Epidermal Growth Factor-mediared Caveolin Recruitment to Early Endosomes and MAPK Activation

ROLE OF CHOLESTEROL AND ACTIN CYTOSKELETON*

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The endocytic compartment of eukaryotic cells is a complex intracellular structure involved in sorting, processing, and degradation of a great variety of internalized molecules. Recently, the uptake through caveolae has emerged as an alternative internalization pathway, which seems to be directly related with some signal transduction pathways. However, the mechanisms, molecules, and structures regulating the transport of caveolin from the cell surface into the endocytic compartment are largely unknown. In this study, normal quiescent fibroblasts (normal rat kidney (NRK)) were used to demonstrate that epidermal growth factor causes partial redistribution of caveolin from the cell surface into a cellubrevin early endocytic compartment. Treatment of NRK cells with cytochalasin D or latrunculin A inhibits this pathway and the concomitant activation of Mek and mitotic-activated protein (MAP) kinase; however, if cells were pre-treated with filipin, cytochalasin D does not inhibit the phosphorylation of MAP kinase induced by epidermal growth factor. From these results we conclude that in NRK cells the intact actin cytoskeleton is necessary for the EGF-mediared transport of caveolin from the cell surface into the early endocytic compartment and the activation of MAP kinase pathway.

Caveolae are 50–100-nm plasma membrane microdomains involved in several crucial cellular functions such as endocytosis, potocytosis, transcytosis, calcium signaling, and cholesterol transport. Biochemical studies revealed the complex molecular composition of caveolae (1). In general, the presence of caveolin, an integral membrane protein (21–24 kDa) (2, 3), and its distinct lipid composition (enrichment of cholesterol, sphingolipids, and glycolipids but the lack of phospholipids) (4, 5) are the main molecular features of caveolae.

According to a growing body of information, caveolae are not static invaginations in the plasma membrane; rather, they are capable of being internalized in a regulated way or under well defined conditions. Several studies have provided evidence for the internalization of different ligands through caveolae into endosomes (6–12). Besides, experiments using phosphatase inhibitors (8) or cholesterol oxidase (13) showed a rapid and reversible modulation of caveolin from the cell surface. The caveolae system is now credited in having its main role in the organization and recruitment of signal transduction machinery, the transport of cholesterol, and the uptake of vitamins, toxins, and other less characterized molecules (1, 14).

Thus, although the clathrin-coated pit-vesicle system is still the best known entrance into cells (15) and may be considered a default system for degradation, recycling, and to some extent, receptor-mediared transcytosis (in polarized epithelial cells), for a number of cell types there is now evidence that uptake of fluid phase and membrane-bound markers also occurs from non-clathrin-coated areas of the membrane. In fact, there is evidence that there may be more than one endocytic mechanism with independent operation and regulation (16, 17).

Since recent data suggest that signal transduction pathways also play a crucial role in the regulation of protein and membrane trafficking, we have addressed the question of the regulation of signal transduction from the caveolae into the endocytic compartment in normal quiescent fibroblasts.

In general, the binding of growth factors to their cognate plasma membrane receptor initiates a kinase cascade that results in the activation of MAP kinase. A key intermediate in this cascade is the GTP-binding protein Ras, which appears to control the recruitment of Raf-1 to the plasma membrane after EGF binding. At the plasma membrane, Raf-1 is activated and becomes available to phosphorylate Mek, the next kinase in the cascade. Recently, we showed that endosomes isolated from quiescent rat liver contain a basal activity of Raf-1 and Mek restricted to the early/sorting endocytic compartment (18) and also that these early endocytic structures were highly enriched with cellubrevin, a protein of the v-SNARE (vesicle-associated SNAP receptor) family (19). In the present study we demonstrate by confocal microscopy and biochemical procedures that the treatment with EGF triggers the recruitment of caveolin from the cell surface into the endocytic compartment. In normal rat kidney (NRK) cells, the integrity of caveolin at the plasma membrane and the intact actin cytoskeleton seem to be crucial requirements for caveolin recruitment into the early, cellubrevin-positive, endocytic compartment and activation of MAP kinase.

