Exosome Uptake Depends on ERK1/2-Heat Shock Protein 27 Signaling and Lipid Raft-mediated Endocytosis Negatively Regulated by Caveolin-1

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Background: Exosome vesicles can transfer molecular information previously shown to stimulate tumor development; however, the mechanism of exosome uptake is unknown.

Results: Mammalian cells internalize exosomes through lipid raft-mediated endocytosis negatively regulated by caveolin-1.

Conclusion: Our findings provide novel insights into cellular uptake of exosomes.

Significance: Our data provide potential strategies for how the exosome uptake pathway may be targeted.

The role of exosomes in cancer can be inferred from the observation that they transfer tumor cell derived genetic material and signaling proteins, resulting in e.g. increased tumor angiogenesis and metastasis. However, the membrane transport mechanisms and the signaling events involved in the uptake of these virus-like particles remain ill-defined. We now report that internalization of exosomes derived from glioblastoma (GBM) cells involves nonclassical, lipid raft-dependent endocytosis. Importantly, we show that the lipid raft-associated protein caveolin-1 (CAV1), in analogy with its previously described role in virus uptake, negatively regulates the uptake of exosomes. We find that exosomes induce the phosphorylation of several downstream targets known to associate with lipid rafts as signaling and sorting platforms, such as extracellular signal-regulated kinase-1/2 (ERK1/2) and heat shock protein 27 (HSP27). Interestingly, exosome uptake appears dependent on unperturbed ERK1/2-HSP27 signaling, and ERK1/2 phosphorylation is under negative influence by CAV1 during internalization of exosomes. These findings significantly advance our general understanding of exosome-mediated uptake and offer potential strategies for how this pathway may be targeted through modulation of CAV1 expression and ERK1/2 signaling.

The process of endocytosis involves multiple mechanisms in mammalian cells differing in the type of cargo and fate of cargo upon internalization. Described mechanisms of classical endocytosis include clathrin-dependent endocytosis, macropinocytosis, clathrin-independent endocytosis pathways such as caveolaemediated uptake associated with lipid rafts (or cholesterol-enriched membrane microdomains) in the plasma membrane and non-classical pathways involving non-clathrin, non-caveolae-mediated endocytosis (1–4). Secreted vesicles, here classified as exosomes (also referred to as microvesicles, shedded vesicles, ectosomes, microparticles, plasma membrane-derived vesicles) are complex biological vehicles enclosed with cytoplasmic components and genetic material (5–8). The putative function of exosomes in cancer are based on the recently described findings of the transfer of genetic material and signaling proteins, resulting in e.g. increased angiogenesis and metastasis (8–12). Exosomes are released from multivesicular bodies (MVBs)2 upon their fusion with the plasma membrane (13). Recent studies suggest that released vesicles may transfer functional RNA as well as transmembrane proteins contributing to the propagation of a transformed cell phenotype (9, 10, 14). Accordingly, exosomes can be regarded as multi-purpose delivery vehicles in analogy with an endogenous virus-like particle infecting cells in the surrounding environment. Importantly, recent reports have documented key processes involved in exosome release and have identified similarities in topology and mechanisms to retroviral budding (15, 16). It is still controversial whether vesicular uptake is cell type specific (17), and whether it involves membrane fusion or endocytosis (18, 19). Thus, studies elucidating the mechanisms involved in exosome uptake remain an important challenge. We have previously reported on microvesicle-induced pro-angiogenic signaling; a process highly relevant in the most aggressive brain tumor type GBM (20). Here we set out to elucidate the unknown mechanisms of the uptake of GBM cell-derived vesicles and the signaling events involved in the internalization process.

2 The abbreviations used are: MVB, multivesicular body; GBM, glioblastoma; CAV, caveolin; HSP, heat shock protein; NTA, nanoparticle tracking analysis; TIRF, total internal reflection fluorescence; TF, tissue factor; TfR, transferrin; Ctx, cholera toxin; MβCD, methyl-β-cyclodextrin; HMG-CoA; 3-hydroxyl-3-methylglutaryl coenzyme A.
EXPERIMENTAL PROCEDURES

Cell Culture—Human umbilical vein endothelial cells (HUVECs, Lonza) were cultured in endothelial basal medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml hydrocortisone, and 20 µg/ml human recombinant EGF. Human cervix adenocarcinoma (HeLa, ATCC), HeLa cells previously generated to stably overexpress CAV1-YFP (21), human GBM cells (U87 MG, ATCC) and wild-type (WT) or CAV1 knock out (cav-1 (−/−)) mouse embryonic...
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fibroblasts (MEF, ATCC) were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (growth medium). CHO-K1 and COS-7 cells were cultured in F12K supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin. U87 MG shRNA negative control (NC) and shRNA CAV1 were routinely cultured in growth medium supplemented with 1000 μg/ml G418. All cells were cultured in a humidified incubator with 5% CO₂ at 37 °C.

