Expression of Caveolin-1 Is Required for the Transport of Caveolin-2 to the Plasma Membrane

RETENTION OF CAVEOLIN-2 AT THE LEVEL OF THE GOLGI COMPLEX*

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Caveolins-1 and -2 are normally co-expressed, and they form a hetero-oligomeric complex in many cell types. These caveolin hetero-oligomers are thought to represent the assembly units that drive caveolae formation *in vivo*. However, the functional significance of the interaction between caveolins-1 and -2 remains unknown. Here, we show that caveolin-1 co-expression is required for the transport of caveolin-2 from the Golgi complex to the plasma membrane. We identified a human erythroleukemic cell line, K562, that expresses caveolin-2 but fails to express detectable levels of caveolin-1. This allowed us to stringently assess the effects of recombinant caveolin-1 expression on the behavior of endogenous caveolin-2. We show that expression of caveolin-1 in K562 cells is sufficient to reconstitute the *de novo* formation of caveolae in these cells. In addition, recombinant expression of caveolin-1 allows caveolin-2 to form high molecular mass oligomers that are targeted to caveolae-enriched membrane fractions. In striking contrast, in the absence of caveolin-1 expression, caveolin-2 forms low molecular mass oligomers that are retained at the level of the Golgi complex. Interestingly, we also show that expression of caveolin-1 in K562 cells dramatically up-regulates the expression of endogenous caveolin-2. Northern blot analysis reveals that caveolin-2 mRNA levels remain constant under these conditions, suggesting that the expression of caveolin-1 stabilizes the caveolin-2 protein. Conversely, transient expression of caveolin-2 in CHO cells is sufficient to up-regulate endogenous caveolin-1 expression. Thus, the formation of a hetero-oligomeric complex between caveolins-1 and -2 stabilizes the caveolin-2 protein product and allows caveolin-2 to be transported from the Golgi complex to the plasma membrane.

Caveolae, the "little caves" first described in electron micrographs of endothelial cells, have emerged in recent years as the site of the important dynamic regulatory events at the plasma membrane (1–4). For example, caveolae have been implicated in signal transduction by both receptor tyrosine kinases and G proteins (2, 3).

Caveolins (Cav-1, -2, and -3)[1] are a gene family of cytoplasmic membrane-anchored scaffolding proteins that: (i) help to sculpt caveolae membranes from the plasma membrane proper and (ii) participate in the sequestration of inactive signaling molecules (2, 3). In the adult, caveolins-1 and -2 are co-expressed and are most abundant in type I pneumocytes, endothelia, fibroblastic cells, and adipocytes (5, 6). In contrast, the expression of caveolin-3 is restricted to striated muscle cells (7). Interestingly, the genes encoding murine caveolin-1 and caveolin-2 are co-localized within the A2 region of mouse chromosome 6 (6-A2) (8). Similarly, human *CAV1* and *CAV2* co-map to 7q31 in a region of conserved synteny with murine 6-A2 (9).

Caveolae-like vesicles can be generated by expressing caveolin-1 or -3 in insect cells or in mammalian cell lines, providing an *in vivo* assay for caveolin-dependent vesicle formation (10–13). In addition, caveolin-induced vesicle formation appears to be isoform-specific. Expression of caveolin-2 alone under the same conditions failed to drive the formation of vesicles, either in insect cells or in mammalian cells (12, 13).

It has been suggested that caveolin-2 may function as an accessory protein in conjunction with caveolin-1 (3, 6). In support of this notion, caveolins-1 and -2 form a stable hetero-oligomeric complex of ~200–400 kDa in cell types where they are co-expressed (6). These caveolin hetero-oligomers are thought to represent the assembly units that drive the forma-

[1] The abbreviations used are: Cav, caveolin; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; Mes, 4-morpholinoethanesulfonic acid.

tion of caveolae membranes in nonmuscle cells (12, 13). However, it has been postulated that caveolin-2 requires caveolin-1 to form high molecular mass oligomers; when caveolin-2 is expressed alone it behaves as a mixture of monomers and dimers (12, 13). In contrast, caveolin-1 forms high molecular mass homo-oligomers of ~350 kDa (14, 15). Thus, it has been hypothesized that caveolin-2 molecules are embedded within or become tightly associated with high molecular mass homo-oligomers formed by caveolin-1 (6).

Given that caveolins-1 and -2 are co-expressed, that they form a hetero-oligomeric complex *in vivo*, and that even their genes are co-localized to the same chromosomal region in mouse and man, it is apparent that this interaction is of vital importance. Despite the emerging importance of caveolins-1 and -2, little is known about the functional significance of their interactions *in vivo*.

Here, we show that caveolin-2 functionally requires co-expression with caveolin-1 for exit from the Golgi complex. In addition, we directly demonstrate that recombinant expression of caveolin-1 allows caveolin-2 to form high molecular mass oligomers that are Triton-insoluble and are targeted to low density Triton-insoluble plasma membrane domains that are enriched in caveolin-1. Thus, the formation of a hetero-oligomeric complex between caveolins-1 and -2 allows caveolin-2 to be transported from the Golgi to the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—Antibodies and their sources were as follows: anti-caveolin-1 IgG (rabbit pAb N-20; Santa Cruz Biotech, Inc.), anti-caveolin-2 IgG (mAb 65; Ref. 6; gift of Dr. Roberto Campos-Gonzalez, Transduction Laboratories), and anti-Cab45 IgG (rabbit pAb; Ref. 16). The anti-GDP dissociation inhibitor antibody was a gift from Dr. Perry Bickel (Washington University, St. Louis, MO) (17). Lissamine rhodamine B sulfonyl chloride-conjugated goat anti-rabbit antibody and fluorescein isothiocyanate-conjugated goat anti-mouse antibody were purchased from Jackson Immunoresearch (West Grove, PA). All other biochemicals used were of the highest purity available and were obtained from regular commercial sources. DNA manipulations, including ligations, bacterial transformation, and plasmid purification were carried out using standard procedures. Protein concentrations of cell lysates were determined using the BCA protein assay (Pierce).

