf{c}, low magnification TEM image of the cells grown at the permissive 33 °C temperature for 9 h showing compromised cellular junctions. \textbf{c}', high magnification TEM image of the boxed region in \textbf{c}, showing a dramatic increase in the number of caveolar vesicles formed after 9 h at the permissive temperature of 33 °C. \textbf{c}'', additional high magnification TEM image of another cell grown at 33 °C for 9 h, also showing a marked proliferation of caveolae. \textbf{d}, comparative quantitation of caveolae-like vesicles formed along the plasma membrane of v-Src\textsuperscript{ts} MDCK cells grown either at the permissive or restrictive temperatures. Similar to EGF-treated cells, v-Src activation increases caveolae number by almost 4-fold. Scale bars: 2 \mu m (\textbf{b} and \textbf{c}); 1 \mu m (\textbf{b}', \textbf{c}', and \textbf{c}''); 0.5 \mu m (\textbf{c}'').
FIGURE 7. Expression of a Cav1Y14F mutant suppresses caveolae formation in EGF-stimulated PANC-1 cells. a and c, phase contrast images of PANC-1 cells grown on coverslips for EM incubated with HRP-cholera toxin to label caveolae before stimulation with EGF and subsequent fixation. Asterisks mark transfected cells. a' and c', immunofluorescence images of the same group of cells as in a and c, respectively, showing cells transfected to express Cav1Y14F-GFP (asterisks). a'' and c'', low magnification TEM images of cells as in a' and c' (the asterisk represents transfected cells). b and d, moderate magnification TEM images of the stimulated cells identified in the lower magnification images. In untransfected cells caveolae formation is substantial, whereas the transfected cells (asterisk) expressing the Cav1Y14F-GFP mutant protein display few, if any caveolae. b', d', and d'', increased magnification micrographs of regions from mutant expressing (asterisk) and non-expressing cells showing the marked contrast in caveolae number. Magnified view of the boxed area of the image (b) shows detailed structures of the cell transfected with Y14F Cav1 mutant DNA (asterisk) and the untransfected PANC-1 cells. d', TEM image of the boxed area in image c' of the cells transfected with mutant GFP-tagged Y14F Cav1 DNA (asterisk) shows the absence of caveolae proliferation after EGF stimulation. d'', magnified view of the untransfected cell in the image (c') demonstrates proliferating caveolae induced by EGF treatment. e, quantitation of caveolae counted to compare the effects of mutant Cav1Y14F-GFP protein on reducing caveolae formation in response to EGF stimulation (n > 8 per condition). Scale bars, 20 μm (a–a'', c–c''); 1 μm (b); 5 μm (d); 0.5 μm (b', d''); 2 μm (d’); YF, Tyr-14→Phe.
for HRP-cholera toxin and label with antibodies to Cav1 by immuno-EM (Supplemental Fig. S1). Finally, live cell imaging of cultured cells with Cav1-GFP exhibited dynamic budding and movements of Cav1-positive vesicles and membrane extensions from the cell borders in response to EGF stimulation (Fig. 5). These EGF-induced dynamics appear to be initiated by the direct action of Src-kinase, as MDCK cells expressing a temperature-sensitive v-Src protein possessed highly phosphorylated Cav1 while forming significant numbers of caveolae when incubated at the permissive temperature for 9 h (Fig. 6). Additionally, after EGF stimulation, treatment of neoplastic tumor PANC-1 cells with pharmacological Src kinase inhibitors dramatically reduced the number of caveolae formed (Supplemental Fig. S3). In support of a role for the phosphorylation of Cav1 by Src kinase, cells expressing a mutant Cav1Y14F protein assembled markedly fewer caveolae at the cell surface compared with cells expressing wild type protein (Fig. 7), suggesting that Src phosphorylation of this key tyrosine residue is crucial in initiating assembly of caveolae. This observation supports that of Pagano and co-workers (26), who have shown that caveolar endocytosis is increased by active Src kinase as well as glycosphingolipids. Furthermore, Pelkmans et al. (27) have shown that SV40-stimulated formation of caveolae before virus internalization requires the participation of a yet to be identified tyrosine kinase (27), potentially Src. These observations along with previous in vitro studies demonstrating a strong phosphorylation of Cav1 tyrosine 14 by Src kinases (28) support the premise of phosphorylation playing a major role in caveolar dynamics.

Because phosphorylation has long been suggested to play an important role in caveolar dynamics (7, 29), several studies have tried to identify changes in the biochemical properties of Cav1 in response to phosphorylation at tyrosine 14 (7, 9). No significant changes were observed in Cav1 Triton X-100 insolubility (7), oligomer formation (7, 9), and its association with Cav2 after Cav1Y14 phosphorylation (7). In our study we used a detergent-free method to assess the Cav1 distribution in lipid fractions after EGF treatment and found that phosphorylation of Cav1 at tyrosine 14 caused Cav1 to shift to lighter, more buoyant lipid membrane fractions (Fig. 1). This is consistent with previous findings by others, where Cav1 was redistributed to lighter density lipid membrane fractions in response to treatment with insulin-like growth factor (8). These results suggest that Cav1Y14 phosphorylation changes Cav1 association with specific lipid membrane microdomains, which may be a consequence of caveolae formation but does not alter other biochemical properties of Cav1. We suggest that the EGF-stimulated lipid membrane microdomain targeting is probably mediated by differential binding of regulatory proteins to phosphorylated tyrosine 14 of Cav1 versus its unphosphorylated form. C-terminal Src kinase has been shown to be specifically recruited to the phosphorylated form of Cav1 (30). Phosphorylation-dependent association of dynamin 2 and Cav1 has also been demonstrated during gp60-dependent Src activation and caveolae-mediated endocytosis (31). How the preferential binding of proteins to the phosphorylated form of Cav1 affects Cav1 targeting to different lipid membrane microdomains and/or detachment of caveolae from the plasma membrane remains to be determined.