Antibodies and Reagents—Antibodies for CAV1 (ab2910), CD63 (ab8219), TSG101 (ab30871), clathrin heavy chain (ab21679), p-HSP27 (ab17937), HSP27 (ab5579), flotillin-1 (ab41927), calnexin (ab2798), β-actin (ab8227), and α-tubulin (ab7291) were from Abcam. TF antibody (10H10) and siRNA for clathrin (sc-35067) were from Santa Cruz Biotechnology. Secondary antibodies conjugated with 5 or 15 nm gold were from Electron Microscopy Sciences, Fort Washington, PA. Total ERK1/2 antibody (9102) and siRNA for HSP27 (#65265) were from Cell Signaling. Normal mouse IgG was from Molecular Probes. siRNA for CD63 (#4392420, ID:s2701), Mission Lentiviral transduction particles (SHCLNV) (shRNA) for CAV1 (NM_001753 clone TRCN000008002) and negative control (NC) (SHCO16V, pLKO.1-puro), phosphorylated (Thr183/Tyr185) ERK1/2 antibody (M8159), PKH67 Green Fluorescent cell linker midi kit, PKH26 red fluorescent cell linker midi kit, CellVive labeling kit, cholera toxin subunit B FITC conjugate, FITC conjugated 10 kDa dextran, FITC-conjugated transferrin, Phalloidin-TRITC, amiloride, methyl-

exosomes were in indicated experiments labeled with PKH67, PKH26 or cellvive midklaret fluorescent labeling kit according to the manufacturer’s protocol (Sigma). Each vesicle preparation was stored at 4 °C and used within 5 days after isolation.

Confocal Laser Scanning Microscopy and Live-cell Imaging of Internalized Exosomes—All experiments were performed in Zeiss LSM 710 confocal scanning equipment using excitation wavelengths of 405, 488, 546, 633 nm, and a Plan-Apochromat 10X/0.8 M27 objective and a C-Apochromat 63X/1.20 W korr M27 glycerol immersion objective. Image analysis was performed using the Zeiss LSM software. Live cell imaging of internalized exosomes was performed with the same microscope equipped with heat incubator set at 37 °C with 5% CO₂. For live cell confocal laser scanning microscopy experiments, cells were grown in glass bottom chamber slides, and 10–20 μg/ml of labeled exosomes were added to subconfluent cells in phenol red-free and serum-free conditions and incubated as indicated in the figure legend. Surface-bound exosomes were removed by extensive washing with 1 × NaCl and serum-free medium, followed by live cell imaging of intracellular exosomes in phenol red-free medium. For colocalization studies, 10 μg/ml CtxB-FITC, 100 μg/ml Dx10-FITC, or 150 μg/ml TfN-FITC was used.
co-incubated with labeled exosomes and incubated for 30 min. Cells were washed as above and fixed using 2% (w/v) paraformaldehyde for 5 min at room temperature. Cells were immediately analyzed. The weighted colocalization coefficients were calculated in representative cells using the Zeiss LSM software, and represent the number of red pixels (exosomes) that colocalize with turquoise pixels (CtxB or Dx10) divided by the total number of turquoise pixels.

**TIRF Microscopy of Cell Surface-bound Exosomes**—Total internal reflection fluorescence (TIRF) microscopy experi-
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Endocytic uptake of exosomes requires intact lipid membrane rafts. While CtxB and exosome uptake are reduced, suggesting specific inhibition of lipid raft-dependent uptake (*p = 0.02), M, simvastatin dose-dependently inhibits exosome uptake (*p = 0.011). N, sequestration of lipid rafts by filipin III substantially inhibits exosome uptake (*p = 0.001). Presented graphs show mean fluorescent values (10,000 events/sample) of one of three representative experiments (n = 3) with similar results ± S.D.