MATERIALS AND METHODS

Reagents and Antibodies—Cell culture media were obtained from Life Technologies, Inc.; fluorescently conjugated antibodies were from Roche Molecular Biochemicals. The affinity-purified polyclonal rabbit

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Growth Factors Trigger the Entry of Caveolin into a Cellubrevin-enriched Endocytic Compartment in Quiescent NRK Cells—In a previous work we demonstrated that in rat liver EGF induces the recruitment of transduction machinery of the MAP kinase cascade from the plasma membrane to the early endocytic compartment where some elements of this machinery are eventually phosphorylated and activated (18). We therefore investigated whether growth factor treatment causes redistribution of caveolin, a marker for caveolae, from the cell surface to the endocytic compartment of NRK cells.

NRK cells were made quiescent following growth in 0.5% FCS for 2–3 days, then 20 ng/ml of EGF (receptor grade), FCS (10%), or 7.5 ng/ml platelet-derived growth factor was added to the medium. After 30 min, cells were fixed and double-labeled for caveolin and cellubrevin as an endosomal marker (12, 19) and analyzed by confocal microscopy (Fig. 1). In non-treated NRK cells the two antibodies labeled distinct compartments; anti-caveolin antibody labeled punctate structures restricted to the perinuclear region of the cells (Fig. 1b), whereas the monoclonal anti-caveolin labeled discrete domains and fine punctate structures on the cell surface (Fig. 1a).

When cells were incubated with EGF (for 30 min), a significant change was observed in the distribution of caveolin (Fig. 1f, in 60–80% of the cells), but not in the pattern of cellubrevin (Fig. 1e). The degree of co-localization after the EGF treatment was higher, especially in the peri-nuclear region (see insets in Fig. 1). Experiments treating the cells with 10% FCS or platelet-derived growth factor gave similar result (not shown). These results suggest that EGF treatment cause caveolin redistribution in endosomal compartments.

Cellubrevin Defines an Early Endocytic Compartment in NRK Cells—To further characterize the cellubrevin endocytic structures, double-labeling experiments with anti-lgp120 (pre-lysosomal) and anti-caveolin antibodies were performed. Fig. 2 shows that cellubrevin is mostly excluded from the pre-lysosomal compartment (Fig. 2a). Although cellubrevin has been characterized as an endosomal marker (recycling endosomes) in different cell types (26–31), it was located in the Golgi region. For this reason we performed double immunolabeling experiments with anti-caveolin and a Golgi marker, the anti-Golgi 58K protein. Confocal microscopy showed very little co-localization in the tubular trans-Golgi membranes (not shown).

We also performed internalization experiments with a fluid-phase ligand (dextran-Texas Red). Fig. 2b shows the internalization of dextran-Texas Red in NRK cells at different times double-labeled with anti-cellubrevin. Dextran was detected only in the cellubrevin-positive structures at earlier time points (5–10 min). In some experiments, we studied the internaliza-
tion of transferrin-FITC (for receptor-mediated endocytosis); most of the transferrin-FITC reached the cellubrevin compartment after 15 min and remained (data not shown).

Finally, sub-cellular fractionation was performed to examine the distribution of cellubrevin-containing structures in NRK cells. Fig. 3 shows the fractions isolated from a 27% Percoll density gradient analyzed by immunoblotting with different markers: lgp120 (pre-lysosomes), Rab5 (early endosomes and plasma membrane), and cellubrevin. Cellubrevin and Rab5 were in the same range of density (fractions 4 to 8), whereas lgp120 was confined to the high density region (fractions 2 to 5). Recently, the same gradient was used to analyze the intracellular location of annexin VI and β-hexosaminidase (32).

Taken together, these observations indicate that in NRK cells cellubrevin is concentrated in an early/sorting endocytic compartment involved in the recycling of transferrin (31). This endocytic compartment is the intracellular target organelle for caveolin after EGF activation.

Fig. 1. Analysis by confocal microscopy of caveolin and cellubrevin in NRK cells after EGF treatment. NRK at 50% of confluence were made quiescent by growing in 0.5% FCS for 3 days. Cells were then treated with EGF or PBS, fixed, and prepared for double-labeling immunocytochemistry with the monoclonal anti-caveolin (rhodamine) and a rabbit anti-cellubrevin (FITC); non-treated (a, b, and c) or cells incubated with 20 ng/ml of EGF for 30 min (d, e, and f) and examined by confocal microscopy. When cells were incubated with EGF the distribution of caveolin was more intracellular, and the degree of co-localization with cellubrevin was higher (see merge in c and f) than in non-treated cells. The cellular distribution of cellubrevin was not affected by the EGF treatment. Insets in c and f show magnified areas. Scale bar is 10 μm.