Cell Culture and Stable Expression of Caveolin-1—K562 cells were grown in RPMI supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% fetal bovine serum. Expression of caveolin-1 was obtained by a described retroviral vector-based gene transfer procedure (18). Briefly, the caveolin-1 cDNA was inserted into the *Bam*HI site of the PINCO plasmid and is under the control of the 5′ Moloney long terminal repeat. This plasmid also separately encodes a form of green fluorescent protein (GFP) under the control of the cytomegalovirus promoter, allowing transduced cells to be conveniently identified and purified by FACS analysis. The plasmid was transfected by the calcium-phosphate/cloroquine method into the amphotropic packaging cell line Phoenix (19, 20); viral supernatants were collected after 48 h. For the infection, K562 cells were resuspended at 1×10^5/ml in 0.45 μM filtered viral supernatant, centrifuged for 45 min at 1, 800 rpm, and placed in the incubator for 2 h. Four infection cycles were performed before the cells were placed in growth medium. Infected cells were analyzed and sorted following standard procedure by FACS scan (FACS-Vantage; Beckton Dickinson, Omaha, CA) with a standard excitation wavelength of 488 nm for GFP.

Transient Expression of Caveolins in CHO Cells—CHO cells were grown in Dulbecco's modified Eagle's medium supplemented with glutamine, antibiotics (penicillin and streptomycin), 1% nonessential amino acids, and 10% fetal calf serum. Cells (~30–50% confluent) were transiently transfected with either the cDNA encoding caveolin-2, caveolin-3, or vector alone (pCB7) using a modified calcium-phosphate precipitation method (21–23). 48 h post-transfection, cells were scraped into lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, and 60 mM octylglucoside). The expression levels of endogenous caveolin-1 were then monitored by SDS-PAGE (12.5% acrylamide) followed by Western blotting.

Triton Insolubility—Infected or uninfected K562 cells were washed twice with PBS and lysed for 30 min at 4 °C in a buffer containing 10 mM Tris, pH 8.0, 0.15 M NaCl, 5 mM EDTA, and 1% Triton X-100 (24).

Samples were centrifuged at 14,000 rpm for 10 min at 4 °C. Pellet (insoluble) and supernatant (soluble) fractions were resolved by SDS-PAGE (12.5% acrylamide) and analyzed by immunoblotting.

Velocity Gradient Centrifugation—Samples were dissociated in Mes-buffered saline containing 60 mM octylglucoside. Solubilized material was loaded atop a 5–40% linear sucrose gradient and centrifuged at 50,000 rpm for 10 h in a SW 60 rotor (Beckman) (5–7, 14). Gradient fractions were collected from above and subjected to immunoblot analysis. Molecular mass standards for velocity gradient centrifugation were as we described previously (5–7, 14).

Preparation of Caveolae-enriched Membrane Fractions—K562 cells were washed with PBS and lysed with 2 ml of Mes-buffered saline (25 mM Mes, pH 6.5, 0.15 M NaCl) containing 1% (v/v) Triton X-100 (22, 23, 25–33). Homogenization was carried out with 10 strokes of a loosely fitting Dounce homogenizer. The homogenate was adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in Mes-buffered saline and placed at the bottom of an ultracentrifuge tube. A 5–30% linear sucrose gradient was formed above the homogenate and centrifuged at 39,000 rpm for 16–20 h in a SW41 rotor (Beckman Instruments). A light scattering band confined to the 15–20% sucrose region was observed that contained caveolin-1 but excluded most other cellular proteins. From the top of each gradient, 1-ml gradient fractions were collected to yield a total of 12 fractions. An equal volume from each gradient fraction was separated by SDS-PAGE and subjected to immunoblot analysis.

Immunoblot Analysis—Cellular proteins were resolved by SDS-PAGE (12.5% acrylamide) and transferred to nitrocellulose membranes. Blots were incubated for 2 h in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween 20) containing 5% powdered skim milk. After three washes with TBST, membranes were incubated for 2 h with the primary antibody in TBST (anti-caveolin-1 IgG diluted 1:1000 or anti-caveolin-2 IgG diluted 1:250) and for 1 h with a peroxidase-conjugated secondary antibody (diluted 1:5000). Immunoreactivity was revealed using an ECL detection kit (Amersham Pharmacia Biotech).

Limits of Detection Analysis—The limit of detection for anti-caveolin-1 IgG was determined experimentally using recombinant caveolin-1 purified after baculo-virus based expression in Sf21 insect cells, as we described previously (11, 34). Serial dilutions of purified recombinant caveolin-1 were performed (such as 25, 50, and 100 pg of protein), separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Blots were incubated with anti-caveolin-1 IgG (a 1:500 dilution) overnight. Bound antibodies were visualized with an horse-radish peroxidase-conjugated secondary antibody (1:10,000 dilution) and detected using an ECL kit (Amersham Pharmacia Biotech). After exposure, the films were scanned and quantitated using NIH Image. Using this approach, we determined that the limit of detection was ~10 pg (defined as twice as much signal as background density). The absolute concentration of recombinant caveolin-1 was determined using a high sensitivity silver staining kit (Waco Chemical, Inc.).

Immunofluorescence Microscopy—K562 cells were washed with PBS, resuspended in PBS containing 1% bovine serum albumin and centrifuged with a Cytospin Shandon (Pittsburgh, PA) onto a slide. Cells were fixed for 30 min at room temperature with 2% paraformaldehyde in PBS. Fixed cells were rinsed with PBS and permeabilized with 0.1% Triton X-100, 0.2% bovine serum albumin for 10 min. Then cells were treated with 25 mM NH_4Cl in PBS for 10 min at room temperature to quench free aldehyde groups. Cells were rinsed with PBS and incubated with the primary antibody for 1 h at room temperature; anti-caveolin-1 IgG (rabbit pAb N-20), anti-caveolin-2 IgG (mouse mAb 65), and anti-Cab45 IgG (rabbit pAb) were diluted 1:200, 1:200, and 1:80, respectively, in PBS with 0.1% Triton X-100, 0.2% bovine serum albumin. After three washes with PBS (10 min each), cells were incubated with the appropriate secondary antibody for 1 h at room temperature: either lissamine rhodamine B sulfonyl chloride-conjugated goat anti-rabbit antibody (5 μg/ml) or fluorescein isothiocyanate-conjugated goat anti-mouse antibody (5 μg/ml). Finally, cells were washed three times with PBS (10 min each wash), and slides were mounted with slow fade anti-fade reagent (Molecular Probes, Eugene, OR) and observed under a Bio-Rad MR 600 confocal microscope. To confirm the specificity of the antibody probes we utilized, we performed a series of critical control experiments, such as omission of primary antibodies, use of caveolin-1 negative and positive K562 cells, and preabsorption of anti-peptide antibodies with the corresponding epitope, as we described previously (6, 16, 23, 35).