Electron Microscopy—Isolated exosomes or cells incubated with or without exosomes were washed twice in Tris-buffered saline (TBS), pelleted, fixed for 1 h at 20 °C, and overnight at 4 °C in 2.5% glutaraldehyde in cacodylate buffer. For uptake of exosomes in HUVECs, cells were incubated with 10 μg/ml exosomes followed by trypsinization, washing in TBS and fixation in 2.5% glutaraldehyde in cacodylate buffer overnight at 4 °C. Some samples were incubated with the primary antibodies TF (10H10 titer 1:50) and tsg101 polyclonal (rabbit, titer 1:100) or flotillin-1 antibody (rabbit, 10 μg/ml) followed by detection with secondary antibodies conjugated with 5 nm gold, TF (1:10), or 15 nm gold (tsg101 and flotillin-1, titer 1:20) and analyzed using a JEOL JEM 1230 transmission electron microscope (JEOL, Peabody, MA) as previously described (20).
RESULTS AND DISCUSSION

Exosomes Enter Cells by Endocytosis and Travel Along Endosomal Cytoskeletal Routes—The GBM cell line U87 MG secretes exosome-like microvesicles or exosomes previously characterized by us and others for features and function (9–11, 20). Nanotrack analysis and electron microscopy showed 50–400 nm-sized vesicles (Fig. 1, A and B) that were further characterized for the presence of the exosomal markers CD63, Tissue Factor (TF), and flotillin-1 and the absence of the ER marker calnexin, demonstrating purity of vesicles (Fig. 1C). The internalization of exosomes was visualized by confocal fluorescence microscopy in human umbilical vein endothelial cells (HUVECs) (Fig. 1D, left panel) and U87 MG cells (supplemental video S1). The specificity of the uptake of fluorescent exosomes

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The uptake mechanism of exosomes has been a matter of debate, i.e. whether exosomes enter cells through direct fusion with the plasma membrane of recipient cells or through endocytosis (18, 19). Here, exosomes displayed time and concentration-dependent uptake kinetics (Fig. 1, E and F), and incubation at 4 °C efficiently attenuated uptake, suggesting an energy-dependent process rather than passive membrane passage (Fig. 1G). Further, several normal and transformed cell-lines were able to internalize exosomes at significant levels (Fig. 1H), which argues against that exosome transfer is restricted to specific cell-types as has previously been suggested (17). Live confocal imaging with tubulin-GFP-expressing cells revealed that internalized exosomes travel along microtubules following their uptake (Fig. 1J and supplemental video S2). The mobility of internalized exosomes was substantially reduced by interference with microtubule polymerization using nocodazole (Fig. 1J and supplemental videos S3 and S4). Importantly, under these conditions nocodazole had no specific effect on cell viability (Fig. 1K). These data are consistent with an endocytic process rather than membrane fusion as exosomes followed a time, concentration, and temperature-dependent pathway. The fact that disruption of microtubule attenuated intracellular exosome transport further indicates that exosomes are not fused with the plasma membrane, but rather are dependent on the regular endosomal transportation machinery for further intracellular sorting. To corroborate these data, tissue factor (TF), previously shown to reside in GBM cell-derived exosomes while absent in HUVECs (20), was used to discriminate between endogenous vesicles and internalized exosomes in electron microscopy analysis. Immunolabeling for TF demonstrated that internalized exosomes are enclosed in double membrane structures and do not merge with the plasma membrane of recipient HUVECs (Fig. 1L).

Exosomes were shown to be sorted to larger compartments positive for the MVB marker TSG101 (tumor susceptibility gene 101 protein) (22) (Fig. 1M). In further support of the sorting of endocytosed exosomes to MVBs, a substantial fraction of internalized vesicles were located in a CD63 (LAMP-3, Tsg30)-positive compartment (Fig. 1N). Experiments with exosomes derived from CD63-GFP transfected cells pre-incubated with PKH-labeled exosomes, suggested that mixing of exosomal constituents can occur during maturation in MVBs, resulting in the generation of compound vesicles (supplemental Fig. S1, A and B). However, siRNA-mediated knockdown of CD63 or antibody-mediated cell-surface blocking of CD63 did not significantly affect exosome uptake (supplemental Fig. S1, C–E). These results, and the fact that exosomes did not colocalize with CD63 at short incubation times (Fig. 1O), suggest that although exosomes are sorted to CD63-positive vesicles in recipient cells, CD63 has no direct role in the endocytic uptake of exosomes.

