Caveolin-1 Null Mice Are Viable but Show Evidence of Hyperproliferative and Vascular Abnormalities*

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From the ‡Department of Molecular Pharmacology and The Albert Einstein Cancer Center, the §Analytical Imaging Facility, the ¶Department of Pathology and Institute for Animal Studies, the **Department of Pathology, ‡‡Departments of Developmental and Molecular Biology and Medicine, and The Albert Einstein Cancer Center, the §§Department of Cell Biology and The Albert Einstein Cancer Center, the ¶¶Departments of Urology, Physiology, and Biophysics, Institute for Smooth Muscle Biology, The Albert Einstein College of Medicine, Bronx, New York 10461

Caveolin-1 is the principal structural protein of caveolae membranes in fibroblasts and endothelia. Recently, we have shown that the human CAV-1 gene is localized to a suspected tumor suppressor locus, and mutations in Cav-1 have been implicated in human cancer. Here, we created a caveolin-1 null (CAV-1−/−) mouse model, using standard homologous recombination techniques, to assess the role of caveolin-1 in caveolae biogenesis, endocytosis, cell proliferation, and endothelial nitric-oxide synthase (eNOS) signaling. Surprisingly, Cav-1 null mice are viable. We show that these mice lack caveolin-1 protein expression and plasmalemmal caveolae. In addition, analysis of cultured fibroblasts from Cav-1 null embryos reveals the following: (i) a loss of caveolin-2 protein expression; (ii) defects in the endocytosis of a known caveolar ligand, i.e. fluorescein isothiocyanate-albumin; and (iii) a hyperproliferative phenotype. Importantly, these phenotypic changes are reversed by recombinant expression of the caveolin-1 cDNA. Furthermore, examination of the lung parenchyma (an endothelial-rich tissue) shows hypercellularity with thickened alveolar septa and an increase in the number of vascular endothelial growth factor receptor (Flk-1)-positive endothelial cells. As predicted, endothelial cells from Cav-1 null mice lack caveolae membranes. Finally, we examined eNOS signaling by measuring the physiological responses of aortic rings to various stimuli. Our results indicate that eNOS activity is up-regulated in Cav-1 null animals, and this activity can be blunted by using a specific NOS inhibitor, nitro-L-arginine methyl ester. These findings are in accordance with previous in vitro studies showing that caveolin-1 is an endogenous inhibitor of eNOS. Thus, caveolin-1 expression is required to stabilize the caveolin-2 protein product, to mediate the caveolar endocytosis of specific ligands, to negatively regulate the proliferation of certain cell types, and to provide tonic inhibition of eNOS activity in endothelial cells.

Caveolin was first identified in 1989 by Glenny and colleagues (1, 2) as a major v-Src substrate in Rous sarcoma virus-transformed chicken embryo fibroblasts. Interestingly, this same protein was found to be the primary structural component of caveolae microdomains, 50–100 nm vesicular invaginations of the plasma membrane (3).

Caveolae were morphologically described as early as the 1950s by Yamada (4) and Palade (5). They are curious structures that can be found individually or in clusters at the surfaces of numerous cell types, the best examples of which are adipocytes, endothelial cells, muscle cells, and fibroblasts. Research in the past decade has shown that caveolae are specialized membrane microdomains formed as a result of localized accumulation of cholesterol, glycosphingolipids, and caveolin (6–8). Caveolin, an integral membrane protein that can directly bind cholesterol, most likely plays a major role in the invagination of caveolae from the plasma membrane proper, although our understanding of the mechanisms behind this process remains rudimentary.

Two other members of the caveolin gene family have recently been identified and cloned, caveolin-2 and caveolin-3 (9, 10); as a consequence, caveolin has been re-termed caveolin-1 (Cav-1).1 Caveolin-2 has the same tissue distribution as and co-localizes with caveolin-1, whereas caveolin-3 is expressed only in cardiac, skeletal, and smooth muscle cells (11, 12).

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‡‡‡ Recipient of a Hirsch/Well-Caulier Career Scientist Award. To whom correspondence should be addressed: Dept. of Molecular Pharmacology and The Albert Einstein Cancer Center, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Tel.: 718-430-8828; Fax: 718-430-8830; E-mail: lisanti@aecom.yu.edu

1 The abbreviations used are: Cav-1, caveolin-1; Cav-2, caveolin-2; Cav-3, caveolin-3; eNOS, endothelial nitric-oxide synthase; FITC, fluorescein isothiocyanate; t-NAME, nitro-L-arginine methyl ester; MAP, mitogen-activated protein; EGF, epidermal growth factor; EGF-R, EGF receptor; VEGF-R, vascular endothelial growth factor receptor; mAb, monoclonal antibody; kb, kilobase pair; bp, base pair; PCR, polymerase chain reaction; MEF, mouse embryonic fibroblast; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PFUs, plaque-forming units; KO, knockout; NO, nitric oxide; NOS, nitric-oxide synthase; PE, phenylephrine; Ach, acetylcholine; pAb, polyclonal antibody; Mes, 4-morpholine-ethanesulfonic acid; ES, embryonic stem; CSD, caveolin-1 scaffolding domain.
Although caveolae function in vesicular and cholesterol trafficking (13, 14), they have also been implicated in signal transduction at the plasma membrane (15, 16). Biochemical and morphological experiments have shown that a variety of lipid-modified signaling molecules are concentrated within these plasma membrane microdomains, such as Src family tyrosine kinases, Ha-Ras, eNOS, and heterotrimeric G-proteins (17–22).

In many ways, caveolin-1 is intricately involved in caveolar functioning. In the years after the discovery that caveolae might serve to compartmentalize signaling molecules and facilitate cross-talk among signaling cascades (the so-called “caveola signaling hypothesis” (16)), Cav-1 has been found to be a key regulator of some of these proteins. Both in vitro and cell culture experiments indicate that Cav-1 can directly interact with and maintain some of these signaling molecules in an inactive conformation (reviewed in Ref. 23). In effect, Cav-1 seems to act as a scaffolding protein, able to negatively regulate the activity of other molecules by binding to and releasing them in a timely fashion.

Research in the past few years has established a recurring theme in this regulation. Many of the proteins that either interact with, transcriptionally repress, or are inhibited by Cav-1 fall under the pro-proliferative, oncogenic, and anti-apoptotic category of molecules. Cav-1 interacts with and negatively regulates the EGF-R, platelet-derived growth factor receptor, and Neu tyrosine kinases (24–26), Ha-Ras (17, 18), c-Src (17), and phosphatidylinositol 3-kinase (27). Conversely, caveolin-1 levels are transcriptionally reduced upon activation of the oncogenes Ha-ras, v-abl, myc, neu, the HPV oncogene E6, among others (26, 28–30). Therefore, it is not surprising that we and others (26, 29, 31–37) observed undetectable or very low expression levels of Cav-1 in numerous tumor-derived cell lines.

For some time, it has been known that a certain locus (D7S522; 7q31.1) is an aphidicolin-induced fragile site in the human genome (38, 39) and a hot spot for deletions in a variety of human tumors including breast, prostate, colorectal, ovarian, pancreatic, and renal cell carcinomas (38, 40–46). Interestingly, determination of the genomic organization of the human CAV-1 locus revealed that it maps to 7q31.1, adjacent to the LOH marker D7S522, and as of yet it still remains the closest known gene to this putative tumor suppressor locus (35, 47).

Taken together, the results described above have led many investigators to propose the possibility that Cav-1 is indeed a “tumor suppressor” whose reduction/deletion in cells would provide growth advantages and expedite tumorigenesis. In support of this idea, the only two methods thus far described to abolish Cav-1 expression have arrived at similar conclusions. Antisense-mediated down-regulation of Cav-1 in NIH-3T3 fibroblasts leads to a hyperactivation of the p42/44 MAP kinase pathway and anchorage-independent growth (48). An RNA interference-based ablation of Cav-1 in Caenorhabditis elegans leads to progression of the meiotic cell cycle, a phenotype that mirrors that of Ras activation (49).