Northern Analysis—Total RNA was extracted and purified according to the manufacturer's instructions (Qiagen). 10 μg of total cellular RNA was separated on a formaldehyde-agarose gel (1%) and subjected to Northern blot analysis with a ^{32}P-labeled probe to detect the human
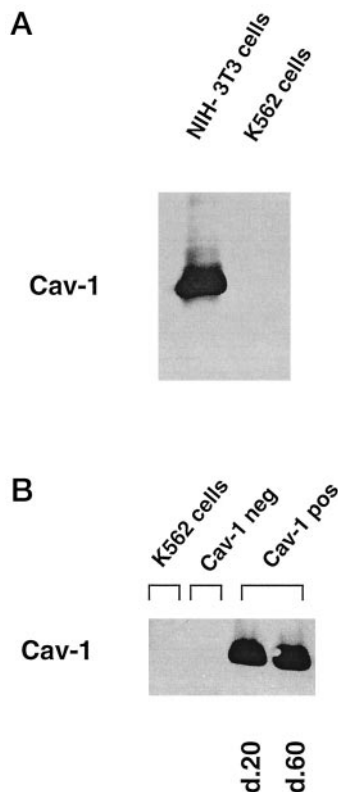
FIG. 1. **Stable expression of caveolin-1 in K562 cells.** *A*, NIH-3T3 and K562 total cell lysates (10 μg of protein) were analyzed for caveolin-1 expression by SDS-PAGE followed by Western blotting. Note that K562 cells were found to be caveolin-1 negative even with longer exposures. *B*, lysates (20 μg of protein) from K562 cells, K562 infected with empty vector (*Cav-1 neg*), or K562 infected with caveolin-1 (*Cav-1 pos*) were subjected to SDS-PAGE and analyzed for caveolin-1 expression. Note that caveolin-1 expression was found to be unaltered even after 60 days post-infection.

FIG. 2. **Caveolin-1 expression induces caveolae formation in K562 cells.** Cav-1 negative (*A*) and Cav-1 positive (*B*) K562 cells were analyzed by electron microscopy. *Arrowheads* point at newly formed caveolae vesicles attached to the plasma membrane. These flask-shaped vesicles of ~50–100 nm in diameter are completely absent in control cells (*A*). *N*, nucleus; *bars*, 500 nm.

caveolin-2 mRNA. The 28 S and 18 S rRNA were visualized by ethidium bromide staining. Hybridization was carried out for 20 h at 42 °C. The blot was then washed sequentially (twice) with 2× SSC/0.1% SDS (30 min, 68 °C) and 0.2× SSC/0.1% SDS (30 min, 68 °C).

Electron Microscopy—Transmission electron microscopy was performed as described previously by our laboratory. Briefly, samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed with 1% osmium tetroxide, followed by 1% uranyl acetate, dehydrated through a graded series of ethanol, and embedded in LX112 resin (LADD Research Industries, Burlington, VT) as detailed in Refs. 25 and 28. Ultrathin sections were cut on a Reichert Ultracut E, stained with uranyl acetate followed by lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope at 180 Kv. The number of caveolae/field was quantitated. >30 fields were subjected to morphometric analysis.

RESULTS

Recombinant Expression of Caveolin-1 in K562 Cells—To stringently assess the effects of caveolin-1 on the behavior of caveolin-2, we searched for a cell line that fails to express detectable levels of endogenous caveolin-1 but still expresses significant levels of endogenous caveolin-2. Through this screening approach, we identified K562 cells as a cell line that fulfills these criteria (Ref. 6 and data not shown). K562 cells are a well characterized human erythroleukemic cell line. Fig. 1*A* shows that these cells clearly fail to express caveolin-1 by Western blot analysis. Thus, we used K562 cells as our model system to assess the behavior of caveolin-2 in the absence or presence of recombinantly expressed caveolin-1.

We derived a pool of K562 cells stably expressing caveolin-1 using an established expression system (Ref. 18; see "Experimental Procedures"). Briefly,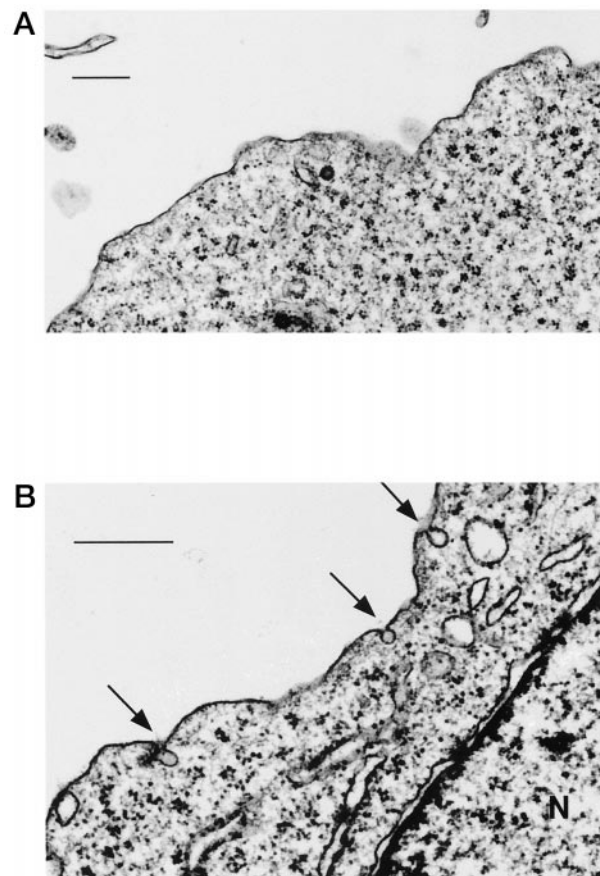 K562 cells were infected with a recombinant retroviral vector encoding two separate protein products, *i.e.* GFP and caveolin-1. This allows for a population of transduced cells to be selected by FACS analysis, using GFP as a marker. These cells will be referred to as Cav-1 positive K562 cells. As a control, we also derived a population of cells harboring vector alone using the same GFP/FACS approach (termed Cav-1 negative K562 cells). This strategy allows us to obtain a high infection efficiency (*i.e.* 30% in K562 cells) and to increase the sorted cell population to virtually 100% of positive expressing cells with the same initial features of the parental cell line without the need for drug selection. Importantly, this approach avoids any possible variation that may occur when selecting clonal cell lines. In this regard, total lysates from parental K562 cells and FACS-sorted caveolin-1 negative or positive K562 were analyzed by Western blotting for caveolin-1 expression. As shown in Fig. 1, Cav-1 positive K562 cells expressed caveolin-1 with no detectable variation after 20 or 60 days post-infection.