Role of Lipid Raft-mediated Endocytosis in Exosome Uptake—We next sought to define the distinct, cellular pathways associated with the endocytic uptake of exosomes. We found no colocalization of exosomes either with transferrin (Tfn) or acetylated LDL (Fig. 2A), i.e. conventional ligands of classical, clathrin-dependent endocytosis (2). Accordingly, 80% knockdown of clathrin (heavy chain) (Fig. 2, B and C) had no effect on exosome uptake (Fig. 2D). Interestingly, when evaluating by confocal microscopy the colocalization coefficient of exosomes and the lipid raft marker cholera toxin subunit B (CtxB) at short-term incubation, we found ~20% colocalization (Fig. 2, E and G). CtxB binds strongly to GM1 (monosialotetrahexosylganglioside), an established constituent of membrane lipid rafts, the integrity of which depends on the high abundance of cholesterol. We found some colocalization of exosomes and the macrophocytosis/liquid-phase marker 10 kDa dextran (Dx10); however, significantly less than between exosomes and CtxB (Fig. 2, F and G). Amiloride, i.e. an inhibitor of Na+/H+ exchange important for macropinocytic uptake expectedly inhibited Dx10 uptake (Fig. 2H); however, amiloride had no significant effect on exosome uptake at any concentration tested (Fig. 2I). In support of a role of membrane rafts, exosome uptake was highly sensitive to membrane cholesterol depletion by MβCD. Exosome internalization was inhibited by MβCD in a dose-dependent manner, and at the highest concentration used (~60% reduction of uptake was shown in HUVECs (Fig. 2J). Importantly, this effect was not restricted to HUVECs, as MβCD similarly inhibited exosome uptake in U87 MG cells (Fig. 2K). The uptake of fluorescently labeled Tfn was intact while CtxB uptake was expectedly inhibited (Fig. 2L), confirming that MβCD preferentially inhibits non-clathrin dependent endocytosis under these conditions. MβCD could potentially also disrupt cholesterol-rich domains of the exosomal mem
brane. We therefore performed experiments with a statin (simvastatin), which acts through inhibition of the rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and that is widely used as a cholesterol lowering drug in humans. Notably, simvastatin was previously shown to reduce intracellular cholesterol levels in HUVECs (23). We show that simvastatin can dose-dependently inhibit exosome internalization in these cells (Fig. 2M).

Non-classical endocytosis pathways (also defined as lipid raft associated pathways) can be either dependent or independent of CAV1 (2, 4). Caveolae-dependent endocytosis is a well-studied clathrin-independent pathway and shares many features with membrane lipid rafts (24, 25). Importantly, filipin III, an
inhibitor of lipid raft-dependent and caveolar endocytosis, was shown to inhibit exosome uptake by ~50% (Fig. 2N, left panel). As controls of filipin specificity, the uptake of Dx10 and Tf was almost unaffected (Fig. 2N, middle and right panels).

CAV1 Negatively Regulates Exosome Uptake—The above data prompted further studies on the role of CAV1 in endocytic uptake of exosomes. Intriguingly, CAV1 knock out cells (MEF cav1 (−/−)) (Fig. 3A) displayed increased levels of exosome uptake as compared with wild type cells (MEF cav1 (+/+), here denoted as MEF WT) as demonstrated by confocal microscopy (Fig. 3B) and flow cytometry analyses (Fig. 3C). Somewhat unexpectedly, these results suggested a negative regulatory role of CAV1 in exosome uptake. This was also true for U87 MG cells, as stable knockdown of CAV1 (Fig. 3D) resulted in significantly increased uptake of exosomes (Fig. 3E, left panel). As important controls, knockdown of CAV1 did not significantly alter the uptake of clathrin-mediated endocytosis, and macropinocytosis was slightly decreased, as evaluated by Tf and Dx10 uptake, respectively (Fig. 3E, middle and right panels). Moreover, rescue experiments in which CAV1-YFP was ectopically expressed in MEF cav1 (−/−) (Fig. 3F), showed reduced exosome uptake by ~50% as compared with control MEF cav1 (−/−) cells (Fig. 3, G–I). In line with these results, HeLa cells stably transfected to highly overexpress CAV1-YFP exhibited significantly reduced uptake of exosomes (Fig. 3, J and K). We could show that these effects were not restricted to MEF and HeLa cells, as U87 MG and CHO-K1 cells transfected with CAV1-YFP plasmid displayed significantly reduced exosome uptake as compared with control plasmid transfected cells (Fig. 3L).