Furthermore, a recent report indicates that the caveolin-1 gene is mutated in up to 16% of human breast cancer samples examined (50). Recombinant expression of the caveolin-1 cDNA harboring this mutation (P132L) was sufficient to transform NIH 3T3 cells (50). As similar results have been obtained previously using an antisense approach to ablate caveolin-1 expression (48), these results indicate that the caveolin-1 (P132L) mutation may behave in a dominant-negative fashion. Interestingly, an analogous mutation occurs within the caveolin-1 gene (P104L) in patients with a novel form of autosomal dominant limb-girdle muscular dystrophy (LGMD-1C) (51).

In order to gain a better understanding of caveolae and caveolin-1 functioning in a mammalian organism, we used a gene targeting strategy to disrupt the Cav-1 locus in the mouse. In this way, we could observe the role Cav-1 plays in animal physiology (i.e., during development and into adult life) as well as molecularly (i.e., caveolar biogenesis, its interaction with caveolin-2, and its functional roles in endocytosis, cellular proliferation, and signal transduction). In this study, we describe the generation of mice lacking the cav-1 gene and determine some of the molecular side effects that result from a deficiency of Cav-1 expression.

Undoubtedly, the generation of viable/fertile Cav-1-deficient mice (and cells derived from these animals) will allow us and others to critically evaluate the many proposed functions of caveolae organelles and the caveolin-1 protein in vivo.

### Experimental Procedures

#### Materials—Antibodies and their sources were as follows: anti-caveolin-1 mAb 2297, anti-caveolin-2 mAb 65, and anti-caveolin-3 mAb 26 (10, 11, 52) (gifts of Dr. Roberto Campos-Gonzalez, BD Transduction Laboratories, Inc.); anti-caveolin-1 pAb N-20 (Santa Cruz Biotechnology); anti-

#### Construction of the Targeting Vector—Genomic clones containing the murine Cav-1 locus were isolated from a 129/SvJ (11) x-phae genome library (53) by using probes corresponding to the murine Cav-1 cDNA. The genomic organization of the locus was determined by subcloning portions of these genomic inserts into the vector pBS-SK+ (Stratagene) and using Southern blotting to determine a detailed restriction map of the region (55). One of the genomic clones (containing the first and second exons of Cav-1) was used to construct the targeting vector. Briefly, a 2.7-kb NotI-EcoRI fragment that is immediately 5’ to the first exon and a 2.1-kb BamHI-BamHI fragment immediately 3’ to the second exon of the cav-1 gene were used to flanking the NEO cassette in the targeting vector pGTV-N29 (New England Biolabs) (as shown in Fig. 1).

#### Screening of Homologously Recombined ES Cells and Generation of Germ Line Chimeras—WW6 ES cells (gift of Dr. Pamela Stanley (56)) were electroporated with the linearized targeting construct (40 µg) and selected with G418 (150 µg/ml of active component, Life Technologies, Inc.) for 20 min at 37 °C.

#### Generation of Cav-1 Knockout Mice—ES clones were microinjected into C57BL/6 blastocysts, and three gave rise to male chimeras with a significant ES cell contribution (as determined by an Agouti coat color). By mating with C57BL/6 females and genotyping of offspring tail DNA via Southern and PCR analysis, germ line transmission was confirmed for two separate clones (Fig. 1). F1 male and female heterozygous animals were interbred to obtain Cav-1-deficient animals. To facilitate the genotyping of all future mice, we also devised a 3-primer PCR-based screening strategy. The wild-type specific forward primer was derived from Cav-1 exon 2 (5’-GTGATG-GACGCCGCAACACAG-3’); the knockout-specific forward primer was derived from the neomycin cassette (5’-CTAGTGACAGCTGCTACT-TCC-3’), and the common reverse primer was derived from Cav-1 intron 2 (5’-CTTACATCTTCTCTGCTAG-3’BamHI). PCR conditions were 95 °C/5 min, 35 cycles of 95 °C/1 min, 56 °C/1 min, 72 °C/1 min 20 s) and then 72 °C/7 min, which resulted in a ~650-bp wild-type band and a ~330-bp knockout band.

Animal’s were analyzed at 2–4.5 months of age. Experiments were conducted under the direct supervision of the trained veterinarians of the Einstein Animal Institute, and animal protocols were approved by the Animal Use Committee.

#### Mouse Embryonic Fibroblast (MEF) Culture and Immortalization Protocol—Primary MEFs were obtained from Day 13.5 embryos essentially as described (58). Briefly, embryos were decapitated, thoroughly minced, and trypsinized in 1 ml of 0.05% trypsin, 0.53 mM EDTA (Life Technologies, Inc.) for 20 min at 37 °C. Ten ml of complete medium (Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and
100 mg/ml streptomycin (Life Technologies, Inc.) was used to inactivate the trypsin and resuspend the dissociated cells. Cells were plated on a 10-cm plate and cultured in a 37 °C, 5% CO2 incubator. These “passage 1” cells were further propagated using a defined 3T3 passing protocol (i.e. 3 × 10^3 cells were plated per 60-mm dish every 3 days). For all experiments, when the cells were confluent, the media was replaced with 1 ml of complete medium containing FITC-conjugated antibody (Sigma) at a final concentration of 10 μg/ml. Cells were incubated at 37 °C for 5, 15, and 30 min, washed in PBS, and fixed in 2% paraformaldehyde for 20 min. The cells were washed for 20 min in PBS and mounted on slides with the Prolong anti-fade reagent (Molecular Probes) and imaged with an Olympus IX 70 inverted microscope. Virtually identical experiments were carried out with FITC-conjugated transferrin (10 μg/ml; Sigma).

Caveolin-1 knockout MEFs were grown on 60-mm tissue culture dishes and transiently transfected with the caveolin-1 cDNA in the pcB7 vector using Lipofectamine PLUS (Life Technologies, Inc.), according to the manufacturer’s instructions. The transfected cells were plated on 18-mm coverslips in 12-well plates after 24 h. FITC-conjugated antibody (Sigma) was applied to the transfected cells for a 10-min incubation. Cells transfected with caveolin-1 were detected by immunostaining with anti-Cav-1 IgG (N-20; Santa Cruz Biotechnology) and a rhodamine-conjugated secondary antibody.

MEF Proliferation Curves and Cell Cycle Analysis—Proliferation curves were conducted essentially as described previously (65). Briefly, 15 × 10^3 cells were seeded in a series of twenty 35-mm dishes and cultured under regular growth conditions (DMEM, 10% FBS). Each day, two plates were counted using a hemocytometer, and the medium was changed for the remaining plates. Growth curves were continued for a 10-day time course.

Cell cycle analysis was conducted by Flow Cytometry essentially as described (66). Briefly, 2 × 10^5 cells were seeded in 60-mm dishes, allowed to adhere, and cultured overnight under regular growth conditions (DMEM, 10% FBS) for 24 h. Cells were trypsinized, washed in PBS, and fixed in 70% ethanol at 4 °C for at least 30 min. Fixed cells were resuspended in PBS containing 0.25 mg/ml RNase A (Sigma) and 10 μg/ml propidium iodide (Sigma) and subjected to univariate cell cycle analysis using a Becton-Dickinson FACScan flow cytometer. The G1/G0, S, and G2/M phases of the cell cycle were quantified with CELLQUEST software.

