Caveolin-1 is a Negative Regulator of Caveolae-mediated Endocytosis to the Endoplasmic Reticulum*

Phuong U. Le‡§, Ginette Guay‡, Yoram Altschuler¶, and Ivan R. Nabi‡‡

From the ‡Department of Pathology and Cell Biology, Université de Montréal, Montréal, Quebec H3C 3J7, Canada and the ¶Department of Pharmacology, Hebrew University of Jerusalem, Jerusalem 91120, Israel

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Caveolae are flask-shaped invaginations at the plasma membrane that constitute a subclass of detergent-resistant membrane domains enriched in cholesterol and sphingolipids and that express caveolin, a caveolar coat protein. Autocore motility factor receptor (AMF-R) is stably localized to caveolae, and the cholesterol extracting reagent, methyl-β-cyclodextrin, inhibits its internalization to the endoplasmic reticulum implicating caveolae in this distinct receptor-mediated endocytic pathway. Curiously, the rate of methyl-β-cyclodextrin sensitive endocytosis of AMF-R to the endoplasmic reticulum is increased in ras- and abl-transformed NIH-3T3 cells that express significantly reduced levels of caveolin and few caveolae. Overexpression of the dynamin K44A dominant negative mutant via an adenovirus expression system induces caveolar invaginations sensitive to methyl-β-cyclodextrin extraction in the transformed cells without increasing caveolin expression. Dynamin K44A expression further inhibits AMF-R-mediated endocytosis to the endoplasmic reticulum in untransformed and transformed NIH-3T3 cells. Adenoviral expression of caveolin-1 also induces caveolae in the transformed NIH-3T3 cells and reduces AMF-R-mediated endocytosis to the endoplasmic reticulum to levels observed in untransformed NIH-3T3 cells. Cholesterol-rich detergent-resistant membrane domains or glycolipid rafts therefore invaginate independently of caveolin-1 expression to form endocytosis-competent caveolar vesicles via rapid dynamin-dependent detachment from the plasma membrane. Caveolin-1 stabilizes the plasma membrane association of caveolae and thereby acts as a negative regulator of the caveolae-mediated endocytosis of AMF-R to the endoplasmic reticulum.

Endocytosis via clathrin-coated vesicles represents the best characterized endocytic pathway, however, other clathrin-independent endocytic mechanisms also exist (1–4). The large GTPase dynamin has been shown to regulate the fission of clathrin-coated pits, and expression of the dynamin K44A (dynK44A) mutant inhibits clathrin-mediated endocytosis (5–7). The dynK44A mutant does not affect fluid phase endocytosis or the clathrin-independent endocytic pathway defined by ricin endocytosis indicating that non-clathrin-coated cell surface invaginations can detach from the plasma membrane in the apparent absence of dynamin-mediated membrane fission (8–11). However, introduction of inhibitory antibodies to dynamin into hepatocytes resulted in the accumulation of both clathrin-coated vesicles and smooth caveolar invaginations and inhibited the endocytosis of cholera toxin (12). In endothelial cells, caveolae budding from isolated membranes was shown to be dynamin-dependent, and caveolae were shown to contain the molecular machinery necessary for vesicle budding (13, 14). Regulation of caveolae budding by dynamin identifies caveolae as endocytosis-competent cell surface invaginations.

Caveolae or smooth plasmalemmal vesicles were first identified in endothelial cells and are morphologically identifiable as smooth flask shaped invaginations of the plasma membrane (15–18). Caveolae are rich in cholesterol and sphingolipids, disrupted by cholesterol extracting agents, and insoluble in Triton X-100 and are therefore considered to form a subclass of cholesterol-rich detergent-resistant membrane domains or glycolipid rafts (19–22). The caveolins (caveolin-1, -2, and -3) are cholesterol binding proteins that form a spiral coat on the cytoplasmic surface of caveolar invaginations and represent caveolae markers (23–25). Caveolar invaginations are not present in cells that express little or no caveolin, and the reintroduction of caveolin-1 into such cells has been shown to induce the formation of caveolae implicating caveolin in the invagination of glycolipid raft microdomains (26–28). Caveolin-1 expression is inversely proportional to cell transformation, and caveolin-1 has been characterized as a tumor suppressor gene (27, 29–31).

