THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 20, Issue of May 17, pp. 17944–17949, 2002
Printed in U.S.A.

Inhibition of Lipid Raft-dependent Signaling by a Dystrophy-associated Mutant of Caveolin-3*

Received for publication, November 13, 2001, and in revised form, February 26, 2002
Published, JBC Papers in Press, March 7, 2002, DOI 10.1074/jbc.M110879200

Amanda J. Carozzi‡, Sandrine Roy‡§, Isabel C. Morrow, Albert Pol, Bruce Wyse§, Jodi Clyde-Smith§, Ian A. Prior§, Susan J. Nixon, John F. Hancock§¶, and Robert G. Parton‖

From the Institute for Molecular Bioscience, Centre for Microscopy and Microanalysis, and School of Biomedical Sciences, University of Queensland, Brisbane 4072, Australia and the §Queensland Cancer Fund Laboratory of Experimental Oncology, Department of Pathology, University of Queensland Medical School, Herston Road, Brisbane 4006, Australia

Specific point mutations in caveolin-3, a predominantly muscle-specific member of the caveolin family, have been implicated in limb-girdle muscular dystrophy and in rippling muscle disease. We examined the effect of these mutations on caveolin-3 localization and function. Using two independent assay systems, Raf activation in fibroblasts and neurite extension in PC12 cells, we show that one of the caveolin-3 point mutants, caveolin-3-C71W, specifically inhibits signaling by activated H-Ras but not by K-Ras. To gain insights into the effect of the mutant protein on H-Ras signaling, we examined the localization of the mutant proteins in fibroblastic cells and in differentiating myotubes. Unlike the previously characterized caveolin-3-DGV mutant, the inhibitory caveolin-3-C71W mutant reached the plasma membrane and colocalized with wild type caveolins. In BHK cells, caveolin-3-C71W associated with caveolae and in differentiating muscle cells with the developing T-tubule system. In contrast, the caveolin-3-P104L mutant accumulated in the Golgi complex and had no effect on H-Ras-mediated Raf activation. Inhibition by caveolin-3-C71W was rescued by cholesterol addition, suggesting that the mutant protein perturbs cholesterol-rich raft domains. Thus, we have demonstrated that a naturally occurring caveolin-3 mutation can inhibit signaling involving cholesterol-sensitive raft domains.

Caveolae are an abundant feature of the sarcolemma of muscle cells. Caveolin-3 (Cav3)[1] is one of the major membrane proteins of skeletal and cardiac muscle caveolae (1, 2). By analogy to the closely related caveolin family member, caveolin-1 (Cav1) (3), Cav3 is thought to play a vital and direct role in the formation of the characteristic flask-shaped pits typical of caveolae. In addition to this structural role, caveolins have been implicated in signal transduction and in lipid transport (4). The role of caveolae and Cav3 in muscle has become clinically relevant with the finding that mutations in the gene for Cav3 are associated with several muscle pathologies including a rare form of limb-girdle muscular dystrophy type 1C and hereditary rippling muscle disease (5–10). To date, one microdeletion and five point mutations (R26Q, A45T or A45V, G55S, C71W, and P104L; note that this nomenclature assumes a single Met at the N terminus) have been described. While four of these mutant proteins cause a decrease in Cav3 expression, in other cases surface expression of the protein appears normal (5, 6). Defining the function of Cav3, identifying its interacting partners, and characterizing the effect of the myopathy-associated mutations on muscle function is therefore of vital importance. Moreover, the specific point mutants of Cav3 that are associated with the skeletal muscle defects may provide powerful new tools in studies of the role of caveolins in both muscle and nonmuscle cells.

Cav3 expression is up-regulated as myoblasts fuse to form myotubes (1, 2, 11). In mature muscle, the sarcolemma is highly organized into regularly spaced Cav3-positive caveolar domains separated by noncaveolar domains (12). Cav3 shows partial colocalization with elements of the dystrophin complex (12) and is proposed to associate with the dystrophin complex at the cell surface (13, 14). In fact, interactions between Cav3 and dystroglycan through a WW-like domain in Cav3 have been suggested to mediate a direct interaction between these two proteins (15). While Cav3 is predominantly associated with the sarcolemma of mature muscle, in differentiating muscle cells Cav3 associates with the developing T-tubule system (11, 16). The functional significance of the association with this extensive plasma membrane subdomain is as yet unknown, but both caveolae and the developing T-tubule system show similar morphology and are sensitive to cholesterol-disrupting agents (11, 17, 18). Recent reports of T-tubule defects in Cav3-deficient mice suggest that Cav3 may be involved in the organization of the T-tubules but is not essential for their formation (19).