It has previously been shown that endocytic uptake of virus particles is negatively regulated by CAV1 by stabilization of specific plasma membrane lipid raft domains (1, 26, 27). We next employed confocal microscopy co-localization studies of CAV1-YFP and PKH-labeled exosomes. As shown in Fig. 3M, we could not detect any colocalization at 30 min of incubation, suggesting that exosomes are not associated with CAV1-positive endosomal structures. Confocal microscopy cannot fully discriminate between newly internalized and cell-surface associated exosomes, i.e. the above data do not exclude the possibility of exosomal association with CAV1-containing lipid rafts at earlier time points than 30 min. We therefore applied TIRF microscopy to visualize exosomes present in the membrane region close to the cell surface, and during early phases of internalization. These analyses confirmed that exosomes do not appear to colocalize with CAV1 upon internalization (Fig. 3N).

Together, these findings indicate that the negative regulatory function of CAV1 does not occur through stabilization of lipid raft domains directly involved in exosome uptake. To corroborate confocal microscopy data, showing that internalized exosomes are associated with lipid rafts (Fig. 2E), we next performed immunoelectron microscopy colocalization studies of exosomes (by tissue factor antibody staining to discriminate from endogenous endosomes) and the lipid raft marker flotillin-1. We found a substantial colocalization at 30 min of internalization, signifying that exosomes indeed are taken up through lipid rafts (Fig. 3O).

Role of ERK1/2-HSP27 Signaling Activation in Exosome Uptake—Our findings that CAV1 negatively regulates membrane raft-dependent exosome uptake without apparent co-localization with exosomes, prompted further mechanistic studies on the role of CAV1. Apart from its role as a structural component of lipid rafts, CAV1 has been shown to modify the activity of several signaling proteins, such as Src family members, epidermal growth factor receptor, and integrins (28–30). Consequently, we set out to explore potential signaling mechanisms involved in exosome uptake. In initial experiments, using phospho-kinase arrays, we found that short-term incubation with exosomes resulted in 2–4.5-fold induction of several lipid raft associated proteins; p-FAK, the heat-shock protein p-HSP27, and p-ERK1/2 and its downstream target p-MSK1/2 (Fig. 4. A and B) (for a complete list of relative phospho-kinase levels, see supplemental Fig. S2). Exosomal induction of p-ERK1/2 and p-HSP27 were validated by Western blotting (Fig. 4, C and F). The mitogen-activated protein kinase (MAPK) pathway may be initiated at the cell surface and continue during endosomal sorting, while more recent studies suggest that MAPK signaling is a required element of endocytosis (31). Interestingly, pharmacological targeting of ERK1/2 signaling using the specific inhibitor, U0126 (Fig. 4C) dose-dependently decreased exosome uptake in HUVECs (Fig. 4D) as well as in HeLa cells (Fig. 4E). We next turned our interest to exosome-dependent induction of p-HSP27 (Fig. 4, A and B) as HSPs besides from being localized in the cytosol, have been associated with the cellular membrane and lipid rafts (32). Accordingly, previous reports have shown a role of p-HSP27 in macromolecular internalization by regulation of actin cytoskeleton dynamics (33, 34). We found that inhibition of ERK1/2 signaling dose-dependently reduced p-HSP27 levels (Fig. 4F), suggesting that, at least in the context...
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A direct role of HSP27 as an effector molecule in exosome internalization was supported by significantly reduced exosome uptake upon siRNA-mediated knock-down of HSP27 (Fig. 4, H and I). In agreement with the previously understood role of HSP27 in cytoskeleton rearrangement, cells transfected with siRNA for HSP27 as compared with non-target siRNA exhibited an abnormal actin cytoskeleton as assessed by phalloidin staining (Fig. 4, J and K). Pharmacological disruption of the actin cytoskeleton (Fig. 4L) using Cytochalasin D or Lantrunculin A inhibited uptake of exosomes under similar conditions (Fig. 4, M and N), reinforcing the notion that an intact actin cytoskeleton is required for efficient exosome uptake. We conclude that exosomes may trigger lipid raft mediated endocytosis through signaling activation of ERK1/2-dependent pathways that include a specific role of HSP27.