Caveolae have long been proposed to be involved in transcytosis across the endothelial cell (18, 32–34). Caveolae- or raft-mediated endocytosis has been reported for cholera toxin-bound GM1 ganglioside, sphingolipids, glycosylphosphatidylinositol-anchored proteins, SV40, and bacteria, as well as the endothelin, growth hormone, interleukin-2, and autocrine motility factor (AMF) receptors (35–46). Autocrine motility factor receptor (AMF-R) is a seven-transmembrane domain receptor localized at steady state to caveolae and the smooth endoplasmic reticulum (ER) that follows an endocytic pathway sensitive to cholesterol extraction with methyl-β-cyclodextrin (mβCD) via caveolae to the smooth ER (43, 44, 47–49). Using AMF as a marker for this caveolae-mediated endocytic pathway, we show that caveolae invaginations and caveolar vesicles

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§ Recipient of a Graduate Student Award from the Cancer Research Society Inc.

¶ Recipient of a CIHR Investigator award. To whom correspondence should be addressed; Dépt. de Pathologie et Biologie Cellulaire, Université de Montréal, C.P. 6128, Succursale A, Montréal, Quebec H3C 3J7, Canada. Tel.: 514-343-6291; Fax: 514-343-2459; E-mail: ivan.robert.nabi@umontreal.ca.

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mediate AMF-R endocytosis in ras- and abl-transformed NIH-3T3 cells that express little caveolin and few caveolae. Adenoviral expression of the dominant negative dynK44A mutant or of caveolin-1 has allowed us to demonstrate that: 1) even when caveolin levels are significantly reduced or absent, caveolae form and rapidly bud from the plasma membrane to form caveolar vesicles that target the ER; and 2) caveolin-1 regulates this endocytic pathway by stabilizing caveolae expression at the plasma membrane thereby slowing down the internalization of caveolar vesicles.

EXPERIMENTAL PROCEDURES

Antibodies, Reagents, and Cells—Monoclonal rat antibody against AMF-R was used in the form of concentrated hybridoma supernatant (50). Rabbit anti-caveolin antibody was purchased from Transduction Laboratories (Mississauga, Ontario, Canada), mouse anti-c-Myc from Santa Cruz Biotechnologies (Santa Cruz, CA), and mouse anti-hemagglutinin (HA) was a gift from Luc Desgroseillers (Department of Biochemistry, Université de Montréal). Horseradish peroxidase, fluorescein isothiocyanate, and gold-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove,
AMF-R cell surface expression in untransformed and ER-associated silver particles were counted from 25 intact cell profiles, and the data represent the average per cell profile. The ER after 30-, 60-, and 120-min incubation with bAMF at 37°C was quantitatively analyzed. Infection with only the tTA adenovirus was used as a control. Where indicated, infection was determined in parallel for each experiment and infection rates were used that resulted in greater than 75% infection rates. The rate of fluorescence labeling with anti-HA or anti-MYC antibodies, and viral titers were used that resulted in greater than 75% infection rates. The rate of infection was determined in parallel for each experiment and infection with only the tTA adenovirus was used as a control. Where indicated, infection rates were determined by immunofluorescence labeling with anti-HA or anti-MYC antibodies, and viral titers were used that resulted in greater than 75% infection rates.