Fig. 2. Characterization of the cellubrevin compartment in NRK cells. Fixed and permeabilized cells were double-labeled with rabbit anti-cellubrevin and mouse anti-lgp120 (a) followed by anti-rabbit FITC-conjugated and anti-mouse TRITC-conjugated secondary antibodies. The degree of overlap was analyzed in optical sections (step size: 0.65 μm) by confocal microscopy. Panel a shows a lack of co-localization between cellubrevin (in green) and lgp120 (in red). The bar is 10 μm. In panel b, NRK cells were incubated with dextran-Texas red for 5 min (pulse) and then chased in normal medium for 5, 10, 30, or 45 min. After fixation and permeabilization, the cells were labeled with anti-cellubrevin followed by FITC-conjugated secondary antibody. Co-localization between dextran-Texas red and cellubrevin was only observed at early time points (5–10 min) of the fluid-phase marker internalization. The bar is 10 μm.
Fig. 3. Differential distribution of lgp120 and cellubrevin in Percoll gradients of NRK cells. A post-nuclear supernatant of NRK cells was layered over a self-generating linear 27% (v/v) Percoll gradient and centrifuged at 21,000 rpm for 90 min. Aliquots were unloaded, electrophoresed (5 μg/lane), and transferred to nitrocellulose membranes. Lgp120, Rab5, and cellubrevin were studied in each fraction by Western blotting using specific antibodies. Although cellubrevin and Rab5 were concentrated in samples of intermediate density in the gradient (aliquots 4–5), lgp120 was concentrated in the heavy density region in the gradient (aliquots 2–5).

Procedures were undertaken: disruption of caveola by treating the cells with agents that interfere with cholesterol and actin cytoskeleton perturbation. The MAP kinase activation was monitored by Western blotting in the post-nuclear supernatant obtained from quiescent NRK cells 15 min after addition of EGF using a specific antibody to the phosphorylated form of MAP kinase, ERK-1 and ERK-2.

Fig. 4 shows the differential effects of treatments on the EGF-mediated MAP kinase activation. Fig. 4a shows the dose-dependent inhibition of MAP kinase (ERK1 and ERK2) by increasing amounts of cytochalasin D in cells incubated with EGF for 15 min. Neither cholesterol oxidase nor filipin has any effect on the EGF-induced activation of MAP kinase; although cytochalasin D had an inhibitory effect in activated cells (+EGF) treated with cholesterol oxidase, the MAP kinase was not inhibited by cytochalasin D in those cells treated with filipin. Cholesterol oxidase activates MAP kinase even in the absence of growth factors; filipin had no effect (Fig. 4).

To establish whether the mechanisms described above are exclusive of the EGF-mediated pathway or may represent a more general cell activation response, the same experiments were performed using medium with 10% FCS (which contains a complex mixture of growth factors) and latrunculin A (as an actin-disrupting agent of the cortical actin cytoskeleton), were performed. Fig. 4c shows that treatment of quiescent cells with 10% FCS has similar effects on the MAP kinase activation as those reported with EGF. Under these conditions cholesterol oxidase treatment was enough to induce the activation of MAP kinase. Latrunculin A as well as cytochalasin D inhibited MAP kinase activation in response to 10% FCS even after cholesterol oxidase pretreatment, but latrunculin A did not inhibit MAP kinase activation in cells pretreated with filipin (data not shown).

Finally, a specific antibody to the phosphorylated form of Mek, the upstream kinase responsible for MAP kinase activation, was used to determine whether the changes described above are restricted to a single step of the MAP kinase cascade. Fig. 4c also shows the Mek activity running in parallel with the activity of its substrate. Taken together, the results of the present study show that agents that disrupt caveolae and interfere with their internalization and function have significant effects on growth factor signaling pathways.

To ascertain whether cytochalasin D or latrunculin A controls the internalization of caveola or merely inhibit MAP kinase activation, two approaches were followed: (i) by immunocytochemistry, to find out whether these actin-disrupting agents or those interfering with cholesterol block the internalization of caveolin after EGF treatment; (ii) by subcellular fractionation to analyze the flotation patterns of caveolin after the same treatments.