Expression of Caveolin-1 Reconstitutes Caveolae Formation in K562 Cells—Next, we compared the morphology of the plasma membrane of Cav-1 positive and negative K562 cells by transmission electron microscopy. Our results indicate that recombinant expression of caveolin-1 is sufficient to reconstitute the formation of caveolae in K562 cells. Fig. 2 shows that Cav-1 negative K562 cells lack any detectable caveolae at the plasma membrane. In striking contrast, Cav-1 positive K562 cells show numerous flask-shaped caveolae at the plasma membrane (~2,000–4,000 caveolae per cell). These results indicate that expression of caveolin-2 alone is not sufficient to

FIG. 4. **Velocity gradient analysis of endogenous caveolin-2 in the absence or presence of caveolin-1 expression.** Approximately 1×10^7 Cav-1 negative or positive K562 cells were solubilized, loaded atop a 5–40% sucrose density gradient, and subjected to centrifugation for 10 h. Twelve fractions were recovered, and a 20 μl aliquot from each fraction was analyzed by SDS-PAGE and Western blotting. *Arrows* mark the positions of molecular mass standards. Note that caveolin-1 expression dramatically increases the molecular mass of caveolin-2 containing oligomeric complexes, *i.e.* from a broad peak of ~29–200 kDa to a discrete peak of ~200–400 kDa.
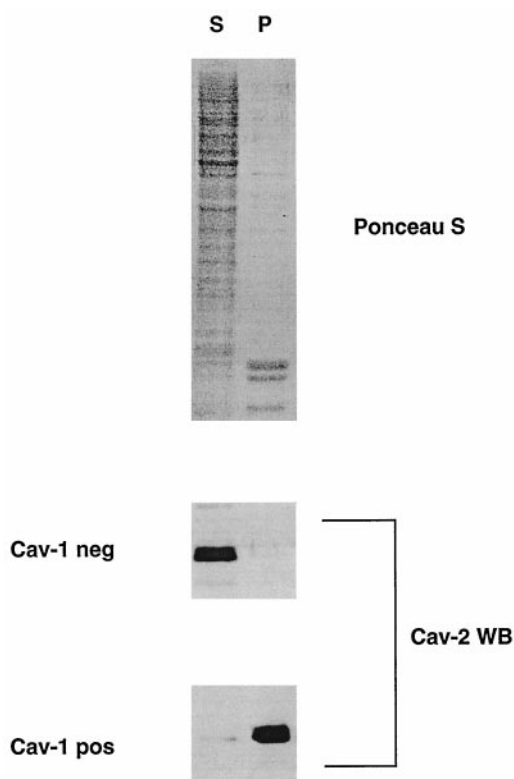

FIG. 3. **Detergent solubility of caveolin-2 in the absence or presence of caveolin-1 expression.** Cav-1 negative and positive K562 cells were lysed in a buffer containing 1% Triton X-100 and centrifuged to obtain insoluble (*P*) and soluble (*S*) fractions. These fractions were adjusted to equal volumes, and an aliquot of each (~20 μl) was analyzed by SDS-PAGE and Western blotting using a caveolin-2-specific monoclonal antibody probe (mAb 65). A representative Ponceau S-stained blot is shown to illustrate the distribution of total protein. Note that expression of caveolin-1 almost quantitatively converts caveolin-2 from a Triton-soluble to a Triton-insoluble protein product.

generate morphologically detectable caveolae in K562 cells.

Expression of Caveolin-1 Allows Endogenous Caveolin-2 to Form Triton-insoluble High Molecular Mass Oligomers That Are Transported to Caveolae Membranes—Caveolins-1 and -2 are normally co-expressed, and they form a stable high molecular mass hetero-oligomeric complex (5, 6). These caveolin hetero-oligomers are transported to the plasma membrane, where they are thought to drive the formation of mature caveolae domains (11). Once these hetero-oligomers reach the plasma membrane, they become Triton-insoluble because of their incorporation into caveolae membranes (24). Resistance to detergent solubilization is thought to reflect the local lipid microenvironment in which these caveolin homo-oligomers are embedded. This lipid microenvironment is rich in cholesterol and sphingolipids and excludes phospholipids, generating a liquid-ordered phase that is reflected by its resistance to solubilization by nonionic detergents at low temperatures (at or below 4 °C) (36). In contrast, caveolin-1 associated with the Golgi complex remains Triton-soluble (27).