Viral Infection—Recombinant adenoviruses expressing the tetracycline-regulatable chimeric transcription activator (tTA), HA-tagged, and dynK44A, and myc-tagged caveolin-1 under the control of the tetracycline-regulated promoter were as previously described (51–53). To enhance infection rates, viral stocks of the tTA and dynK44A or caveolin-1 adenoviruses were diluted in 320 μl of sterile PBS and preincubated with 72 μl of 1 μg/ml polylysine for 30 min at room temperature. Infection with only the tTA adenovirus was used as a control. 2.5 × 10^5 untransformed, ras-transformed or abl-transformed NIH-3T3 cells were plated onto 10-cm dishes for 10 h, rinsed with PBS before addition of the adenovirus polylysine mixture in 3 ml of serum-free media for 1 h at 37°C. After removal of the adenovirus mixture, the cells were rinsed twice with serum-free media and then incubated for 36 h in regular culture media. For the EM studies, infection rates of the three cell lines were determined by immunofluorescence labeling with anti-HA or anti-MYC antibodies, and viral titers were used that resulted in greater than 75% infection rates. The rate of infection was determined in parallel for each experiment and infection with only the tTA adenovirus was used as a control. Where indicated, infection rates were determined by immunofluorescence labeling with anti-HA or anti-MYC antibodies, and viral titers were used that resulted in greater than 75% infection rates.

Electron Microscopy—All three cell types were pulsed with bAMF for the indicated times and processed for electron microscopy, and bAMF was revealed with nanogold-streptavidin followed by silver enhancement as previously described (44). For the double labeling with anti-AMF-R or polyclonal anti-caveolin antibodies, bAMF was first revealed with nanogold-streptavidin and silver amplification, and then the sections were labeled with the primary antibodies following by the appropriate gold-conjugated secondary antibodies. The sections were contrasted with uranyl acetate and lead citrate and examined in a Zeiss.

**Fig. 3.** Increased rate of AMF-R endocytosis to the ER in ras- and abl-transformed cells. A, NIH-3T3 (white bars) or ras (gray bars)- and abl (black bars)-transformed NIH-3T3 cells were pulsed with bAMF for 60 min at 37°C in the absence or presence of mβCD. bAMF was revealed by postembedding labeling with nanogold-streptavidin and silver amplification. The numerical density of silver particles associated with the ER, endosomes, and mitochondria was determined from 36 images (×12,000) from two different experiments for each condition. Control values are presented as ±S.E.). The data shows that bAMF targeting to the ER is selectively inhibited by mβCD. B, quantitative analysis of AMF delivery to the ER after 30-, 60-, and 120-min incubation with bAMF at 37°C in NIH-3T3 (circles), NIH-ras (squares), and NIH-abl (triangles) cells. ER-associated silver particles were counted from 25 intact cell profiles, and the data represent the average per cell profile. C, FACS analysis of AMF-R cell surface expression in untransformed and ras- and abl-transformed NIH-3T3 cells shows that AMF-R cell surface expression is decreased following cell transformation. The data are presented as relative fluorescence intensity in percentage compared with NIH-3T3 cells.

PA. Rabbit phosphohexose isomerase (referred to as AMP) was purchased from Sigma Chemical Co. (Oakville, Ontario, Canada) and biotinylated with long chain N-hydroxyl-succinimido-biotin (Pierce, Rockford, IL) according to the manufacturer's instructions. Nanogold-streptavidin and the HQ-Silver Enhancement kit were purchased from Nanoprobes, Inc. (Stony Brook, NY). MβCD, poly-L-lysine, and propidium iodide were purchased from Sigma.

An NIH-3T3 fibroblast clone (43) and H-ras- and v-abl-transformed NIH-3T3 cells (29) were grown in complete medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% calf serum, non-essential amino acids, vitamins, glutamine, and a penicillin-streptomycin antibiotic mixture (Invitrogen Canada Inc., Burlington, Ontario, Canada). Treatment of cells with 5 mM mβCD was performed as previously described (44).