Caveolins have been shown to interact *in vitro* with a number of signaling molecules including trimeric G protein subunits, Src kinases, and Raf through direct interactions of these proteins with a conserved region of caveolin termed the caveolin scaffolding domain (20). More recent studies have shown that this domain of caveolin can interact with, and apparently insert into, lipid bilayers (21, 22) raising questions regarding the specificity of these protein-protein interactions *in vivo*. An additional hypothesis for the role of caveolins has emerged with the finding that Cav1 is a lipid-binding protein. Cav1 binds both cholesterol (23) and fatty acids (24), and its expression is regulated by cholesterol at the transcriptional level (25–27). Cav1 expression also increases cholesterol transport (28). Consistent with these findings, we have shown that exogenous

* This research was supported by grants from the National Health and Medical Research Council of Australia (to R. G. P. and J. F. H.). The Centre for Functional and Applied Genomics is a Special Research Centre of the Australian Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Both authors contributed equally to this work.
¶ Also supported by the Royal Children's Hospital Foundation, Queensland.
‖ To whom correspondence should be addressed. Tel.: 61-7-3365-6468; Fax: 61-7-3365-4422; E-mail: R.Parton@imb.uq.edu.au.

[1] The abbreviations used are: Cav, caveolin; HA, hemagglutinin; WT, wild type; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; YFP, yellow fluorescent protein; GFP, green fluorescent protein; MES, 4-morpholineethanesulfonic acid.

expression of a Cav3 truncation mutant, Cav3-DGV, in fibroblastic cells disrupts lipid transport and causes inhibition of specific signaling pathways (29). Rather than a direct interaction with signaling proteins, these studies suggest that the mutant protein affects lipid homeostasis and so causes disruption of cholesterol-rich surface domains, termed lipid rafts, with which specific signaling pathways are associated (4). The muscle cell surface has been shown to be arranged into highly organized arrays of caveolae raft domains (12). In view of the finding that artificial Cav3 truncation mutants can inhibit signal transduction through raft domains (29), we speculated that naturally occurring Cav3 mutants may also affect signaling through sarcolemmal raft domains and that these mutants may provide excellent tools to dissect caveolin function. In the present study, we have characterized the mutant Cav3 proteins functionally using two different assays to monitor raft- and non-raft-mediated Ras-dependent signaling events. We show that one Cav3 point mutant reaches the cell surface and specifically inhibits H-Ras-dependent signaling pathways with the inhibition being rescued by cholesterol addition. These findings give new insights into the effect of specific point mutations on caveolin function, the role of caveolins in regulating raft-dependent signaling events, and the potential effect of caveolin dysfunction in muscle.

MATERIALS AND METHODS

Cell Culture—BHK cells and C_2C_{12} cells were maintained as described previously (17, 29). PC12 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) horse serum, 10% (v/v) fetal calf serum, and 2 mM L-glutamine.

Antibodies and cDNAs—Affinity-purified rabbit antibodies against p23 (30) were kindly provided by Professor Jean Gruenberg (University of Geneva, Switzerland). A rabbit polyclonal antibody against the hemagglutinin (HA) tag was a generous gift from Professor David James (University of Queensland, Australia). A rabbit polyclonal antibody to the conserved region of Cav3 (αCon-Cav) has been characterized previously (32). A polyclonal antibody to Cav1 was from Transduction Laboratories (catalog no. C13630). Other reagents have been described previously (31). HA-tagged Cav3 point mutants were generated by PCR using mouse HA-tagged Cav3 as template. Cav3-DGV has been described previously (32). Cav3-LLS comprises residues 74–150 of Cav3. All constructs were confirmed by sequencing.

Transfections—BHK cells were transiently transfected using LipofectAMINE or LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's instructions. C_2C_{12} cells were transiently transfected using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. Briefly, myoblasts were transfected at confluence in differentiation medium (Dulbecco's modified Eagle's medium with 1% (v/v) Serum Supreme (BioWhittaker) and 2 mM L-glutamine) using 4 μg of total DNA/14 μl of LipofectAMINE 2000 in a total volume of 2.4 ml of medium (Invitrogen). Cells were incubated overnight with the DNA mixture before it was replaced by fresh differentiation medium, which was subsequently replaced every 2 days prior to fixation, which routinely occurred 4–6 days postremoval of the transfection reagent (at which point the myoblasts had morphologically fused to form multinucleated myotubes). PC12 cells were transiently transfected using LipofectAMINE according to the manufacturer's instructions. Cells were left with the DNA transfection mix for 5 h prior to addition of medium containing 20% (v/v) calf serum. This medium was replaced the following day by standard PC12 maintenance medium, and the cells were incubated a further 24 h prior to fixation. In BHK cells, the HA-tagged wild type (WT) and mutant Cav3 proteins were expressed at comparable levels. Note that no correlation was found between expression level of the HA-tagged proteins and ability of the mutants to inhibit H-Ras function.