Lung tissue—The lungs were dissected in MEM—25°C. The lungs were minced by 300 mg of liver tissue was placed in 2 ml of PBS (25 mM Mes, pH 6.5, 150 mM NaCl) containing 1% Triton X-100 and solubilized by using quick 10-s bursts of a rotor homogenizer and passing 10 times through a loose fitting Dounce homogenizer. The sample was mixed with an equal volume of 80% sucrose (prepared in MBS lacking Triton X-100), transferred to a 1.2 ml ultracentrifuge tube, and overlaid with a discontinuous sucrose gradient (4 ml of 30% sucrose, 4 ml of 5% sucrose, both prepared in MBS, lacking detergent). The samples were subjected to centrifugation at 200,000 × g (39,000 rpm in a Sorval rotor TH-641) for 16 h. A light scattering band was observed at the 5–30% sucrose interface. These fractions were collected, and 50-μl aliquots of each fraction were subjected to SDS-PAGE and immunoblotting.

Immunofluorescence Microscopy—The procedure was performed as we described previously (17). MEFs (either un-transfected or transfected with the caveolin-1 cDNA) were fixed in 30 min in PBS containing 2% paraformaldehyde, rinsed with PBS, and quenched with 50 mM NH4Cl for 10 min. The cells were then incubated in methanol/acetone, washed with PBS, and double-labeled with a 1:400 dilution of anti-caveolin-1 pAb (Roche Molecular Biochemicals). Protein concentrations were determined using the BCA reagent (Pierce), and the volume required for 10 mg of protein was determined. Samples were separated by SDS-PAGE (12.5% acrylamide) and transferred to nitrocellulose. The nitrocellulose membranes were stained with Pierce CoS (to visualize protein bands) followed by immunoblot analysis. All subsequent wash buffers contained 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20, which was supplemented with 1% bovine serum albumin (BSA) and 2% nonfat dry milk (Carnation) for the blocking solution and 1% BSA for the antibody diluent. Primary antibodies were used at a 1:500 dilution. Horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution, Pierce) were used to visualize bound primary antibodies with the Supersignal chemiluminescence substrate (Pierce).

Purification of Caveolin-enriched Membrane Fractions—Caveolin-enriched membrane fractions were purified essentially as we described previously (59). 200 mg of liver tissue was placed in 2 ml of PBS (25 mM Mes, pH 6.5, 150 mM NaCl) containing 1% Triton X-100 and solubilized by using quick 10-s bursts of a rotor homogenizer and passing 10 times through a loose fitting Dounce homogenizer. The sample was mixed with an equal volume of 80% sucrose (prepared in MBS lacking Triton X-100), transferred to a 1.2 ml ultracentrifuge tube, and overlaid with a discontinuous sucrose gradient (4 ml of 30% sucrose, 4 ml of 5% sucrose, both prepared in MBS, lacking detergent). The samples were subjected to centrifugation at 200,000 × g (39,000 rpm in a Sorval rotor TH-641) for 16 h. A light scattering band was observed at the 5–30% sucrose interface. These fractions were collected, and 50-μl aliquots of each fraction were subjected to SDS-PAGE and immunoblotting.

Immunoblot Analysis—Cells were cultured in their respective media and allowed to reach 80–90% confluence. Subsequently, they were washed with PBS, and incubated with lissamine-rhodamine-conjugated secondary antibody (Jackson ImmunoResearch) for a period of 60 min. Cells were washed with PBS (3 times) and slides were air-dried for 1 h. Cells were further propagated using a defined 3T3 passaging protocol (i.e. 105 cells were plated per 60-mm dish every 3 days). For all experiments, when the cells were confluent, the media was replaced with 1 ml of complete medium containing FITC-conjugated antibody (Sigma) at a final concentration of 10 μg/ml. Cells were incubated at 37 °C for 5, 15, and 30 min, washed in PBS, and fixed in 2% paraformaldehyde for 20 min. The cells were washed for 20 min in PBS and mounted on slides with the Prolong anti-fade reagent (Molecular Probes) and imaged with an Olympus IX 70 inverted microscope. Virtually identical experiments were carried out with FITC-conjugated transferrin (10 μg/ml; Sigma).

Endocytosis Assays—Wild-type and Cav-1 null mouse embryonic fibroblast (MEFs) were plated on 18-mm glass coverslips (Fisher) in 12-well plates. Cells were grown in complete medium (DMEM supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.)). When cells reached ~75–85% confluence, they were further propagated using a defined 3T3 passaging protocol (i.e. Passage 25) then lysed and subjected to immunoprecipitation. MEFs were then lysed and subjected to immunoprecipitation.
Immunostaining of Lung Paraffin Sections—Sections of wild-type and Cav-1 null mouse lung were de-paraffinized in xylene for 4 min and rehydrated through a graded series of ethanol and placed in PBS. Sections were pre-blocked with 2% horse serum for 20 min and then washed with PBS for 10 min. The sections were then incubated with a given primary antibody overnight at 4°C. An HRP-conjugated or biotinylated secondary antibody was added to the sections after a 10-min wash in PBS. After a 30-min incubation with the secondary antibody, the sections were washed in PBS for 20 min. Prolong anti-fade reagent was then added to prevent bleaching of the fluorochrome. Sections were imaged with an Olympus IX 70 inverted microscope. Negative control experiments were also performed using pre-immune serum or normal mouse serum.

Assessment of Exercise Tolerance—A 4-liter beaker filled with water (25°C) was used as a “swimming pool” to assess the exercise tolerance of male mice. Briefly, a very light weight (a paper clip; 0.4 g; ~1.25% of their body weight) was attached to the tail of a mouse with a body weight of ~32 g. The mouse was gently placed in the water and carefully observed. The time at which the mouse was initially unable to maintain complete buoyancy was recorded and the mouse was immediately removed from the pool. No mice were injured in these experiments; 5 animals were tested for each genotype.

Aortic Ring Studies of Vasoconstriction and Vasorelaxation—Wild-type and Cav-1 null male mice (4.5 months old) were obtained from C57BL/6J and reared in a controlled environment with a 12-h light/12-h dark cycle and ad libitum access to food and water. The animal experiments were performed in accordance with the principles outlined in the US National Research Council’s “Guide for the Care and Use of Laboratory Animals.”

RESULTS

Generation of Caveolin-1-deficient Mice via Targeted Disruption of the Cav-1 Locus—We previously determined the genomic organization of the Cav-1 (Cav-1) locus and found that exons 1 and 2 are spaced within 2 kb of each other, whereas exon 3 is ~10 kb downstream (47). Therefore, we generated a targeting vector designed to replace the first two exons and a small portion of the 5’ promoter sequence with the neomycin resistance cassette (neo) (Fig. 1A).

WW6 embryonic stem (ES) cells (56) were electroporated with the targeting vector, and 360 clones were selected with G418. Homologous recombination at this locus is predicted to create two new restriction sites, PstI and XhoI, both of which can be used to identify positive ES cell clones by Southern blot analysis (Fig. 1A); four clones were determined to be positive in this manner. Germ line chimeras were derived from only two of these clones, as shown in the positive Southern blots in Fig. 1B. We subsequently mated these chimeras with C57Bl/6 mice to yield F1 heterozygous offspring, a cohort of which was interbred to produce the Cav-1 null progeny. Southern blot and PCR-based methods of assessing the targeted locus were performed on the first series of live offspring, confirming the predicted loss of a wild-type 8.0-kb band on Southern blot and the 500-bp band on PCR analysis (Fig. 1C). Genotyping of offspring from such heterozygous inter-crosses revealed that there is no reduced viability of the Cav-1-null mice and that mice of all three genotypes are present at the expected Mendelian frequency (Cav-1+/+ 25.2%, Cav-1 +/− 49.2%, Cav-1−/− 25.6%; n = 305 animals).

Although Cav-1 is expressed in numerous tissues at varying levels, it is found in abundance in certain terminally differentiated cells (i.e. adipocytes, endothelial cells, type I pneumocytes, and fibroblasts) (9, 11). Furthermore, Cav-1 expression is completely absent in skeletal and cardiac muscle cells, and in contrast, caveolin-3 (Cav-3), another member of the caveolin gene family, is selectively expressed (10, 12). In order to verify whether the targeted disruption of the Cav-1 locus led to a truly null mutation, we determined the expression of the Cav-1 protein in adipose, lung, and heart tissues from mice of all three genotypes (wild-type, heterozygote, and knockout; Fig. 1C). In all tissues examined, ablation of the Cav-1 locus leads to a concomitant loss of Cav-1 protein expression; β-tubulin is shown as an equal protein loading control.