**Fig. 4.** bAMF and AMF-R are localized to caveolin-positive caveolae. NIH-3T3 (A, B) or NIH-3T3 cells infected with dynK44A (C–H) were pulse-labeled with bAMF for 60 min at 37°C. Postembedding labeling with nanogold-streptavidin and silver amplification first revealed the localization of bAMF to caveolae, and then caveolin distribution was determined using polyclonal anti-caveolin antibodies and 12-nm gold-conjugated anti-rat IgM and 18-nm gold-conjugated anti-rabbit secondary antibodies, respectively (B, D, F, and H). In A, C, E, and G, the arrow indicates caveolin and the arrowheads bAMF. In B, D, F, and H, the arrows indicate AMF-R and the arrowheads caveolin. Bars = 0.2 μm.
CEM902 electron microscope. In the absence of nanogold-streptavidin labeling, silver particles due to nonspecific silver enhancement were not observed.

For the quantification of internalized bAMF, the number of silver particles localized to ER, endosomes, and mitochondria were counted, and the surface area of the indicated organelles was measured using a Sigma Scan measurement system. ER labeling included both ribosomes studded rough ER profiles as well as morphologically identified smooth ER (43, 47–49). To ensure that the smooth membranous organelles were not early endosomes, only smooth membrane-bound structures wider than 75 nm and longer than 200 nm were considered to be ER tubules. Similarly, smooth caveolar invaginations and clathrin-coated vesicles within 100 nm of the plasma membrane were counted per unit membrane (43). The average and standard error from 36 images obtained from two separate experiments are presented. Alternatively, bAMF expression at the plasma membrane, in endosomes and in the ER, and caveolin expression at the plasma membrane (including membrane invaginations) and in morphologically identifiable smooth caveolar invaginations (E, F, G, H) and to clathrin-coated vesicles (I, J). Bar = 0.2 μm.

FACS Analysis—Cells were detached from the dish with EDTA and resuspended in bicarbonate-free medium supplemented with 25 mM Hepes and 5% calf serum. The cells were then incubated with anti-AMF-R antibodies at 4 °C for 1 h, washed three times with cold media, incubated with fluorescein isothiocyanate-conjugated anti-rat IgM at 4 °C for 1 h, and then washed three more times with cold media and twice with PBS. The cells were then incubated with 0.5 μg/ml propidium iodide for 10 min at 4 °C. Cell-associated fluorescence intensity was analyzed on a Beckman FACScan. Cells stained for propidium iodide were discarded from the analysis, and cell surface AMF-R expression was determined only on intact cells.

Immunoblot—Cells cultured at ~70% confluency were scraped, lysed, and sonicated in lysis buffer containing 1% SDS, 5 mM EDTA, and protease inhibitors. Protein content was assayed using the BCA protein assay (Pierce, Rockford, IL), and 40 μg of protein was separated by SDS-PAGE and blotted onto nitrocellulose paper. The blots were

![Figure 5: Expression of dynK44A induces caveolae in ras- and abl-transformed NIH-3T3 cells.](http://www.jbc.org/)

![Figure 6: Quantitative analysis of caveolae and clathrin-coated vesicles at the plasma membrane following dynK44A infection.](http://www.jbc.org/)
blocked with 5% milk in PBS, incubated with rabbit anti-caveolin antibody together with mouse anti-HA, and then with horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies. The labeled bands were revealed by chemiluminescence and exposed to preflashed Kodak XRP-1 film.