Thus, we next examined the effects of recombinant caveolin-1 expression on the (i) Triton-insolubility; (ii) oligomeric state; and (iii) caveolar targeting of endogenous caveolin-2. In Cav-1 negative K562 cells, caveolin-2 remained greater than 95% Triton-soluble and partitioned with the majority of cellular proteins (~85%) (Fig. 3). In striking contrast, in Cav-1 positive cells, caveolin-2 behaved as a Triton-insoluble protein, with greater that 90% of caveolin-2 partitioning with the Triton-insoluble fraction that represents the minority of cellular proteins (~15%). Thus, recombinant expression of caveolin-1
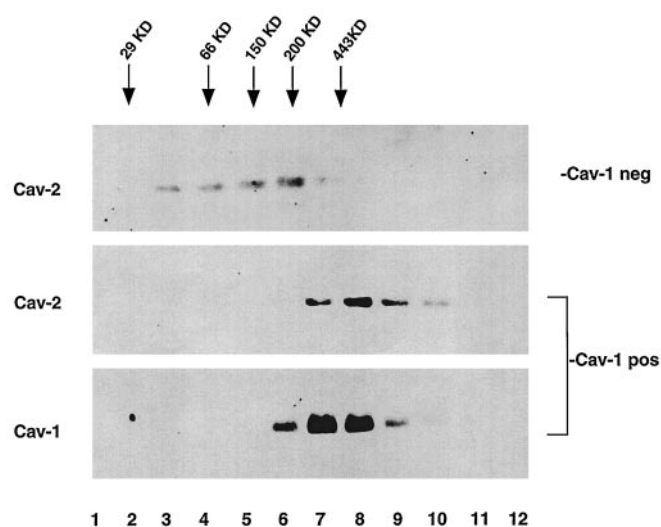
was sufficient to almost quantitatively convert caveolin-2 from a Triton-soluble to a Triton-insoluble protein.

To assess the oligomeric state of caveolin-2, we employed an established top-loaded velocity gradient system that we used previously to assess the oligomeric state of homo-oligomers of caveolins-1, -2, and -3 (5–7, 14). Under these conditions, recombinant caveolins-1 and -3 form homo-oligomers of ~350 kDa, whereas recombinant caveolin-2 migrates as a mixture of monomers or homo-dimers. Interestingly, when caveolins-1 and -2 are endogenously co-expressed they form a high molecular mass hetero-oligomeric complex of ~200–400 kDa. This implies that caveolin-2 can form high molecular mass oligomers in conjunction with caveolin-1, although this hypothesis has never been formally tested. Fig. 4 shows that in Cav-1 negative K562 cells, endogenous caveolin-2 behaves as a heterogeneous species, migrating as a broad peak of ~29–200 kDa. In contrast, in Cav-1 positive K562 cells, endogenous caveolin-2 migrates as a discrete high molecular mass complex of ~200–400 kDa and follows the distribution of recombinant caveolin-1. These results indicate that co-expression with caveolin-1 is required for caveolin-2 to form a high molecular mass oligomer.

To separate membranes enriched in caveolae from the bulk of cellular membranes and cytosolic proteins, an established equilibrium sucrose density gradient system was utilized (22, 23, 25–33). In this fractionation scheme, immunoblotting with anti-caveolin IgG can be used to track the position of caveolae-derived membranes within these bottom-loaded sucrose gradients. Using this procedure, caveolin-1 is purified ~2000-fold relative to total cell lysates as ~4–6 μg of caveolin-rich domains (containing ~90–95% of total cellular caveolin-1) are obtained from 10 mg of total cellular proteins (22, 30). We and others have shown that these caveolae-enriched fractions exclude >99.95% of total cellular proteins and also markers for noncaveolar plasma membrane, Golgi, lysosomes, mitochondria, and endoplasmic reticulum (25, 28, 29).

Fig. 5 illustrates that in Cav-1 negative K562 cells, caveolin-2 is quantitatively excluded from these low density Triton-
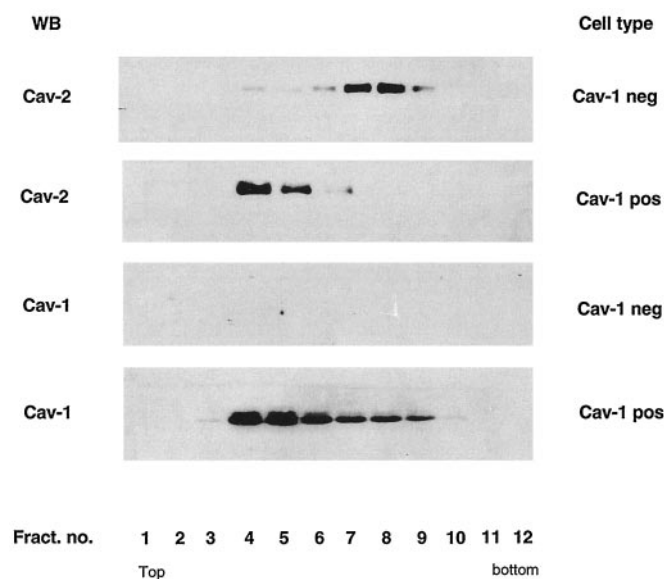
WB   Cell type

Cav-2   Cav-1 neg

Cav-2   Cav-1 pos

Cav-1   Cav-1 neg

Cav-1   Cav-1 pos

Fract. no.   1   2   3   4   5   6   7   8   9   10   11   12

Top   bottom

FIG. 5. **Targeting of endogenous caveolin-2 to low density Triton-insoluble membrane domains in the absence or presence of caveolin-1 expression.** Approximately 1.8×10^7 Cav-1 negative or positive K562 cells were homogenized in a buffer containing 1% Triton X-100, adjusted to 40% sucrose, and placed at the bottom of an ultracentrifuge tube. A 5–30% linear sucrose gradient (a flotation gradient) was formed above the homogenate and centrifuged at 39,000 rpm for 16–20 h in a SW41 rotor. Twelve 1-ml fractions were collected, and an aliquot of each fraction ($\sim 20~\mu l$) was resolved by SDS-PAGE and subjected to immunoblot analysis with anti-caveolin-1 or anti-caveolin-2 IgG. As expected, recombinant caveolin-1 is highly enriched in fractions 4 and 5, which are the caveolae-enriched membrane fractions. Note that in the absence of caveolin-1, endogenous caveolin-2 is confined mainly to the Triton-soluble fractions (7–9); in contrast, in the presence of caveolin-1, endogenous caveolin-2 is found predominantly in fractions 4–5 that represent Triton-insoluble caveolae-enriched membrane domains.

insoluble membranes (fractions 4 and 5). However, caveolin-2 still attained partial buoyancy, migrating predominantly in the noncaveolar fractions 7–9; this may be due to the fact that caveolin-2 is still tightly associated with membrane-derived lipids even in its Triton-soluble form. In contrast, recombinant expression of caveolin-1 was sufficient to recruit caveolin-2 quantitatively to the low density Triton-insoluble fractions that contain caveolae membranes (fractions 4 and 5). Taken together, these results indicate that the expression of caveolin-1 allows endogenous caveolin-2 to form Triton-insoluble high molecular mass oligomers that are correctly transported to caveolae membranes.