In addition, several points are worth noting. 1) These mice are deficient in both Cav-1 isoforms (the full-length 178-amin acid α-form and the shorter 146-amino acid β-isoform (52)). 2) The ablation of the Cav-1 locus in only one chromosome, as in the heterozygous animals, is sufficient to reduce protein levels by approximately half. 3) Although Cav-1 is expressed in the cardiac tissue of wild-type and heterozygous mice (Fig. 1D), Cav-1 expression is derived from endothelial and fibroblastic cells within the heart and not the cardiac myocytes themselves (10, 12).

We also assessed Cav-1 expression in cultured mouse embryonic fibroblasts (MEFs), another cell type with abundant Cav-1 expression (67). Two different clones of MEFs for each possible genotype were extracted and cultured from day 13.5 embryos. Immunoblot analysis of Cav-1 levels indicated similar findings to those above, namely a complete ablation of Cav-1 expression in the knockout and a significant reduction in Cav-1 expression in heterozygous MEFs (Fig. 1E).

Phenotype and Histopathological Examination of Cav-1−/− Mice—Caveolin-1 null mice are both viable and fertile. We initially established a large cohort consisting of mice from all genotypes, the eldest of which are now 9 months of age. Although no overt phenotypic abnormalities (including tumors) have been detected, only two mice have thus far died of unknown causes, both of which were Cav-1-deficient (autolysis prevented a pathological work-up). Follow-up of this cohort in the coming months will establish whether Cav-1 deficiency can precipitate tumorigenesis and/or a reduction in life span. A routine histopathological examination of Cav-1 null mice at 4–5 months of age (n = 4 male, n = 4 female) failed to show any evidence of abnormalities, with the exception of lung tissue (see below). We have noticed, however, that although in the first few months of life there is no overt difference between wild-type and knockouts, older Cav-1-deficient mice are more likely to be smaller in size than their wild-type littermates.

The Absence of Caveolin-1 Is Sufficient to Abrogate Caveolae Formation—The molecular components required for caveolar biogenesis have been studied by numerous investigators. From the following observations, the general consensus remains that caveolin-1 plays an essential role in caveolae formation. 1) Cholesterol is required for caveolar invagination, because treatment with cholesterol-depleting agents (e.g. filipin and methyl-β-cyclodextrin) ablates caveolar structures (68). 2) Caveolin-1 is a cholesterol-binding protein, possibly facilitating the concentration of the critical mass of cholesterol required for invagination (8, 69). 3) Down-regulation of caveolin-1 in Ha...
Ras and v-Abl-transformed NIH 3T3 fibroblasts or by antisense strategies results in a concomitant reduction of caveolae at the membrane (28, 48). 4) Overexpression of caveolin-1 in a lymphocytic cell line, cells that do not endogenously express the protein, is sufficient to allow the formation of caveolae (6). Thus, the generation of caveolin-1 null mice provided an opportunity to test this assertion under physiological conditions. In this manner, nonspecific effects due to chemical treatments, cellular transformation, and overexpression would not confound such a study. MEFs derived from Cav-1+/+ and −/− embryos were cultured to near 100% confluency, conditions that have been shown to result in optimal caveolin-1 expression and caveolae formation. Standard transmission electron microscopy was used to visualize the plasma membrane (Fig. 2). While wild-type MEFs have numerous uniformly sized caveolae, the Cav-1-deficient cells are conspicuously devoid of caveolae. An exhaustive search of the plasma membrane from Cav-1 knockout MEFs failed to show any invaginations resembling Cav-1-deficient Mice Show Proliferative and Vascular Defects
However, we did observe the occasional clathrin-coated pit (invaginations typically 5 times larger than caveolae) in MEFs of both genotypes (data not shown), indicating that their number or presence is not affected by a Cav-1 deficiency.

The Absence of Caveolin-1 Leads to Degradation and Redistribution of Caveolin-2—Currently, the caveolin gene family is composed of caveolin-1, -2, and -3. All known terminally differentiated tissues that express caveolin-1 also express the closely related family member caveolin-2 (Cav-2) (9, 11). In contrast, caveolin-3 (Cav-3), the protein with the highest homology to Cav-1, is expressed specifically in muscle cells (including cardiac, skeletal, and smooth muscle). Therefore, we were interested to determine any possible counter-regulatory or compensatory behavior by Cav-2 and Cav-3 in Cav-1 null tissues. We immunoblotted the same tissue samples used to compare Cav-1 expression in mice of different genotypes (Fig. 1D) for Cav-2 and Cav-3. To our surprise, Cav-2 expression was greatly reduced in all the Cav-1(-/-) tissues examined (Fig. 3A). A longer exposure of the same blots shows that caveolin-2 is in fact expressed, albeit at 5% of wild-type levels. Cav-3 levels remained unperturbed, however, and showed the expected expression pattern (i.e. muscle-specific expression) (Fig. 3A). A β-tubulin immunoblot indicates equal protein loading in all lanes (Fig. 3A). More importantly, in heterozygous animals, Cav-2 levels remain unperturbed despite the reduction in caveolin-1 (Fig. 1, D and E). Similar results were observed in Cav-1-null MEFs (Fig. 3B). Therefore, it seems that the absence, but not the reduction of Cav-1, is sufficient to cause a near-total deficiency in Cav-2.

The in vivo relationship between these two proteins (Cav-1 and Cav-2) goes beyond simple co-expression however. Although Cav-1 is able to form homo-oligomers consisting of 14–16 individual molecules (70, 71), it also is capable of forming similar size hetero-oligomers with Cav-2 (11, 72) and co-localizes with Cav-2 in caveolae microdomains (11). Additionally, it appears that Cav-2 requires the presence of Cav-1 for oligomerization and plasma membrane localization; when Cav-2 is overexpressed alone, it behaves as a mixture of monomers and dimers and is found in the Golgi complex (73–75). However, down-regulation of caveolin-1 either by antisense strategies or by oncogenic transformation (conditions where Cav-1 is reduced to undetectable levels) has no effect on Cav-2 levels or their localization (48). The generation of Cav-1-deficient mice provided us the opportunity to definitively resolve the relationship between Cav-1 and Cav-2 in vivo.

Due to the abundance of Type I pneumocytes and endothelial cells, lung tissue is a great source for the purification and molecular analysis of caveolae (60). In order to determine the localization of Cav-2 in Cav-1 null mice, we subjected mouse

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Fig. 2. A deficiency in Cav-1 is sufficient to disrupt caveolae formation. Cav-1 wild-type and knockout MEFs were grown to near-confluence on 60-mm dishes and prepared for transmission electron microscopy as described under "Experimental Procedures." All analyses were performed at ×16,000 magnification (for ease of view, images shown are further magnified to ×43,500). The plasma membranes of numerous cells were exhaustively scanned for caveolae, defined as uniform 50–100-nm flask-shaped membrane invaginations. The scale bar is shown at the lower left corner (bar, 150 nm).

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lung tissue to extraction and sucrose gradient ultracentrifugation, a procedure with which we have previously separated caveolar microdomains from other cellular constituents. Via this method, it is possible to dramatically concentrate Cav-1, the caveolae marker protein, with respect to total cellular protein (60, 62). The outputs of this centrifugation consist of 12 equal fractions (of which fractions 4 – 5 and 8 – 12 are considered of caveolar and non-caveolar origin, respectively). As shown in Fig. 3C, Cav-1 and -2 are enriched heavily in the caveolar fractions of wild-type lungs. Interestingly, however, in Cav-1-deficient lungs, Cav-2 is almost entirely excluded from such fractions. We also obtained similar results in cultured MEFs (data not shown), indicating that a lack of Cav-1 alters the fractionation of Cav-2.