Fig. 5 shows the immunocytochemical patterns of caveolin in NRK cells after different treatments with (panels d, f, h, and l) or without EGF (a, e, g, and i) or with cholesterol (a, e, g, and i). Using a polyclonal anti-caveolin antibody in non-treated cells (control, without EGF), caveolin was localized at the cell surface and in the perinuclear (Golgi) region of NRK cells. When cells were pre-treated with agents that disrupt the actin cytoskeleton no changes in the caveolin distribution were observed in response to EGF (see Fig. 5, panels c, d, e, and f), and most of the caveolin remained at the cell surface. This indicates that disorganization of actin interferes with the internalization of caveolin induced by EGF.

However, when cells were treated with cholesterol oxidase or filipin (Fig. 5, panels g, h, i, and j) and then activated with EGF, fixed, labeled with anti-caveolin, and analyzed at the confocal microscope, it can be observed that in both cases the treatment did not interfere with internalization of caveolin triggered by EGF.

Finally, to confirm the immunocytochemical data and further demonstrate changes in caveolin distribution in response to EGF, we used OptiPrep™ density gradients. Membrane rafts are relatively insoluble in non-ionic detergents (e.g. Triton X-100) on ice and can be recovered with certain membrane proteins (e.g. caveolin) as insoluble complexes (36, 37). Some of these cholesterol-sphingolipid membrane rafts float at low density compared with detergent-insoluble cytoskeleton or detergent-soluble complexes, which remain at higher densities.

Caveolin floats in OptiPrep™ gradients (up to 20% OptiPrep™) after Triton X-100 solubilization. However, endosomes, detergent-soluble proteins, and cytoskeleton-associated structures remain in the heavy fractions of the gradient (38). After EGF treatment, caveola detach from the membranes and become lighter in the gradient (up to 12% OptiPrep™). Fig. 6 shows that there was a shift in the density of caveolin in OptiPrep™ gradients in those samples treated with EGF (Fig. 6b) compared with the control (no EGF) (Fig. 6a). On the other hand and in agreement with previous studies (38), flotation of caveolin was not affected when the cells were treated with latrunculin A. However, when those cells treated with latrunculin A were activated with EGF, a more homogeneous distribution of caveolin was detected throughout the gradient, but certain accumulation of caveolin peaking in the same fractions as the control without EGF can be observed (Fig. 6c). Finally, for those cells treated with filipin (plus EGF), although caveolin was detected all over the gradient, a peak with an increased amount can be observed in the lightest fractions, similar to the control plus EGF (Fig. 6d).

In addition to caveolin, we also analyzed the behavior of Mek (total) and Mek-P in the same OptiPrep™ density gradients (Fig. 6, g–j). Both were detected at the bottom of the gradients in cells not treated with EGF and at the same density as caveolin (of non-treated cells) in those cells stimulated with EGF; since Mek interacts with caveolin (in the caveolae), it is likely that it can be transported “ensemble” to the endocytic compartment where it can be phosphorylated, a situation similar to that shown in the liver where Mek is mainly in caveolae but Mek-P was found in early endosomes. Therefore, caveolin in low density fractions of the OptiPrep™ gradients could correspond to the vesicles that move from the cell surface to the early endosomes, transporting Mek to this endocytic compartment where it can be eventually phosphorylated (activated) (18). These results are in agreement with the immunocytochemical studies in Fig. 5. As mentioned above, in these gradients early endosomes were located at the bottom of the OptiPrep™ gradients (Fig. 6e, Rab5). Interestingly, after treatment of EGF, Rab5 showed a slight displacement similar to Mek-P.
Finally, we analyzed the expression of Mek after latrunculin A or filipin treatments. In all cases the distribution of Mek parallels with that of caveolin (data not shown).

Whether caveolin found in densities 12–20% OptiPrep™ after EGF treatment represents caveolae detached and on the way to the endocytic compartment is still uncertain. However, it seems that phosphorylation of Mek does not occur in these intermediate structures nor in plasma membrane caveolae, since Mek-P does not float with caveolin.

**DISCUSSION**

In this study we demonstrate that, in normal quiescent fibroblasts, caveolin is transported from the cell surface into cellubrevin-enriched early endosomes in response to EGF; in addition, the concomitant MAP kinase activation depends on the intact actin cytoskeleton and the integrity of caveolae at the plasma membrane.