Retention of Caveolin-2 at the Level of the Golgi Complex: Caveolin-1 Expression Is Required for the Transport of Caveolin-2 to the Plasma Membrane—In Cav-1 negative K562 cells, endogenous caveolin-2 was excluded from caveolae membranes (Fig. 5). Thus, we next determined the subcellular localization of caveolin-2 by immunofluorescence using confocal microscopy. Fig. 6 shows the localization of endogenous caveolin-2 in Cav-1 negative and positive K562 cells. Interestingly, in Cav-1 negative K562 cells, endogenous caveolin-2 was primarily retained at the level of a perinuclear compartment and did not reach the plasma membrane (Fig. 6*A*). We identified this perinuclear compartment as the Golgi complex by performing double-labeling experiments with antibodies directed against the resident Golgi marker protein, Cab45, that is endogenously expressed (16). In contrast, in Cav-1 positive K562 cells, endogenous caveolin-2 was efficiently targeted to the plasma membrane under these conditions (Fig. 6*B*), whereas Cab45 remained within the Golgi complex.

NIH 3T3 cells co-express caveolins-1 and -2 where they are

co-localized and form a stable hetero-oligomeric complex *in vivo*, and both are targeted to caveolae membranes (6). Thus, we compared the subcellular distribution of recombinant caveolin-1 with the distribution of endogenous caveolin-2 in Cav-1 positive K562 cells. Fig. 6*C* shows the distribution of recombinant caveolin-1 and endogenous caveolin-2. The localization of endogenous caveolin-2 was revealed by immunostaining with specific antibodies that recognize only caveolin-2 and not caveolin-1. These antibodies have been extensively characterized in a previous report (6). Note that both recombinant caveolin-1 and endogenous caveolin-2 show strict co-localization at the level of the plasma membrane.

Fig. 6*D* shows that in caveolin-1 positive K562 cells, all three antibodies used for immunocytochemistry (directed against caveolin-1, caveolin-2, or Cab45) recognize a single major band of the expected size by immunoblot analysis. These results using K562 cells directly confirm the high specificity of these well characterized antibody probes (6, 16, 23).

Recombinant Expression of Caveolin-1 Increases the Expression Levels of Endogenous Caveolin-2—Given that endogenous caveolin-2 is retained in an intracellular compartment in Cav-1 negative K562 cells, this may affect the stability of the caveolin-2 protein product. Thus, we examined the steady-state expression levels of the endogenous caveolin-2 protein in Cav-1 negative and positive K562 cells by Western blot analysis. Fig. 7 shows that recombinant expression of caveolin-1 greatly up-regulates the steady-state expression levels of endogenous caveolin-2. This result cannot be explained by anti-body cross-reactivity, as we have previously shown that these antibodies are isoform-specific and only recognize either caveolin-1 or caveolin-2 selectively (6, 23).

To examine the possibility that up-regulation of endogenous caveolin-2 occurs via transcriptional control, we performed Northern blot analysis using the cDNA for human caveolin-2 as the probe. Our results indicate that the levels of caveolin-2 message remain the same in Cav-1 negative and positive K562 cells (Fig. 8). These results are consistent with the idea that caveolin-1 expression stabilizes the caveolin-2 protein product.

Does Recombinant Expression of Caveolin-2 Up-regulate Endogenous Caveolin-1 Expression?—To address this issue, we transiently transfected CHO cells with the cDNA for caveolin-2. As controls, we also transfected CHO cells with vector alone or with the cDNA for caveolin-3. Caveolin-3 does not form a complex with caveolin-1 or with caveolin-2 and serves as a highly selective and appropriate negative control for these studies. Interestingly, we find that transient expression of recombinant caveolin-2, but not caveolin-3, up-regulates the expression of endogenous caveolin-1 (Fig. 9). No changes in endogenous caveolin-1 expression were observed with the empty vector control (pCB7). These data support the notion that caveolins-1 and -2 can act to stabilize each other. Immunoblotting with IgG directed against GDP dissociation inhibitor served as an additional control for equal loading.