We next attempted to uncover more definitively the subcellular localization of Cav-2. Fig. 3D shows a series of micrographs of wild-type and knockout MEFs co-immunostained with anti-Cav-1 polyclonal and anti-Cav-2 monoclonal antibodies. There is a distinct and overlapping membrane localization for Cav-1 and -2 in wild-type cells; in contrast, we found Cav-2 only in the perinuclear Golgi compartments of Cav-1-deficient MEFs (Fig. 3D). We reasoned that transient transfection of these Cav-1 knockout cells with the Cav-1 cDNA should rescue and redistribute Cav-2 away from the Golgi, restoring its plasma localization.
Caveolin-1 null MEFs show defects in the endocytosis of albumin but not transferrin—Albumin has been used extensively to monitor caveolae-mediated endocytosis (78). Thus, we next examined the ability of Caveolin-1-deficient MEFs to endocytose the fluorescent tracer, FITC-albumin. Fig. 5, A and B, shows that Caveolin-1-deficient MEFs fail to accumulate FITC-albumin even after 30–60 min of continuous incubation. In contrast, wild-type MEFs show cell surface labeling with FITC-albumin after only 5 min, with significant intracellular accumulation by 15 min of incubation. These results support the idea that caveolae clearly participate in endocytosis of specific ligands, such as albumin. Importantly, transient expression of the caveolin-1 cDNA in Caveolin-1-deficient MEFs was sufficient to restore the uptake of FITC-albumin (Fig. 5B).

To ensure that a lack of caveolin-1 expression did not globaly infect Cav-1(−/−) MEFs with Ad-Cav-1 and Ad-tTA at varying titers (100, 400, and 1000 PFUs/cell). Controls included infection with Ad-Cav-1 alone (1000 PFUs/cell) or co-infection with Ad-GFP and Ad-tTA (1000 PFUs/cell). C and D, in the absence of Cav-1, Cav-2 is partially degraded through the proteasomal pathway. Caveolin-1(−/−) MEFs grown to near-confluence were treated with the proteasomal inhibitors MG-132 (1 μM) and MG-115 (1 μM) for a series of time points (8, 16, and 24 h) or with vehicle (Me2SO). Whole cell lysis and subsequent SDS-PAGE allowed the comparison of Cav-2 levels with that of wild-type MEFs. D, Caveolin-1(−/−) MEFs grown to near-confluence, were treated with the lysosomal inhibitors chloroquine (50 μM) and NH4Cl (10 mM) or vehicle (Me2SO) for 24 h. Whole cell lysis and subsequent SDS-PAGE allowed comparison of Cav-2 levels with that of wild-type MEFs. Equal protein loading was assessed in A and B with anti-β-actin mAb (clone AC-15).

To gain insight into possible mechanisms for the reduction in Cav-2 levels, we focused on the cellular degradative machinery. The obvious requirement of Cav-1 for both the expression and membrane trafficking of Cav-2 indicates that protein misfolding, a hang-up in the Golgi, and subsequent degradation (proteasomal pathway) or an increase in retrograde trafficking from the membrane (lysosomal pathway) are possible areas of investigation. Therefore, we treated Caveolin-1 knockout MEFs with MG-132 and MG-115 (two classically used proteasomal inhibitors (76, 77)) and chloroquine and NH4Cl (two lysosomal inhibitors) for a period of up to 24 h (Fig. 4, C and D). We discovered that only the proteasomal inhibitors have a positive effect on Cav-2 expression. Over a time course of 8, 16, and 24 h, Cav-2 levels increase substantially from base line. It is interesting to note, however, that the increase in Cav-2 expression is not complete. This could be due to the following: 1) sub-optimal dosages of proteasomal inhibitors, a condition not rectifiable in such experiments as higher dosages have toxic effects on the MEF cells or 2) the presence of other degradative processes not fully abrogated by our repertoire of chemical inhibitors.

Caveolin-1 Null MEFs Show Defects in the Endocytosis of Albumin but Not Transferrin—Albumin has been used extensively to monitor caveolae-mediated endocytosis (78). Thus, we next examined the ability of Caveolin-1-deficient MEFs to endocytose the fluorescent tracer, FITC-albumin. Fig. 5, A and B, shows that Caveolin-1-deficient MEFs fail to accumulate FITC-albumin even after 30–60 min of continuous incubation. In contrast, wild-type MEFs show cell surface labeling with FITC-albumin after only 5 min, with significant intracellular accumulation by 15 min of incubation. These results support the idea that caveolae clearly participate in endocytosis of specific ligands, such as albumin. Importantly, transient expression of the caveolin-1 cDNA in Caveolin-1-deficient MEFs was sufficient to restore the uptake of FITC-albumin (Fig. 5B).

To ensure that a lack of caveolin-1 expression did not globaly...
bally affect endocytosis, we also examined the fate of a second fluorescent tracer, FITC-transferrin, which is endocytosed via clathrin-coated pits. Fig. 5C demonstrates that FITC-transferrin was rapidly endocytosed in both wild-type and Cav-1-deficient MEFs, with no apparent differences. After 30 min, FITC-transferrin accumulated in a perinuclear compartment in both wild-type and Cav-1-deficient MEFs.

Thus, Cav-1-deficient MEFs show a selective defect in the following figure:

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** Caveolin-1-deficient MEFs show defects in the endocytosis of albumin but not transferrin. A, wild-type and Cav-1 null MEFs were incubated in normal medium supplemented with FITC-albumin (10 μg/ml). After 5, 15, and 30 min at 37 °C, cells were formaldehyde-fixed and visualized by fluorescence microscopy. Note that Cav-1 null MEFs fail to internalize FITC-albumin. Left panels, wild-type MEFs (WT); right panels, Cav-1 null MEFs (KO). B, Cav-1 null MEFs were transiently transfected with the full-length cDNA encoding caveolin-1. Thirty-six hours post-transfection, cells were allowed to continuously endocytose FITC-albumin for 30 min, as in A. Cells were then formaldehyde-fixed and immunostained with anti-Cav-1 IgG (rabbit pAb N-20). Note that in cells recombantly expressing the caveolin-1 cDNA that uptake of FITC-albumin is clearly restored (left panels). However, untransfected cells in the same cell population failed to internalize FITC-albumin (right panels). Upper panels, FITC-albumin uptake; middle panels, Cav-1 immunostaining; lower panels, phase images. C, wild-type and Cav-1 null MEFs were incubated in normal medium supplemented with FITC-transferrin (10 μg/ml). After 5, 15, and 30 min at 37 °C, cells were formaldehyde-fixed and visualized by fluorescence microscopy. Note that both wild-type and Cav-1 null MEFs internalize FITC-transferrin, without any apparent differences. Left panels, wild-type MEFs (WT); right panels, Cav-1 null MEFs (KO).
FIG. 6. Growth properties and cell cycle analysis of Cav-1−/− MEFs. A–C, Cav-1-deficient MEFs proliferate faster and have increased S phase fractions. A, six independent MEF cultures, consisting of two Cav-1+/−, two Cav-1+/−, and two Cav-1−/− genotypes, were plated at a density of 15 × 10^3 cells/dish on a series of 35-mm dishes. Duplicate plates from each MEF culture were then counted each day for a period of 10 days. Cell numbers at the indicated time points (Days 1–10) are the average of duplicate plates. B, wild-type and knockout MEFs were plated at a density of 2 × 10^5 in 60-mm dishes. At the exponential phase of growth they were ethanol-fixed, stained with propidium iodide, and analyzed by flow cytometry for the G0/G1, S, and G2/M phases of the cell cycle. Parameters indicated are the percentage of cells in each phase of the cell cycle out of a total of 10,000 cells analyzed. C, wild-type and knockout MEFs were plated at a density of 2 × 10^5 in 60-mm dishes. Upon adherence to the plates they were supplemented with 1 μCi/ml [3H]thymidine and cultured overnight. Incorporated tritium was determined by scintillation counts of alkaline lysed cells/trichloroacetic acid-precipitated DNA (see “Experimental Procedures”). Data shown are the average and standard deviation of counts from five plates. D and E, the increased S phase in Cav-1 null cells can be rescued by re-introduction of Cav-1. D, Cav-1 knockout MEFs were transfected with either GFP alone or GFP-Cav-1. Untransfected plates of wild-type and knockout cells were similarly cultured. Thirty-six hours post-transfection, all cells were trypsinized, live-stained with Hoechst 33342, and subjected to flow cytometry. Transfected (i.e. GFP-positive) cells were distinguished from non-transfected cells (GFP-negative) by using appropriate fluorescence channels. Cell cycle
uptake of a known caveolar ligand, i.e. albumin. This is consistent with our observation that Cav-1-deficient MEFs lack morphological caveolae, as seen by transmission electron microscopy (Fig. 2).