Raf-1 Kinase Assays—Membrane fractions (P100) from transfected BHK cells were normalized for Raf protein content via Western blot analysis and assayed for Raf activity using a two-stage coupled MEK/ERK assay with phosphorylation of myelin basic protein as readout exactly as described previously (33). Cholesterol supplementation was carried out for 1 h using a mix of 16 μg/ml cholesterol with 0.4% cyclodextrin in Dulbecco's modified Eagle's medium exactly as described previously (34).

Sucrose Gradients and Western Blots—Sucrose gradients of transfected BHK cells and subsequent Western blot analysis were performed exactly as described previously (31, 35, 36).

Light and Electron Microscopy—For immunofluorescence studies, cells were routinely fixed with 4% paraformaldehyde and immunolabeled according to Pol *et al.* (37). Electron microscopic localization of Cav3 mutants in plasma membrane sheets was performed exactly as described previously (31).

RESULTS AND DISCUSSION

A Caveolin-3 Point Mutant Specifically Inhibits H-Ras-mediated Signaling—We used a model fibroblast transfection system to examine the possible effect of Cav3 myopathy mutants on H- or K-Ras-mediated Raf activation. Ras recruits Raf from the cytosol to the plasma membrane where Raf activation proceeds through a complex pathway involving interactions with lipids, displacement of 14-3-3, and phosphorylation (38). By co-expression of the mutants with activated Ras (H- or K-Ras) and with Raf, we examined the effect of the Cav3 mutants on two aspects of Ras function: association of Raf with membranes (Raf recruitment) and activation of Raf (Raf specific activity). It was found that, as has been published previously (29), neither Ras expression nor recruitment of Raf to the membrane by constitutively activated H-Ras (H-RasG12V) or K-Ras (K-RasG12V) was affected by co-expression of the mutant proteins (data not shown). Moreover, Raf activation by K-Ras was also unaffected by co-expression of the WT or mutant Cav3 proteins (Fig. 1). In striking contrast, one of the mutants, Cav3-C71W, showed a strong inhibition of H-Ras-mediated Raf activation. The level of inhibition was comparable to that seen with the previously documented Cav3-DGV N-terminal truncation mutant (which lacks amino acids 1–54) (29) (Fig. 1). Cav3-WT and the Cav3-G55S and Cav3-P104L mutants had no significant effect on H-Ras-mediated Raf activation. Since the caveolin scaffolding domain (amino acids 55–73), in which three dystrophy-associated mutations occur, has been implicated in the direct binding of signaling molecules, we also examined whether a caveolin truncation mutant that lacks this region would inhibit H-Ras-mediated Raf activation as effectively as the Cav3-DGV mutant. In fact, the Cav3-LLS mutant, which lacks the entire N-terminal domain of Cav3 (amino acids 1–73), was a specific and potent inhibitor of H-Ras-mediated Raf activation showing that the scaffolding domain was not required for the inhibitory effect.

To validate the use of the Ras/Raf transfection assay for studies of the caveolin mutants, we examined whether the Cav3-C71W mutant would inhibit signaling in a completely different Ras assay system. For this we took advantage of the well characterized role of Ras in regulating neurite outgrowth in PC12 cells (39). Introduction of activated H-Ras (Fig. 2) or K-Ras (not shown) into undifferentiated PC12 cells caused extensive neurite outgrowth, although the extent of neurite outgrowth induced by K-Ras was not as marked as that induced by H-Ras. Activated H-Ras was then transfected into PC12 cells together with Cav3-WT, Cav3-DGV, or the Cav3 point mutants. As shown in Fig. 2, Cav3-DGV and Cav3-C71W caused a dramatic inhibition of H-Ras-mediated neurite extension. The same mutant proteins had no effect on K-Ras-mediated neurite outgrowth (results not shown). Thus, we have identified a point mutation in Cav3 that inhibits H-Ras signaling in two independent assays.