The internalization of EGF is a complex process in which caveolae and clathrin-coated pits are involved (39). The presence of EGFR in caveolae-rich membrane fractions from human fibroblasts (40) and a direct interaction of EGFR with caveolin in vitro could facilitate the spatial interaction and activation of Rac/Raf-1 and the MAP kinase pathway (41, 42). Thus, EGF may interact first with its receptor in a caveolae, where it is phosphorylated, then it migrates to a clathrin-coated pit (39), where the EGFR interacts with AP2 (43), completes its phosphorylation, and is internalized. The subsequent events leading to the activation of the Ras/Raf-1, Mek, and MAP kinase may occur in caveolae (44) and/or in the endocytic compartment (18, 45–47).

The potential involvement of caveolae in cellular trafficking is controversial. Whereas morphological evidence for the role of caveolae in endocytosis has been provided in several cell types (6–8, 11, 48–51) and also for transcytosis in endothelial cells (52–55), other authors considered caveolae more stable structures on the cell surface (56, 57); this also may include potocytosis (58). Caveolae are believed to be dynamic vesicular carriers capable of budding, docking and fusion. Besides, since caveolae are also sites where signal transduction molecules are concentrated, their transport into the cell may provide a crucial regulatory mechanism for cell activation (59). In liver, we showed that the immediate destination of caveolae is the endocytic compartment, the early/sorting endosomes (compartment of uncoupling receptors and ligands (CURL)) (12), where Raf-1 is active and Mek is phosphorylated (18). Exogenous administration of EGF, through the portal vein, led to redistribution of Raf-1 from the plasma membrane into the endosomes (18). Recently Gilbert et al. (60) demonstrate in a cell-free system the ability of caveolae to fission and form vesicles detached from the plasma membrane of 3T3 fibroblasts.

It has been shown that normal endocytic trafficking of EGFR was important for the full activation of MAP kinases (61). To investigate whether the activation of the signal transduction machinery (MAP kinase cascade) in the endocytic compartment was dependent on the physical entry of caveolae/caveolin in response of EGF, two different approaches were considered: the inhibition of endocytosis and cholesterol perturbation.

Actin-disrupting agents such as cytochalasin D (or latrunculin A) inhibit the endocytosis (clathrin-independent and -dependent) (34, 35) and significantly decrease the MAP kinase activity in EGF- or FCS-treated cells. Results shown in the present study indicate that the actin cytoskeleton is required for the activation of Mek and MAP kinase activities and caveolin redistribution; therefore, the entry of caveolae seems necessary for Mek and MAP kinase (hyper)activation. Inhibition of MAP kinase activity by cytochalasin D was dose-dependent with a maximum at 10 µg/µl.

The connection between actin filaments and caveolin might be through actin-binding proteins (e.g. spectrin or α-actinin) or...
by means of GTP-binding proteins such as RhoA, which was found located in the caveola-enriched membrane domains of endothelial cells and fibroblasts (62, 63). Interestingly, the Rho family of small GTPases, which include Rac1, Cdc42, and RhoA, regulate the rearrangement of actin cytoskeleton when cells are exposed to growth factors and cytokines (64). Whether this reorganization of the actin cytoskeleton is responsible for the regulated migration of EGFR (65) from the caveolae to the clathrin-coated pits and the subsequent initiation of EGF/EGFR is unknown.

On the other hand, cholesterol is required for the maintenance of caveolar integrity and function. It has been demonstrated that although filipin flatten the caveolae, inhibit the entry of cholera toxin, and release several proteins of the cortical cytoskeleton such as annexin II, α-actinin, ezrin, moesin, and membrane-associated actin (66), cholesterol oxidase is involved in the recruitment of caveolin from the caveolae to the Golgi structures (67). It has also been demonstrated that cholesterol depletion causes hyperactivation of MAP kinase (ERK) (68, 45, 69).

Thus, this study extends our previous data in isolated endosomes from rat liver (18, 12, 70) to normal quiescent fibroblasts...
(the same was observed in A431 cells, where a clear re-organization of cell surface caveolin into intracellular stores was observed after EGF treatment) and seems to indicate that EGF can modulate the location and perhaps the function of some caveolin-associated-proteins at the cell surface. Redistribution of caveolin into the endosomal compartment may trigger the signal transduction machinery and therefore participate in cell activation.

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