The Growth Properties and Cell Cycle Analysis of Cav-1−/− MEFs—In the past decade, several important observations have implicated Cav-1 as a negative regulator of signaling pathways involved in pro-proliferative responses; as a result, Cav-1 has been suggested to function as a putative tumor suppressor. Caveolin-1 levels are drastically reduced upon oncogenic transformation of several cell lines (28–30, 33–37, 61). More importantly, overexpression of Cav-1 is sufficient to abrogate anchorage-independent growth in these transformed cells (29–32, 34, 61). Cav-1 also interacts with and negatively regulates several pro-proliferative signaling molecules, such as certain receptor-tyrosine kinases (including EGF-R, platelet-derived growth factor receptor, and Neu), Ha-Ras, c-Src, and phosphatidylinositol 3-kinase, among others (17, 18, 24–27). Finally, down-regulation of Cav-1 by an antisense strategy in NIH-3T3 fibroblasts leads to a tumorigenic phenotype, enabling the these cells to grow in soft agar and nude mice (48).

However, all of this work has been conducted in immortalized cell lines (i.e. cells that have perturbations in one or more genes important for controlling cell cycle progression), a situation that may confound the physiological behavior of Cav-1. Therefore, we attempted to study Cav-1 function in cellular proliferation in cultured primary MEFs. We first determined the growth potential of six independent MEF cultures (two wild-type and two knockout) were propagated according to the 3T3 protocol (i.e. 3 x 10^5 cells were plated in 60-mm dishes every 3 days). At each passage (2–16), the combined cell counts from three 60-mm dishes were determined for each MEF culture by hemocytometer. B, wild-type and Cav-1 knockout MEFs were propagated according to the 3T3 protocol (i.e. 3 x 10^5 cells were plated in 60-mm dishes every 3 days). Cell lysates were prepared from Passage 4 and Passage 14 MEFs, subjected to SDS-PAGE, and immunoblotted with anti-Cav-1 mAb (clone 2297), anti-Cav-2 mAb (clone 65), or anti-β-actin mAb (clone AC-15).
from each possible genotype) (also shown in Figs. 1E and 3B) over a 10-day period. Cells were plated sparsely in a series of 35-mm dishes, two of which were counted each day. In each case, the Cav-1-deficient cells proliferated approximately 2-fold faster and to higher saturation densities than their wild-type counterparts (Fig. 6A). Remarkably, cells heterozygous for Cav-1 show intermediate proliferation rates that seem to inversely correlate with their levels of Cav-1 expression (see Fig. 1E for a comparison of Cav-1 protein levels). These experiments were also performed with MEFs derived from 2 other knockout and wild-type embryos with similar results (data not shown).

We next conducted a more quantitative growth comparison of the Cav-1−/+ and −/− cells. Fig. 6B shows a representative flow cytometric analysis of the cell cycle of wild-type and knock- out MEFs in their exponential phase of growth. Note that there is a relatively resistant to high transfection efficiencies, the follow- ing strategy was devised. We have described previously (63) the characterization of a GFP-Cav-1 chimera that behaves indis- tinguishably from wild-type Cav-1. Transient transfection of this GFP-Cav-1 chimera or GFP alone, followed by flow cyto- metric analysis of GFP-positive cells, allowed us to compare the cell cycle responses of transfected and untransfected cells. Fig. 6D summarizes the results from such an experiment, whereas Fig. 6E shows the relative changes in S phase between each group of cells. Note that expression of GFP alone was insufficient to complement the cell cycle defect in Cav-1−/− MEFs, whereas expression of GFP-Cav-1 successfully complemented this defect. Thus, we conclude that the observed increase in cell proliferation in Cav-1 null MEFs reflects a decrease in the number of cells in the G0/G1 phase of the cell cycle, with a corresponding proportional increase in the number of cells in the S phase.

The relative inability of Cav-1-deficient cells to control cel-
Cav-1-deficient Mice Show Proliferative and Vascular Defects

Caveolin-1-deficient mice show proliferative and vascular defects. Fig. 8B shows a dramatic increase in Cav-1 and p2 expression at the higher passage, even though this phenomenon apparently does not appear to be important for age-dependent cellular senescence and growth arrest.

Caveolin-1-deficient Mice Show Lung Abnormalities, with Thickened Alveolar Septa and Hypercellularity, and Exercise Intolerance—As a consequence of the hyperproliferative phenotype we observed with Cav-1 null MEFs, we extensively examined our pathology specimens for evidence of hypercellularity. Interestingly, the lung appeared abnormal, and thin sections (1 μm) were cut to better evaluate this possible phenotype.

Fig. 9A shows toluidine blue-stained thin sections of lung parenchyma from wild-type and caveolin-1-deficient animals. Note that in caveolin-1-deficient mice, the alveolar spaces appeared significantly smaller or appeared constricted, with thickened alveolar septa and hypercellularity. In caveolin-1-deficient mice, reticulin staining showed increased basement membranes in the thickened alveolar walls (Fig. 9B). There was increased density and thickness of the basement membranes and loose arrays of reticulin fibers. However, lung fibrosis was not detected with trichrome staining (not shown). The presence of thickened alveolar septa and hypercellularity was indeed confirmed by transmission electron microscopy at low magnification. Montages of images taken of wild-type and Cav-1 null alveoli are shown in Fig. 9C.

As these lung tissue sections appeared hypercellular, we next quantitated the number of nuclei per high power field using hematoxylin and eosin-stained paraffin sections. Our results indicate that Cav-1 null mice lung sections show an ~2-fold increase in cellularity. This is consistent with our observation that Cav-1-deficient MEFs proliferate ~2-fold faster and to higher saturation densities than their wild-type counterparts (Fig. 5D).

As endothelial cells are one of the major cell types in lung tissue, and they are known to normally express high levels of...
In Cav-1 null animals, we examined the status of lung endothelial cells in wild-type and Cav-1-deficient mice. Transmission electron microscopy revealed that endothelial cells from Cav-1 null mice lack caveolae, whereas their normal counterparts in wild-type mice showed abundant caveolae (Fig. 10A). We also used antibodies to VEGF-R (Flk-1) as a marker for endothelial cells. Immunostaining of paraffin sections with anti-VEGF-R revealed that the number of lung endothelial cells were increased in Cav-1 null animals. For example, in wild-type animals, we routinely observed 1–2 VEGF-R-positive endothelial cells per 60× field, whereas in Cav-1-deficient animals there were 6–10 VEGF-R-positive endothelial cells per 60× field. Also, in Cav-1-deficient animals, the VEGF-R-positive endothelial cells were sometimes present in discrete clusters, i.e., reminiscent of a focus of cellular growth. Two representative images for each genotype are shown in Fig. 10, panels B and C.