RESULTS

Caveolae-mediated Endocytosis of AMF-R to the ER—In NIH-3T3 cells, AMF-R is localized to smooth caveolar invaginations and is endocytosed via a mβCD-sensitive caveolae-mediated pathway to the smooth ER tubules defined by AMF-R expression (43, 44). To specifically assess the specific role of caveolae in this endocytic route, endocytosis of biotinylated AMF (bAMF) was followed in ras- and abl-transformed NIH-3T3 cells that exhibit significantly reduced expression of caveolae and caveolin (Fig. 1). An 80-min bAMF pulse at 37°C, endocytosed bAMF detected by nanogold-streptavidin and silver amplification was localized to smooth and rough ER tubules of NIH-3T3 and ras- and abl-transformed NIH-3T3 cells (Fig. 1). Endocytosis of bAMF to multivesicular bodies (MVBs) was also detected in all three cell lines (Fig. 1) as previously reported in NIH-3T3 cells (44). Double labeling of the cells for bAMF (nanogold and silver amplification) and for AMF-R (12-nm gold particles) (Fig. 4A) or for AMF-R (12-nm gold particles) and caveolin (18-nm gold particles) (Fig. 4B). Both Quantification of bAMF labeling of the ER, endosomes, and mitochondria (see “Experimental Procedures” for details) showed increased bAMF internalization to the ER in ras- and abl-transformed NIH-3T3 cells compared with control NIH-3T3 cells (Fig. 3A). Clathrin-dependent endocytosis to endosomal structures (44) was detected at similar levels between the three cell types. Nonspecific labeling of mitochondria and control labeling performed in the absence of endocytosed bAMF are also presented. The significantly reduced expression of caveolae and caveolin in the transformed NIH-3T3 cell lines does not therefore prevent endocytosis of bAMF to the ER.

Kinetic analysis of bAMF endocytosis to the ER in the three cell lines showed that the rate of accumulation of bAMF in the ER was equivalent in the ras- and abl-transformed NIH-3T3 cells and approximately 2-fold greater than that in untransformed NIH-3T3 cells (Fig. 3B). Over the 2-h time course of the experiment, delivery of bAMF to the ER was maintained and not saturable in the three cell lines. FACS analysis showed that cell surface expression of AMF-R is reduced following transformation of NIH-3T3 cells (Fig. 3C), although total AMF-R expression as determined by immunoblot was equivalent or increased in ras- and abl-transformed NIH-3T3 cells, respectively, compared with untransformed NIH-3T3 cells (data not shown). The increased rate of delivery of bAMF to the ER is therefore not a consequence of increased receptor expression at the plasma membrane but rather due to rapid receptor recycling.

Incubation of NIH-3T3 cells with 5 mM mβCD selectively blocks bAMF endocytosis to the ER but not the clathrin-dependent endocytosis of bAMF to multivesicular endosomes (44). As can be seen in Fig. 3A, mβCD also blocks bAMF delivery to the ER in both untransformed and ras- and abl-transformed NIH-3T3 cells without significantly affecting bAMF endocytosis to endosomes. High concentrations (10 mM) of mβCD have been shown to block clathrin-dependent endocytosis (54, 55); however, the lack of an effect on the clathrin-dependent endocytosis of bAMF to endosomes serves as an internal control demonstrating that at the 5 mM concentration used, mβCD is selectively inhibiting the caveolae-like pathway of bAMF to the ER. The ability of mβCD to inhibit bAMF endocytosis to the ER in ras- and abl-transformed NIH-3T3 cells confirms the similar nature of this pathway in the three cell lines.

The caveolar distribution of AMF-R is based on the EM localization of AMF-R to smooth plasmalemmal invaginations and its partial colocalization with caveolin by immunofluorescence labeling (43). To ensure that the smooth invaginations to which AMF-R is localized are indeed caveolin-positive and therefore correspond to accepted definitions of caveolae, NIH-3T3 cells were double-labeled by postembedding EM for bAMF (nanogold-streptavidin and silver amplification) and caveolin (12-nm gold particles) (Fig. 4A) or for AMF-R (12-nm gold particles) and caveolin (18-nm gold particles) (Fig. 4B). Both
bAMF- and AMF-R-positive invaginations are labeled for caveolin.