Localization of Heterologously Expressed Caveolin-3 Mutants in Fibroblasts and Differentiating Muscle Cells—We have postulated that Cav3-DGV specifically inhibits H-Ras signaling through an effect on cellular cholesterol. Cav3-DGV accumulates intracellularly and does not reach the cell surface. We therefore examined the localization of the inhibitory and non-inhibitory Cav3 point mutants in fibroblasts and in differentiating muscle cells. Cav3-WT localizes to surface caveolae and

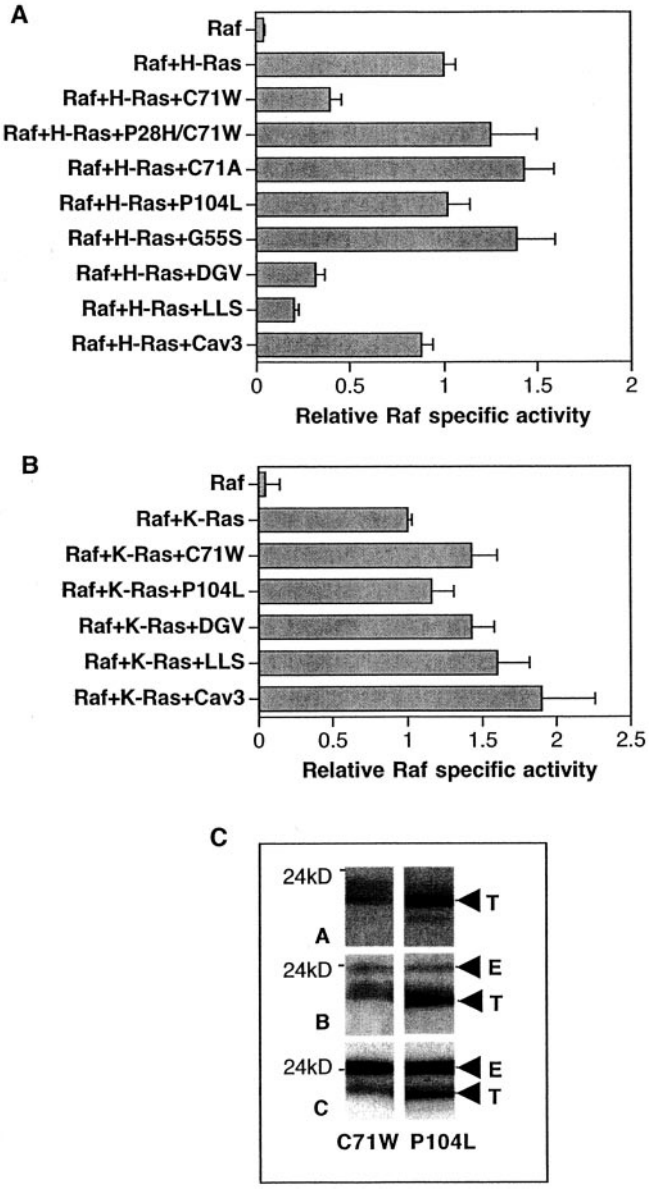

FIG. 1. **A caveolin-3 point mutant specifically inhibits H-Ras-dependent Raf activation.** BHK cells were transfected with the indicated combinations of activated H- or K-Ras (G12V), Raf, and Cav3 mutant constructs. The cells were then fractionated, and 20 μg of each membrane (P100) fraction were immunoblotted for Ras and Raf-1. P100 fractions from H-Ras (*A*) and K-Ras transfection series (*B*) were then normalized for Raf content and assayed for Raf specific activity using a coupled MEK/ERK assay. The results show relative Raf specific activity ± S.E. from three to five independent experiments for each *bar*. Each assay set was normalized against the Raf activity of cells transfected with Ras and Raf alone (=1). *C* shows the relative expression levels of two of the HA-tagged Cav3 constructs compared with that of endogenous Cav1 in BHK cells. Extracts from BHK cells transfected with Cav3-C71W or Cav3-P104L were processed for Western blot analysis and probed with antibodies to the HA tag (*upper panel A*) to detect transfected proteins (*T*) with an antibody (αCon-Cav) to the conserved region of Cav3 (*middle panel B*) or with a polyclonal antibody (αCav1) to Cav1 (*bottom panel C*). The αCon-Cav and αCav1 antibodies recognize both endogenous Cav1 (*E*) and ectopically expressed Cav3 (*T*) with varying affinities. Densitometric analysis of blots obtained with the αCon-Cav antibody showed that, after correction for transfection efficiency, Cav3-P104L levels were approximately 6-fold and Cav3-C71W levels were approximately 3-fold greater than those of endogenous Cav1.