The Ki67 "proliferation" antigen is a nuclear protein that is highly expressed in proliferating cells (late G1, S, G2, and M phases of the cell cycle) and is undetectable in cells in the G0 phase of the cell cycle (83, 84). Fig. 11 shows that Ki67 immunoreactivity is dramatically increased in lung tissue sections from Cav-1 null mice, as compared with wild-type controls. This is consistent with the idea that a lack of caveolin-1 can lead to hyperproliferation, as we have shown with Cav-1 null MEFs in culture (Fig. 6A).

To grossly assess the possible physical consequences of these lung abnormalities, we examined the exercise tolerance of wild-type and Cav-1-deficient mice. For this purpose, we subjected these animals to a "swimming test" (see "Experimental Procedures"). Fig. 12 shows that wild-type animals were able to swim for up to 40 min, whereas Cav-1-deficient animals only swam for 10 min. Thus, Cav-1-deficient mice clearly show exercise intolerance when compared with their wild-type littermates.

Caveolin-1-deficient Mice Show Abnormal Vasoconstriction and Vasorelaxation Responses—Caveolin-1 is highly expressed in endothelial cells where caveolae are abundant. In addition, in vitro studies have shown that caveolin-1 functions as a tonic inhibitor of eNOS (85–87). Thus, we next assessed the vascular tone of isolated mouse aortic rings by using an established physiological method that measures tension in response to vasoconstriction or vasorelaxation.

For this purpose, we employed phenylephrine (PE; an α1-adrenergic receptor agonist) as a vasoconstrictor and acetylcholine (Ach) to induce NO-dependent relaxation. To demonstrate a role for eNOS in these physiological responses, we took advantage of the availability of a well characterized arginine-based NOS inhibitor, known as L-NAME (nitro-L-arginine methyl ester).

As shown in Fig. 13, aortic rings isolated from the Cav-1 null mice were significantly different from their wild-type counterparts in all parameters examined. The results of a representative experiment on an aortic ring from each genotype (WT versus KO) are shown in Fig. 13A. As illustrated, PE was first used to elicit a contractile response. Upon achieving steady-state, relaxation was induced by adding acetylcholine (Ach) in gradually increasing doses (from 10−8 to 10−4 M), thereby creating a dose-response curve. Finally, in order to dissociate the PE-induced contractility from NO-mediated relaxation, the NOS inhibitor L-NAME was added to all rings.

There are several important observations to note. 1) The steady-state maximal tension response to PE in the wild-type aortic rings was nearly 2-fold greater than that observed for the Cav-1 null aortic rings (Fig. 13B, p < 0.05). It should be noted that over the same experimental time course, there was a less than 10% variation in tension development in wild-type and Cav-1 null aortic rings. 2) Although Ach induced a concentration-dependent relaxation response in aortic rings from both wild-type and Cav-1 null mice, significantly greater relaxation was observed in Cav-1 null aortic rings at all Ach concentrations examined (Fig. 13C). 3) After addition of L-NAME, the steady-state contractile response in the continuing presence of PE was significantly greater in aortic rings from both the wild-type and Cav-1 null mice; however, the percent increase was significantly greater for the Cav-1 null mice (see Fig. 13, A and B). Moreover,
FIG. 13. Caveolin-1-deficient mice show abnormal endothelium/NO-dependent modulation of mouse aortic contraction. A, representative trace of concentration-dependent acetylcholine (10⁻⁶–10⁻⁴ M)-induced relaxation, followed by addition of L-NAME (100 μM), which induced further constriction of the mouse aorta. Note the appearance of spontaneous oscillatory contractions present in the Ach concentration response curve in aortic rings from the wild-type mouse but largely absent from tracings obtained on aortic rings from the Cav-1 null mouse. As such, for comparative purposes, the % relaxation (see C) was calculated from the “steady-state” trough of relaxation observed for each Ach concentration. The asterisks indicate the times of addition of increasing amounts of Ach (10⁻⁶, 3 × 10⁻⁶, 10⁻⁷, 3 × 10⁻⁷, 10⁻⁸, 3 × 10⁻⁸, 10⁻⁹, 3 × 10⁻⁹, and 10⁻¹⁰ M (molar)). B, L-NAME (100 μM) modulation of PE-induced constriction in mouse aorta from wild-type (WT) and Cav-1 null (KO) mice. Points represent the mean ± S.E. of 5 (KO) and 6 (WT) rings from 3 mice each. *, p < 0.05 versus control WT; ***, p < 0.0001 versus KO; two-way analysis of variance for repeated measures. Note that Cav-1 null mice showed (i) an impaired vasoconstrictor response to PE, that observed in the wild-type mice (Fig. 13, WT, open squares) and Cav-1 null (KO; black squares) mice. Points represent mean ± S.E. of 5 (KO) or 6 (WT) rings from 3 mice each. ***, p < 0.0001 versus WT. Note that Ach-induced relaxation (a NO-dependent phenomenon) of the aortic rings was clearly potentiated by loss of caveolin-1 expression.

DISCUSSION

The discovery of caveolae by pioneering cell biologists in the 1950s added yet another major organelle to the cellular repertoire. Although the field remained relatively dormant for several decades, the advent of caveolar biology occurred in 1992 with the discovery of Cav-1 as the marker protein for such microdomains. It has become clear over the ensuing years that caveolar function is intimately linked to this marker protein.

In this study, we describe the generation of a new mouse model with an ablation of the gene encoding the Cav-1 protein. We show that the cells derived from these mice are deficient in caveolae, as determined ultrastructurally, thereby conclusively demonstrating that Cav-1 is required for caveolar formation in primary cells. Surprisingly, despite a lack of such prevalent and conspicuous organelles, these mice are both viable and fertile.

Cav-1 null MEFs are perturbed in several other ways, however. First, we show that Cav-2, a protein that is co-expressed, co-localizes, and hetero-oligomerizes with Cav-1 is severely affected in Cav-1 null cells. In the absence of caveolin-1, Cav-2 levels are reduced by ~95%. In addition, the remaining Cav-2 no longer targets to the plasma membrane but instead is sequestered within the Golgi complex. We further show that re-introduction of Cav-1 in these deficient cells can rescue this effect by elevating Cav-2 levels and recruiting it to the plasma membrane. Thus, the reduction of Cav-2 protein seems to be independent of transcriptional repression and is rather mediated by proteasomal degradation, as two inhibitors of the 26 S proteasome are able to partially reverse this effect. Second, we demonstrate the Cav-1 null MEFs fail to endocytose a known caveolar ligand, i.e. FITC-albumin, but show no defects in the uptake of FITC-transferrin, a marker for clathrin-mediated endocytosis. Importantly, transient expression of the caveolin-1 cDNA in Cav-1-deficient MEFs was sufficient to restore the uptake of FITC-albumin. Third, we show that Cav-1 null MEFs reveal a hyperproliferative phenotype. Cav-1 null MEFs are able to grow approximately 2-fold faster during the exponential phase and reach higher densities at confluence. These effects are due to an increase of ~25–30% in the S phase fraction. Furthermore, we demonstrate a reversion of this excess proliferation to wild-type levels by re-expressing Cav-1 in knockout cells. However, we do not find any evidence that the observed growth augmentation is due to a hyperactivation of the p42/44 MAP kinase cascade, a signaling pathway reported by many investigators to be intimately linked to caveolae/caveolin functioning. Furthermore, although we show that Cav-1 levels increase in higher passage cells (i.e. cells at or near senescence), a deficiency in Cav-1 is not sufficient to expedite immortalization in primary fibroblasts.