Numerous in vitro and in vivo studies convincingly demonstrated that Cav1 expression is essential for caveolae formation in fibroblasts, adipocytes, epithelial, and endothelial cells (32–34). Recently, Verkade and co-workers (35) have indicated an involvement of Cav2 in caveolar biogenesis. They showed that in normal canine kidney cells Cav1 and Cav2 were found together on basolateral caveolae, whereas the apical membrane, where only Cav1 was present, lacked caveolae. Additionally, MDCK cells overexpressing Cav2 exhibited an increase in the number of caveolae attached to the plasma membrane (35). Another study has demonstrated that phosphorylation of Cav2 on serine 23 and 36 could modulate Cav1 caveolae assembly versus nonattached vesicles in normal canine kidney and prostate cancer cells (36). Although it was shown that caveolae are still present in ultrathin sections from lung capillary endothelium and in adipose tissue of Cav2 (−/−) knock-out mice (37), we cannot exclude that Cav2 may exert cell- or tissue-specific functions on caveolae proliferation during EGF stimulation.

Our study utilized three distinct cell types including PANC-1 cells, which are from a human pancreatic carcinoma (25), as well as the widely used NRK cells and MDCK cells expressing a temperature sensitive v-Src (24). Our goal was to test if the rapid formation of caveolae after stimulation was cell type-specific or more prevalent in neoplastic cells. Because formation of caveolae was markedly induced in all three cell types used, we assume that this phenomenon is common in many cells. Furthermore, the NRK cells formed a greater number of caveolae in response to EGF (8–12-fold that of resting) than did the PANC-1 cells, which exhibited a 3–5-fold increase compared with resting cells. PANC-1 cells did seem to respond more quickly to EGF treatment, as these cells increased the number of caveolae formed in just 5 min, whereas the NRK cells required longer time periods. Such a rapid response by the PANC-1 cells may reflect an amplified and highly active EGF signaling network in these tumor cells which is prevalent in many pancreatic tumor types (21).

Specific Cellular Processes Require Dynamic Caveolae Formation—Regulated alterations in caveolar number and dynamics have been predicted to play a role in a variety of distinct cellular processes, including cell migration and metastasis (9), internalization of adherens junction proteins (38), endocytosis of receptor/ligand complexes (for review, see Ref. 2), and regulated uptake of pathogens (for review, see Ref. 1). Intriguing differences in caveolar dynamics have been observed in migrating cells where caveolin and assembled caveolae appear to reside at different cellular locations during migration (6, 39). This disparity in the cytoplasmic locations of Cav1 protein with assembled caveolae-like vesicles provides insight into a highly regulated assembly process that could affect migration and/or invasion, possibly through regulating lamellipodial extension (40), adhesion plaque recycling (9), or perhaps metalloproteinase activation/secretion (41). Indeed, caveolae have been implicated in the binding to and regulation of membrane type 1 matrix metalloproteases (41, 42). For a nice review, see Ref. 43. The ultrastructural observations in our study (Figs. 2 and 3) show a large number of caveolae forming at the cell-cell interface, suggesting a participation in the disassembly and internalization of cell junctions as cells disseminate before migration. Such images are consistent with predictions that caveolae formation mediates internalization of E-cadherin (38).

There is an extensive body of literature implicating caveolae with the internalization of a variety of receptor-tyrosine kinases and serpentine G-protein-coupled receptors. Endothelin ETa-R (44), chicken gonadotropin-releasing hormone receptors (45), angiotensin AT1-R (46), adenosine A1-R (47), and bradykinin B2-R (48) receptors involved in different signaling pathways may all be internalized, at least in part, via caveolae.

EGFR desensitization has been proposed to involve a preliminary sequestration of the EGFR in caveolae, then an exchange from caveolae to clathrin-coated pits in response to EGF (49). Thus, caveolar assembly may be amplified in response to EGF stimulation as an initial mechanism for receptor internalization. Recent observations by Di Fiore and co-workers (50) have suggested that low concentrations of EGF (5–10 ng/ml) lead to receptor internalization by traditional clathrin-mediated endocytosis, whereas higher concentrations (50–100 ng/ml) stimulate an endocytic uptake by caveolae (50). These observations are consistent
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Caveolae Formation Induced by EGF

with a model that caveolae formation could precede and facilitate receptor-tyrosine kinase internalization. Thus, caveolar dynamics participate in multiple cellular signaling pathways, and temporal caveola formation is likely to play an important regulatory role.