DISCUSSION

Here, we succeeded in stably expressing caveolin-1 through a retroviral vector, leading to the formation of caveolae vesicles at the plasma membrane in K562 cells. We have shown that caveolin-1 co-expression is required for the transport of caveolin-2 from the Golgi complex to the plasma membrane. Expression of caveolin-1 in K562 cells dramatically up-regulated the expression of endogenous caveolin-2. Northern analysis revealed that caveolin-2 mRNA levels remain constant under these conditions, suggesting that the expression of caveolin-1 stabilizes the caveolin-2 protein. Interestingly, we also observed that transient expression of recombinant caveolin-2 up-regulates the expression of endogenous caveolin-1 in CHO
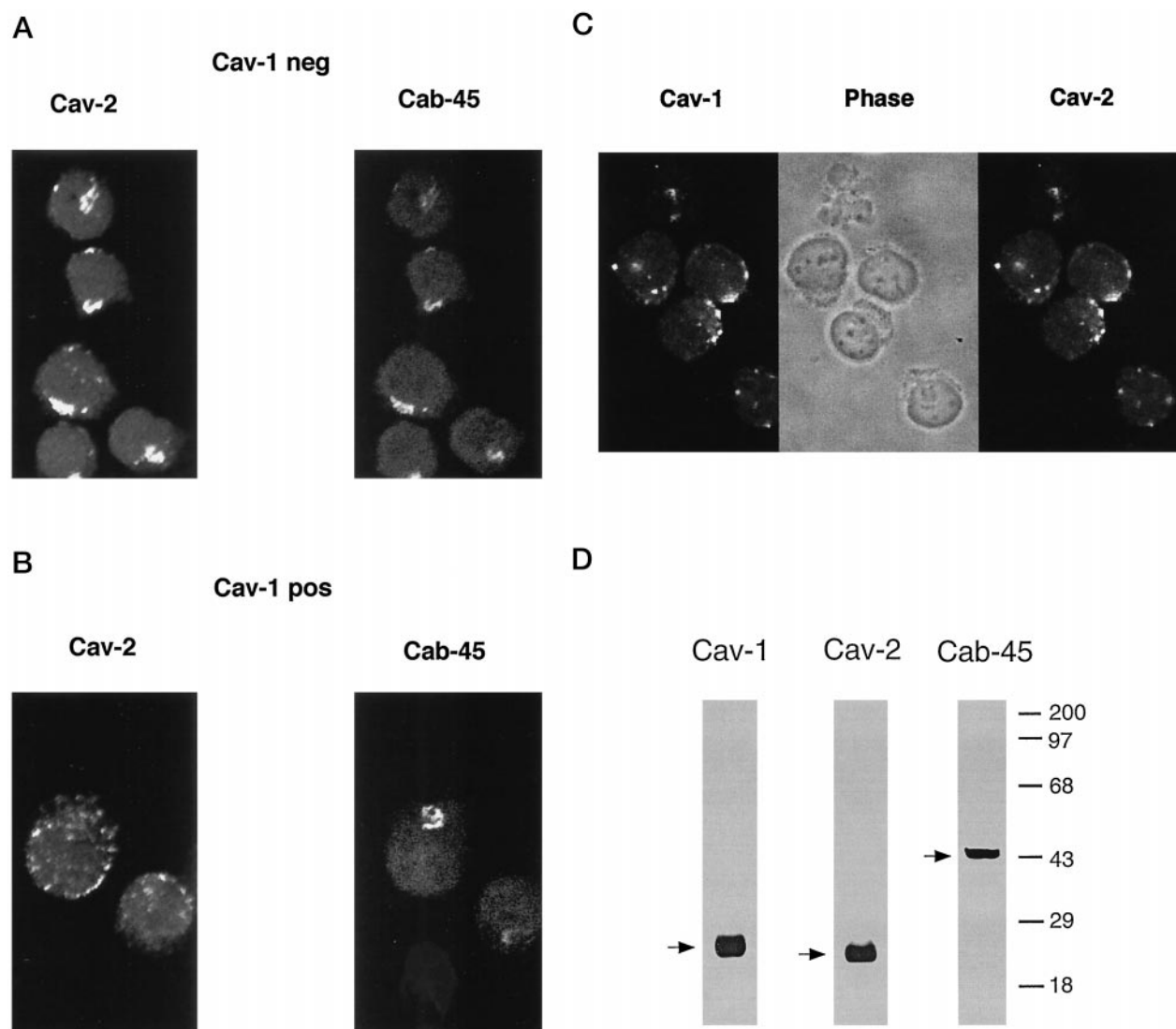
FIG. 6. **Retention of caveolin-2 at the level of the Golgi complex: Caveolin-1 expression is required for the transport of caveolin-2 to the plasma membrane.** *A* and *B*, Cav-1 negative (*A*) and Cav-1 positive (*B*) K562 cells were doubly immunostained with a mouse mAb directed against caveolin-2 and a rabbit pAb directed against the Golgi marker protein, Cab-45. Bound primary antibodies were visualized by incubation with distinctly tagged fluorescent secondary antibodies (see "Experimental Procedures"). In the absence of caveolin-1, note that caveolin-2 and Cab-45 are co-localized to the same area of a given cell that corresponds to the Golgi complex. In contrast, in the presence of caveolin-1, caveolin-2 is localized to the plasma membrane, and Cab-45 is still retained within the Golgi complex. *C*, Cav-1 positive K562 cells were doubly immunostained with a mouse mAb directed against caveolin-2 and a rabbit pAb directed against the unique N terminus of caveolin-1 (residues 2–21). *Left panel*, caveolin-1 immunostaining; *middle panel*, phase image; *right panel*, caveolin-2 immunostaining. Note that caveolin-1 and caveolin-2 are co-localized to the same areas of the plasma membrane, yielding the typical punctate pattern that is characteristic of caveolar localization. *D*, lysates from caveolin-1 positive K562 cells were used to confirm the specificity of the antibody probes used for immunofluoresence by Western blot analysis. Note that all three antibodies (directed against caveolin-1, caveolin-2, or Cab-45) recognize a single major band by immunoblotting; these results directly confirm the high specificity of these well characterized antibody probes (6, 16, 23).

cells. This stabilizing effect appeared to be isoform-specific because expression of recombinant caveolin-3 does not up-regulate endogenous caveolin-1 expression.

Why is caveolin-2 retained intracellularly within the Golgi in the absence of caveolin-1 expression? There are several possibilities: (i) Other membrane protein complexes, such as the T cell receptor (37, 38) or the asialo-glycoprotein receptor (39, 40), require the co-expression of multiple subunits; if these subunits are overexpressed individually, they are retained in an intracellular compartment, such as the endoplasmic reticulum, and degraded. (ii) Alternatively, caveolin-2 may be retained in the Golgi complex via a novel Golgi retention signal; little is currently known about the signals that govern stable residence within the Golgi complex. (iii) In addition, caveolin-2 may stably interact with a resident Golgi protein, and this interaction may be disrupted by the interaction of caveolin-1 with caveo-

lin-2. Because we show here that caveolin-2 expression is up-regulated by expression of caveolin-1 and visa versa, we favor the first possibility that caveolins-1 and -2 are simply part of a multi-subunit complex that requires interaction at the level of the Golgi for the transport of the caveolin-2 protein to the plasma membrane. Further studies will be necessary to determine whether caveolin-2 specifically interacts with any resident Golgi proteins.