Adenoviral Expression of dynK44A Induces Caveole and Inhibits AMF Endocytosis—Infection of NIH-3T3 cells with an adenovirus expressing the dynK44A mutant enhanced our ability to identify double-labeled caveolae. NIH-3T3 cells expressing this mutant show numerous caveolin-positive caveolae at the plasma membrane (Fig. 4, C–H), including those exhibiting the typical long neck associated with dynamin inhibition (Fig. 4G) (12). Caveolae double-labeled for caveolin and either bAMF (Fig. 4, C and E) or AMF-R (Fig. 4, D, F, and H) are readily detected. bAMF is therefore localized with its receptor to caveolae in NIH-3T3 cells.

ras- and abl-transformed NIH-3T3 cells exhibit significantly fewer caveolae relative to NIH-3T3 cells (Fig. 5, A and B), as reported previously (29), and introduction of the dynK44A mutant by adenoviral infection (51, 52) into ras- and abl-transformed NIH-3T3 cells induced the expression of numerous smooth invaginations morphologically similar to caveole (Fig. 5, C–I). Treatment of dynK44A-infected ras- and abl-transformed NIH-3T3 cells with mβCD prior to fixation resulted in the complete absence of smooth caveolar invaginations (Fig. 5, K and L), as observed for uninfected cells (Fig. 5, A and B). The caveolar invaginations induced by dynK44A are, therefore, sensitive to cholesterol depletion and represent a cholesterol-rich membrane domain or class of glycolipid rafts. Quantification of the expression of caveolae and clathrin-coated pits in uninfected and dynK44A-infected cells demonstrated the significantly increased expression per micron of membrane of morphological caveolae but not of clathrin-coated pits in all three cell types (Fig. 6).

Adenoviral expression of dynK44A blocked both the clathrin-dependent endocytosis of bAMF to endosomes and the caveole-like pathway to the smooth ER (Fig. 7A). Expression of the tTA adenovirus alone did not influence either of the AMF endocytic pathways indicating that inhibition of AMF endocytosis is specifically due to expression of the dynK44A mutant and not to adenoviral infection (Fig. 7B). Dynamin-mediated budding of caveolar vesicles from the plasma membrane therefore regulates AMF-R endocytosis to the ER.

To ensure that dynK44A expression is not inducing caveolin expression and thereby affecting the expression of caveolae, we quantified plasma membrane-associated caveolin labeling by postembedding immunoelectron microscopy of whole cell profiles. As presented per micron of membrane in Fig. 6, the number of caveolar invaginations per cell is dramatically reduced in ras- and abl-transformed cells, and dynK44A expression induces the stable expression of a large number of smooth caveolar invaginations (Fig. 8A). Caveolin labeling associated with the plasma membrane, including caveolae (Fig. 8B) or specifically with caveolae (Fig. 8C) is significantly reduced in ras- and abl-transformed NIH-3T3 cells relative to untransformed NIH-3T3 cells. Expression of dynK44A does not affect total plasma membrane-associated caveolin expression (Fig. 8B) indicating that increased expression of caveolin or its increased recruitment to the plasma membrane is not responsible for the dynK44A-mediated induction of smooth caveolar invaginations. A slight increase in caveolin labeling of caveolar invaginations is observed in all three cell lines (Fig. 8C) but is minimal relative to the increased number of caveolae expressed (Fig. 8A). Immunohistological analysis reveals that caveolin expression in the ras and abl transformants remains significantly below that in NIH-3T3 cells even after adenoviral expression of HA-tagged dynK44A (Fig. 9). Expression of dynK44A has not therefore induced the formation of caveolar invaginations by increasing caveolin expression levels.

FIG. 8. Expression of caveolin at the plasma membrane is not increased following dynK44A infection. Quantification of the number of morphologically identifiable caveolae (A), caveolin labeling at the plasma membrane (including smooth caveolar invaginations) (B), and caveolin labeling specific to smooth caveolar invaginations (C) was determined for 50 complete cell profiles from anti-caveolin-labeled EM grids for untransformed and ras- and abl-transformed NIH-3T3 cells either uninfected (white bars) or expressing dynK44A via adenoviral infection (black bars). The data were obtained from two distinct experiments and represent the average per cell profile.