CAV1 Regulates Uptake of Exosomes through Suppression of ERK1/2 Signaling—From the above findings, we next explored the hypothesis that CAV1 negatively regulates exosome uptake through interference with ERK1/2 signaling. We could appreciate that the induction of p-ERK1/2 during exosome uptake was enhanced in MEF cav-1 (−/−) as compared with MEF WT cells at all time points tested (Fig. 5A). Previous studies suggested CAV1-mediated down-regulation of MAPK signaling in rodents but not in human fibroblasts (35); however, in the context of exosome uptake we observed CAV1-mediated suppression of p-ERK1/2 induction in both mouse (MEF) and human (HeLa)-derived cells (Fig. 5, A and D). Consistent with the findings in HUVECs (Fig. 4D) and HeLa cells (Fig. 4E), reduced levels of p-ERK1/2 by U0126 treatment in MEF cells (Fig. 5B) resulted in decreased internalization of exosomes (Fig. 5C). More importantly, enhanced exosome uptake by CAV1-deficiency could be directly linked to ERK1/2 signaling, as U0126 treatment efficiently counteracted exosome uptake in MEF cav-1 (−/−) cells (Fig. 5C). Accordingly, in HeLa-CAV1-YFP overexpressing as compared with wild-type HeLa cells, we found reduced capability of exosomes to induce p-ERK1/2 signaling and subsequent p-HSP27 induction at multiple time points of exosome stimulation (Fig. 5D). We conclude that exosome uptake commences through lipid raft-mediated endocytosis associated with and dependent on signaling activation of ERK1/2 and HSP27. Further, our data indicate that CAV1 localized in the plasma membrane negatively regulates endocytic uptake of exosomes at least partly through suppression of ERK1/2 signaling activation (for a schematic overview, see Fig. 5E).

Here, we present several significant findings that advance our understanding of exosome uptake at the level of membrane uptake pathways and signaling regulation. First, we establish that exosome uptake mainly occurs through non-clathrin dependent, lipid raft-mediated endocytosis. To circumvent limitations caused by the high sensitivity of labeled exosomes to detergents and fixation solutions, we have in our studies control by CAV1. Additional signaling proteins involved are ERK1/2 and HSP27, and probably additional ERK1/2 downstream targets. HSP27 is known to be involved in rearrangement of the actin cytoskeleton important for the invagination of the plasma membrane during endocytosis.
applied live microscopy using fluorescent fusion protein constructs, fluorophore-labeled ligands and/or mild fixation procedures in combination with electron microscopy studies. Data from these studies strongly suggest that exosomes are internalized by endocytosis rather than by membrane fusion at the plasma membrane. More importantly, we identify specific signaling mechanisms implicated in the uptake process, and find an unexpected and significant role of CAV1 in regulating exosome uptake. Our data suggest that negative regulation of endocytic uptake of exosomes occurs through stabilization of lipid rafts by CAV1. However, these results do not exclude multilevel signaling through the described or other pathways regulated by or independent of CAV1. Further studies investigating other related pathways are of high interest.

Although we provide convincing evidence that ERK1/2 is activated by exosomes especially in the context of CAV1 deficiency, and that ERK1/2 activity is required for efficient exosome uptake, our studies do not fully elucidate the protein interactions involved. A reciprocal regulation of ERK1/2 and CAV1 has previously been described in other systems, where the activation of ERK1/2 was suppressed by CAV1, and the up-regulation of ERK1/2 in turn was shown to suppress CAV1 mRNA levels (36). Moreover, ERK1/2 was shown to localize to caveolae in the plasma membrane, and the caveolar localization of ERK1/2 negatively regulated further signal transduction to the nucleus (36, 37). In the context of exosome internalization, however, we failed to demonstrate a direct protein interaction of CAV1 and ERK1/2, as determined by confocal microscopy analyses and anti-CAV1 antibody pulldown experiments (Supplemental Fig. S3, A and B). The protein interactions responsible for CAV1-mediated negative regulation of ERK1/2 signaling during exosome uptake remains has to be determined in future studies. Based on our findings of a role for CAV1 and ERK1/2 in exosome internalization, it may be speculated that uptake of extracellular vesicles is governed by the signaling status of recipient cells. In the context of the tumor microenvironment, this may be determined by specific oncogenic events in malignant cells and the availability of e.g. growth factors, cytokines, and their respective receptors in the stromal compartment. Thus, future studies should explore the possibility of differential transfer of vesicles in the context of CAV1 and ERK1/2 expression, as these proteins are frequently deregulated at various stages of tumor development (38, 39). Moreover, as exosome-based delivery of mRNA and miRNA has been proposed as a feasible, therapeutic approach in cancer and other pathological conditions (40), further comprehensive understanding of vesicular uptake mechanisms should offer a more rational design of exosomes as drug delivery vehicles.

Acknowledgment—We thank Maria Baumgarten for preparation and immunostaining of EM sections.

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