Does caveolin-1 require caveolin-2 co-expression for transport to the cell surface? Currently, this question remains unresolved. Thus far, all the mammalian cell lines that we have examined either express both caveolins-1 and -2 (*e.g.* normal NIH 3T3 cells), express caveolin-2 and reduced levels of caveolin-1 (*e.g.* Ras-transformed NIH 3T3 cells), or express caveolin-2 and undetectable levels of caveolin-1 (*e.g.* K562 cells) (6, 13, 41). This is consistent with the general observation that
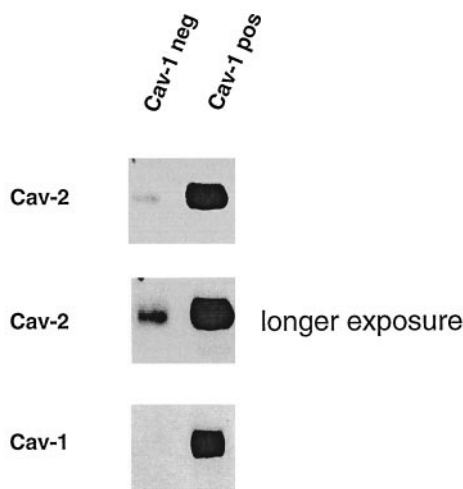
FIG. 7. **Recombinant expression of caveolin-1 up-regulates the endogenous expression levels of caveolin-2 in K562 cells.** Total cell lysates from Cav-1 negative or Cav-1 positive K562 were subjected to immunoblot analysis with either anti-caveolin-1 or anti-caveolin-2 IgG. Note the dramatic increase in the expression level of caveolin-2 in cells expressing caveolin-1. Each lane contains an equivalent amount of total protein (10 μg).

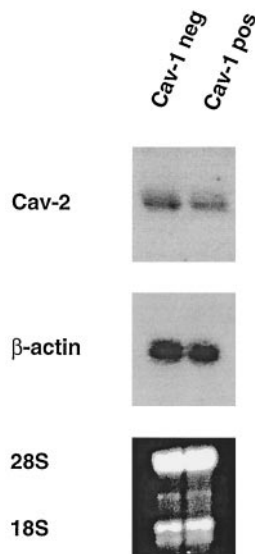
FIG. 8. **Northern blot analysis of caveolin-2 mRNA levels in Cav-1 negative or Cav-1 positive K562.** Total RNA (10 μg) purified from K562 cells was separated using a formaldehyde-agarose gel, transferred to a hybridization membrane, and probed with the ^{32}P-labeled human caveolin-2 cDNA. Note that the levels of caveolin-2 mRNA remain the same in Cav-1 negative and positive K562 cells. Molecular sizes were derived from the ethidium bromide stained RNA marker. The distribution of β-actin mRNA (2 kilobases) is shown as a control for equal loading. The positions of the 28 S and 18 S rRNA species were visualized by ethidium bromide staining.

caveolin-1 levels are down-regulated in response to cell transformation, whereas caveolin-2 levels remain relatively unchanged by comparison (6). However, to date, we have not identified a cell line that expresses caveolin-1 and reduced levels of caveolin-2 or a cell line that expresses caveolin-1 and undetectable levels of caveolin-2. Thus, this will remain an open question until a cell line that fulfills these criteria can be obtained. However, we favor the possibility that caveolin-1 does not require caveolin-2 for transport to the cell surface. This idea is indirectly supported by the observation that recombinant expression of mammalian caveolin-1 alone in insect cells leads to the formation of caveolae-sized vesicles (~50–100 nm in diameter) (11); in contrast, recombinant expression of caveo-
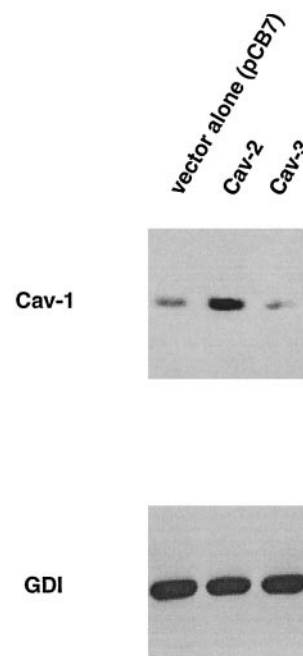

FIG. 9. **Transient expression of recombinant caveolin-2 up-regulates the endogenous expression levels of caveolin-1 in CHO cells.** CHO cells were transiently transfected with the cDNAs encoding caveolin-2, caveolin-3, or empty vector alone (pCB7). Total cell lysates were prepared and subjected to immunoblot analysis with anti-caveolin-1 IgG. Each lane contains an equivalent amount of total protein (50 μg). Interestingly, we find that transient overexpression of caveolin-2, but not caveolin-3, up-regulates the expression of endogenous caveolin-1. No changes in caveolin-1 expression were observed with the empty vector control (pCB7). Immunoblotting with IgG directed against GDP dissociation inhibitor served as an additional control for equal protein loading.

lin-2 alone in insect cells does not result in the formation of any caveolae-sized vesicles (12). Finally, co-expression of caveolins-1 and -2 in insect cells leads to the formation of a much more uniform population of caveolae-sized vesicles (~45–65 nm in diameter) (12). This is consistent with the hypothesis that caveolin-2 functions as an "accessory protein" in conjunction with caveolin-1 to modulate the size of caveolae.

Previously, we attempted to assess the effects of caveolin-1 co-expression on the behavior of caveolin-2 by employing v-Abl and Ras-transformed NIH 3T3 cell lines that expressed dramatically reduced levels of caveolin-1 and virtually normal levels of caveolin-2. However, the expression of caveolin-1 in these cell lines was still clearly detectable, in contrast with K562 cells in which caveolin-1 is undetectable even on long overexposures. Using these transformed NIH 3T3 cells, we observed modest effects of caveolin-1 induction on the behavior of caveolin-2 (6, 12). However, our current results are consistent with the general trend of these previous observations. This suggests that even low levels of caveolin-1 expression can serve to allow caveolin-2 to exit from the Golgi complex and to form large hetero-oligomeric complexes with caveolin-1.

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Expression of Caveolin-1 Is Required for the Transport of Caveolin-2 to the Plasma Membrane: RETENTION OF CAVEOLIN-2 AT THE LEVEL OF THE GOLGI COMPLEX

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