Adenoviral Expression of Caveolin-1 Negatively Regulates AMF Endocytosis to the ER—Infection of the three cell types with tTA and caveolin-1 adenoviruses induces increased levels of caveolin-1 expression significantly above those in uninfected NIH-3T3 cells (Fig. 9). As previously reported (27), the reintroduction of caveolin-1 into ras- and abl-transformed NIH-3T3 cells induces numerous caveolae at the plasma membrane (Fig. 10) that are morphologically indistinguishable from the caveolae induced by dynK44A infection (Fig. 5). Quantitatively, a dramatic increase in caveolar expression and in anti-caveolin labeling at both the plasma membrane and in caveolae was observed in ras- and abl-infected cells such that caveolae and caveolar levels were equivalent to or greater than those of uninfected NIH-3T3 cells (Fig. 11, A–C). Infection of NIH-3T3 cells with the caveolin-1 adenovirus induced lesser (1.5- to 2-fold) increases in the number of caveolae and in caveolin expression at the cell surface (Fig. 11, A–C). Caveolin-1 overexpression in the transformed cells reduced bAMF endocytosis to the ER to levels comparable to uninfected NIH-3T3 cells but did not affect bAMF internalization to endosomes; the increased expression of caveolin-1 in NIH-3T3 cells also selectively decreased bAMF endocytosis to the ER (Fig. 11, D and
SV40 is also internalized via cell surface caveolae to the smooth endoplasmic reticulum (ER), defines at least two distinct non-clathrin endocytic pathways. Inhibition of AMF endocytosis to the ER represents the first identified endocytic pathway from the clathrin-independent pathway (9, 10, 54) and the existing one.

Indeed, the term caveolae was invoked long before the identification of cholesterol-rich and detergent-resistant membrane domains or glycolipid rafts in the absence of caveolin is quite significant. Glycolipid rafts are therefore dynamic endocytic microdomains (19, 21). Distinct dominant negative caveolin mutants differentially affect SV40 endocytosis and RAS signaling and suggest that caveolin may act to regulate caveolae function and endocytosis by controlling the cholesterol content of glycolipid rafts and perhaps caveolar vesicles (58).

Caveolin Is Not Necessary for Caveolae Invagination and Budding of Caveolar Vesicles—The caveolae-mediated endocytic pathway of AMF-R to the ER is still present in ras- and abl-transformed NIH-3T3 cells that exhibit reduced levels of caveolin and caveolae. Overexpression in these cells of the dynK44A dominant negative mutant using an adenoviral expression system induces the expression of morphologically identifiable caveolae and inhibits AMF-R endocytosis to the ER, confirming previous reports that dynamin regulates the budding and endocytic function of caveolae (12, 13). The smooth caveolar invaginations formed in dynK44A-infected ras- and abl-transformed NIH-3T3 cells are not enriched for caveolin yet are still sensitive to cholesterol depletion with mβCD. In cells that express limited amounts of caveolin, cholesterol-rich and detergent-resistant membrane domains or glycolipid rafts invaginate to form caveolae that rapidly give rise to endocytosis-competent vesicles such that caveolae are visible only when budding is inhibited. Caveolin is not essential for caveolae invagination or endocytosis, and, indeed, the endocytic potential of cholesterol-rich and detergent-resistant membrane domains or glycolipid rafts in the absence of caveolin is quite significant. Glycolipid rafts are therefore dynamic endocytic structures (59) that, upon invagination and budding from the plasma membrane, are equivalent, if only transiently, to the morphological definition of caveolae. The absence of caveolae at the plasma membrane does not preclude the presence of a caveolar endocytic pathway.

The induction of caveolae by caveolin expression in caveolin-minus cells, as described here and in other reports (26–28), is due to the stabilization of caveolae by caveolin at the plasma membrane, permitting their visualization by electron microscopy of fixed samples, as previously suggested (43). Caveolin association with rafts may modify their functional properties by regulating the protein and lipid composition of individual plasma membrane microdomains (19, 21). Distinct dominant negative caveolin mutants differentially affect SV40 endocytosis and RAS signaling and suggest that caveolin may act to regulate caveolae function and endocytosis by controlling the cholesterol content of glycolipid rafts and perhaps caveolar vesicles (58).