Caveolae are thought to form as a result of a local accumulation of cholesterol, glycosphingolipids, and caveolin-1 (8, 88, 89). Caveolin-1 can bind cholesterol in vitro (8); also, Cav-1 is a major protein bound to photoactivable forms of both cholesterol and glycolipids in vivo (88, 90). Although in this study we have demonstrated that physiological levels of Cav-1 protein are required for caveola formation (in corroboration of previous overexpression studies), the mechanisms underlying this process remain entirely unknown. Primarily, this is due to the fact that the Cav-1 protein is not readily amenable to mutational analysis. Due to its ability to form a large oligomeric complex with itself and with Cav-2 (11, 70, 72, 91), its ability to coalesce
into even larger macrostructures (62), its binding to cholesterol and glycosphingolipids (8, 88–90), and its membrane-spanning properties, any deletion/mutation of the protein can confound an analysis of caveola formation in numerous ways. For example, baculovirus-mediated expression of Cav-1 proteins lacking their oligomerization domain or C-terminal domains (i.e. Cav-1 Δ61–100 and Δ140–178) in SF21 insect cells can induce vesicle formation albeit with sizes 10× normal caveolae. Our establishment of Cav-1-deficient cells can aid future studies in several ways. First, determinations of the composition of the plasma membrane in Cav-1-deficient cells could possibly establish whether the absence of caveolae is due to relative reductions in cholesterol/glycosphingolipid content or to simply the Cav-1 protein itself. Second, overexpression of Cav-1 mutants in these cells will establish an elegant screening strategy for de novo caveola formation.

Based on the current study, the intricate dependence of Cav-2 on the presence of Cav-1 is obvious. Cav-2 is present at astonishingly lower levels (~5% of wild-type) in knockout tissues, is localized in the Golgi compartment, and is degraded by the proteasomal pathway. However, the degradation of Cav-2 is perhaps not entirely surprising. Rather elaborate mechanisms of quality surveillance have developed at various levels of the secretory pathway. Incompletely folded or assembled proteins are often sequestered at the endoplasmic reticulum where they are eventually degraded by the 26 S proteasome (reviewed in Refs. 92 and 93). The few molecules that “escape” detection, traffic to the Golgi where they can again be detected and re-routed to the endoplasmic reticulum for degradation. Since Cav-2 cannot homo-oligomerize but rather hetero-oligomerizes with Cav-1, it is possible that in the absence of Cav-1, several critical hydrophobic regions remain exposed, thereby affecting not only folding of Cav-2 but increasing the probability of recognition by the proteasomal apparatus. To date, however, much less is known about Cav-2 function than is for Cav-1 function. Cav-2 does not contain a scaffolding domain (the primary proposed region of interaction between Cav-1 and signaling molecules) and has rarely been implicated in signal transduction processes. Although there is no overt reason to believe that its severely reduced levels in Cav-1-deficient cells can compound any phenotypic analyses, the fact remains that Cav-1 knockout mice are in effect deficient in two caveolins. The generation of Cav-2 knockout mice will ultimately resolve this issue.

Based on the growth curves of MEFs and the corresponding cell cycle analyses, we have shown in this study that a deficiency in Cav-1 leads to higher proliferation rates. This is the first direct demonstration of a relationship between Cav-1 and the cell cycle under physiological circumstances. Our results corroborate previous data showing that Cav-1 overexpression can reduce cell proliferation and/or abrogate anchorage-independent growth in several cancer cell lines (29, 31, 61). Surprisingly, we found that this proliferation is not due to a hyperactivation of the p42/44 MAP kinase cascade, a signaling pathway that had been shown in numerous ways to be reciprocally regulated by Cav-1 (28, 48, 49, 61, 81, 94).

The main difference in our study lies in the use of embryonic fibroblasts, instead of immortalized and transformed cell lines. This type of discrepancy is not unusual and necessitates the analysis of proteins in primary culture systems, such as this one. For example, the ras oncogene, a potent transforming agent when used in immortalized cells (95, 96), actually induces cell cycle arrest and premature senescence in MEFs (97, 98). In the same way, the mechanistic explanation of the excessive proliferation of Cav-1-deficient primary cells may depend on other signal transduction processes. Although several other pro-proliferative signaling molecules have been shown to be regulated by Cav-1, further work is required to determine more closely their physiological relevance. Analysis of these pathways in knockout versus wild-type MEFs will eventually shed light on the detailed mechanism for the observed hyperproliferation. In addition, although we have not noticed any spontaneous tumors in Cav-1 null mice at 9 months of age, they may have a higher susceptibility than wild-type mice to tumors induced either chemically or by breeding with other tumor-prone mice (e.g. the p53- or INK4a-deficient mice (79, 99)).

It should be noted that the lack of spontaneous tumor formation and the modest proliferation defect observed in the Cav-1 null setting is reminiscent of several previously described mice lacking inhibitory cell cycle proteins. For example, mice deficient in the cyclin-dependent kinase inhibitor, p21 (which functions in G1 phase progression (100, 101) and is a major target of p53 (102)), do not develop tumors, and their MEFs display only a modest proliferative advantage over the wild-type counterparts (65). The ablation of the p15INK4a, a member of the INK4 family of proteins, also does not predispose mice to tumors or cell cycle defects (103). A deficiency of p15INK4b predisposes only a small percentage of mice to tumors (104). Although mice lacking p27, another important cyclin-dependent kinase inhibitor, can develop pituitary tumors, cells derived from these mice only show subtle cell cycle defects (105, 106). In many instances, the lack of an overt phenotype can be due to compensatory proteins (i.e. compensation derived from parallel-acting cell cycle and checkpoint control pathways). We assessed a possible up-regulation of caveolin-3, the highly homologous muscle-specific caveolin family member, in several Cav-1 null tissues, and we found it to remain unperturbed. Therefore, if there are any counter-regulatory mechanisms involved, they are independent of the caveolin gene family.

In accordance with the hyperproliferative phenotype we observed with Cav-1 null MEFs, the lung parenchyma of Cav-1 null animals appeared hypercellular with thickened alveolar septa. Quantitation of the number of nuclei per high power field using hematoxylin-eosin-stained paraffin sections revealed an ~2-fold increase in cellularity. The Ki67 “proliferation” antigen is a nuclear protein that is highly expressed in proliferating cells and is undetectable in cells in the G0 phase of the cell cycle (83). Interestingly, Ki67 immunoreactivity was also dramatically increased in lung tissue sections from Cav-1 null mice. This is consistent with our observation that Cav-1 deficient MEFs proliferate faster and to higher saturation densities. We also found that the number of VEGF-R-positive lung endothelial cells were increased in Cav-1 null animals. These VEGF-R-positive endothelial cells were sometimes present in discrete clusters, i.e. reminiscent of a focus of cellular growth. Transmission electron microscopy revealed that lung endothelial cells from Cav-1 null mice lack caveolae, whereas their normal counterparts in wild-type mice showed abundant caveolae. Taken together, these findings are consistent with the idea that a lack of caveolin-1 expression and caveolae organelles can lead to hyperproliferation in certain cell types. These lung abnormalities appeared to have physical consequences, as the Cav-1-deficient mice clearly showed exercise intolerance.

Several in vitro studies employing recombinant expression and peptide-based analyses have strongly suggested that caveolin-1 can function as an endogenous negative regulator of eNOS, by providing tonic inhibition of eNOS enzymatic activity (85–87). Here, by using isolated mouse aortic rings, we evaluated the effect of loss of caveolin-1 expression on the vasoconstrictor actions of PE, an α1-adrenergic receptor agonist. We observed that Cav-1 null mice showed an impaired vasocon-
Cav-1-deficient Mice Show Proliferative and Vascular Defects

M. P. Lisanti, P. E. Scherer, M. Sargiacomo, F. Galbiati, and M. P. Lisanti

Cav-1-deficient mice show increased proliferation and vascular defects, suggesting a role for Cav-1 in regulating cell behavior.

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\section*{References}
Cav-1-deficient Mice Show Proliferative and Vascular Defects


Science 286, 533–539

Nature 304, 648–651


Science 286, 1882–1886

Science 286, 1888–1893


J. Biol. Chem. 274, 16240–16247

J. Biol. Chem. 272, 25078–25071


J. Biol. Chem. 272, 25078–25071


J. Cell Biol. 138, 867–873
Caveolin-1 Null Mice Are Viable but Show Evidence of Hyperproliferative and Vascular Abnormalities

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