Our study therefore demonstrates that morphological flask-shaped caveolae form independently of caveolin-1 expression. Indeed, the term caveolae was invoked long before the identi-
fication of caveolin (15). Nevertheless, because few caveolae are visualized at the plasma membrane in the absence of caveolin, stably expressed caveolae are necessarily caveolin-associated, and caveolin is therefore a reliable marker for caveolae expression. The caveolin-1 knockout mouse is viable, and the phenotype is relatively minor suggesting that if caveolae function is essential for development and survival of the organism, it is not dependent on caveolin-1 expression (60, 61). Furthermore, although caveolae expression was dramatically reduced in the caveolin-1 knockout mice, a few caveolar invaginations were still identified (61).

Caveolin Is a Negative Regulator of Caveolae Internalization—Similar to the AMF endocytosis to the ER reported here in ras- and abl-transformed NIH-3T3 cells, prior studies have also reported the internalization of cholera toxin, glycosylphosphatidylinositol-anchored proteins, or the interleukin 2 receptor via non-clathrin cholesterol-dependent pathways in cells that do not express caveolin (42, 45, 62). The significant overexpression of caveolin-1 obtained using adenoviral infection significantly reduced but did not completely inhibit AMF internalization (Fig. 11D) indicating that caveolin-1 stabilization of caveolae at the plasma membrane slows but does not prevent caveolae-mediated endocytosis.

Caveolae-mediated endocytosis in endothelial cells that express significant amounts of caveolin-1 is well documented (63). Although caveolin-1 knockout mice did not exhibit altered serum albumin levels (61), lung endothelial cells of caveolin-1 knockout mice exhibit reduced albumin uptake (64), and reintroduction of caveolin-1 into caveolin-1 knockout fibroblasts induced albumin internalization (60). Internalization of albumin by gp60 or alboncid requires gp60 activation and interaction with caveolin-1; albumin endocytosis was disrupted by caveolin-1 overexpression, which resulted in the sequestration of Gαi preventing gp60 activation (34). Caveolin-1 expression and association with caveolar domains may regulate not only their rate of internalization but also select the cargo that follows this endocytic route.

It is possible that all glycolipid raft domains, currently defined biochemically, can invaginate to form caveolar vesicles. However, it is more likely that different classes of rafts exist with differential abilities to invaginate and bud from the plasma membrane and to form functionally distinct caveolar vesicles. For instance, caveolae and raft domains mediate both endocytosis to the ER (41, 43, 44, 46) and to endosomes and the Golgi (35–37, 59, 65, 66) and in endothelial cells, distinct caveolar vesicle populations have been shown to mediate transcytosis of albumin and insulin (67).

Caveolin-1 is shown here to be a negative regulator of caveolar endocytosis that acts to slow detachment of caveolar vesicles from the plasma membrane. Reduced caveolin-1 expression is associated with different forms of cancer in vivo, and decreased caveolin-1 expression in vitro is associated with cell transformation and tumorigenicity identifying caveolin-1 as a tumor suppressor gene (27, 29, 30, 53, 68–71). The increased rate of internalization of AMF-R to the ER in transformed NIH-3T3 cells corresponds to decreased cell surface expression...
of AMF-R suggesting that, in cells lacking caveolin-1, AMF-R rapidly transits the plasma membrane. Similarly, FACS analysis of B16 melanoma and K1755 fibrosarcoma metastatic variants reported decreased cell surface AMF-R expression in the high metastatic variants (72). It is therefore conceivable that decreased expression of caveolin-1 results in the destabilization of AMF-R cell surface expression and the deregulation of AMF-R trafficking.

The well-characterized association of AMF-R expression with tumor malignancy (73–79) implicates this caveolea-mediated endocytic pathway in AMF-R function in tumor cell motility and metastasis.

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