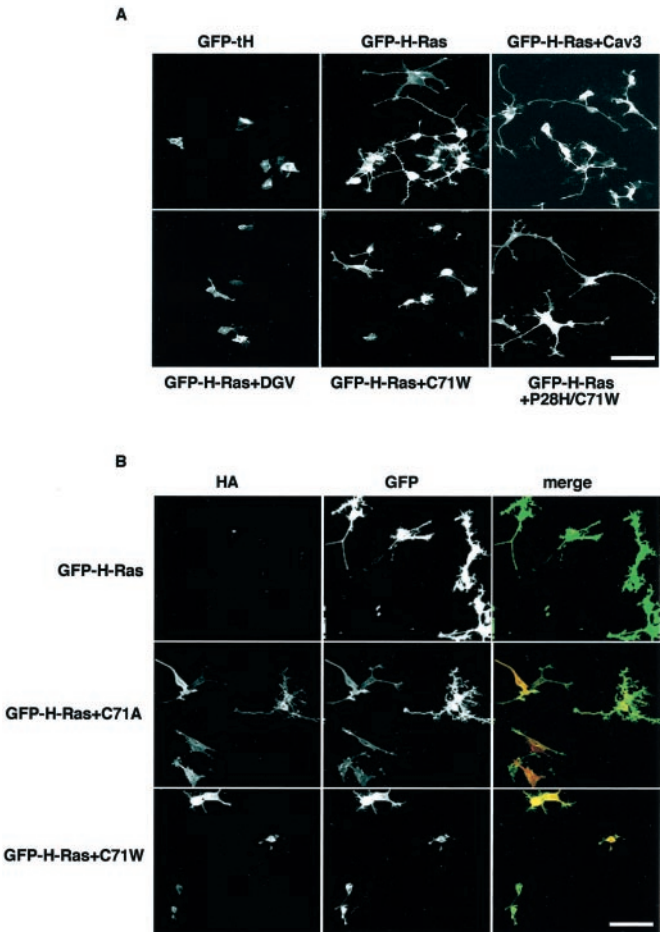

FIG. 2. **Specific inhibition of H-Ras-stimulated neurite outgrowth in PC12 cells by caveolin-3 mutants.** PC12 cells were transfected with the indicated constructs. After 48 h the cells were fixed and examined by fluorescent microscopy. *A*, cells transfected with the control vector (*GFP-tH*) have no neurites, whereas cells transfected with GFP-H-RasG12V show extensive neurite outgrowth. In cells coexpressing H-RasG12V and Cav3-C71W or Cav3-DGV neurite outgrowth is severely inhibited. Negligible effects on neurite outgrowths are seen when H-RasG12V is co-expressed with Cav3-WT or Cav3-P28H/C71W. *B*, cells co-transfected with GFP-H-RasG12V and the indicated HA-tagged Cav3 mutants were labeled with antibodies to HA and imaged for both constructs as shown. Unlike Cav3-C71W, Cav3-C71A had no impact on H-RasG12V-induced neurite outgrowth. *Bars*, 25 μm. The experiments shown are representative of three independent experiments.

the Golgi complex of nonmuscle cells (32) and to surface caveolae and T-tubules of differentiating muscle cells (11). We therefore examined whether the Cav3 point mutants showed aberrant localization by expression of the HA epitope-tagged forms of the protein in BHK cells. By immunofluorescence microscopy, the labeling patterns for Cav3-G55S and Cav3-C71W were indistinguishable from Cav3-WT-HA. In contrast, Cav3-P104L was predominantly (although not exclusively) in the Golgi complex, consistent with previous studies (40). We then examined the localization of the mutant proteins in C_2C_{12} cells, a model muscle cell line. As in the nonmuscle cells, the labeling patterns for Cav3-G55S and -C71W were largely indistinguishable from Cav3-WT, labeling the cell surface and putative T-tubules. The Cav3-P104L mutant, in contrast, gave a striking perinuclear labeling, which colocalized with the cis Golgi marker protein, p23, in all the transfected cells (Fig. 3).

In view of the inhibitory effect of the Cav3-C71W mutant, we examined the localization of this protein in more detail. By confocal microscopy the Cav3-C71W mutant colocalized with Cav3-WT-YFP in co-transfected C_2C_{12} cells (Fig. 3). In BHK cells, Cav3-C71W localized to caveolae as shown by immunoelectron microscopy on plasma membrane sheets (Fig. 3).

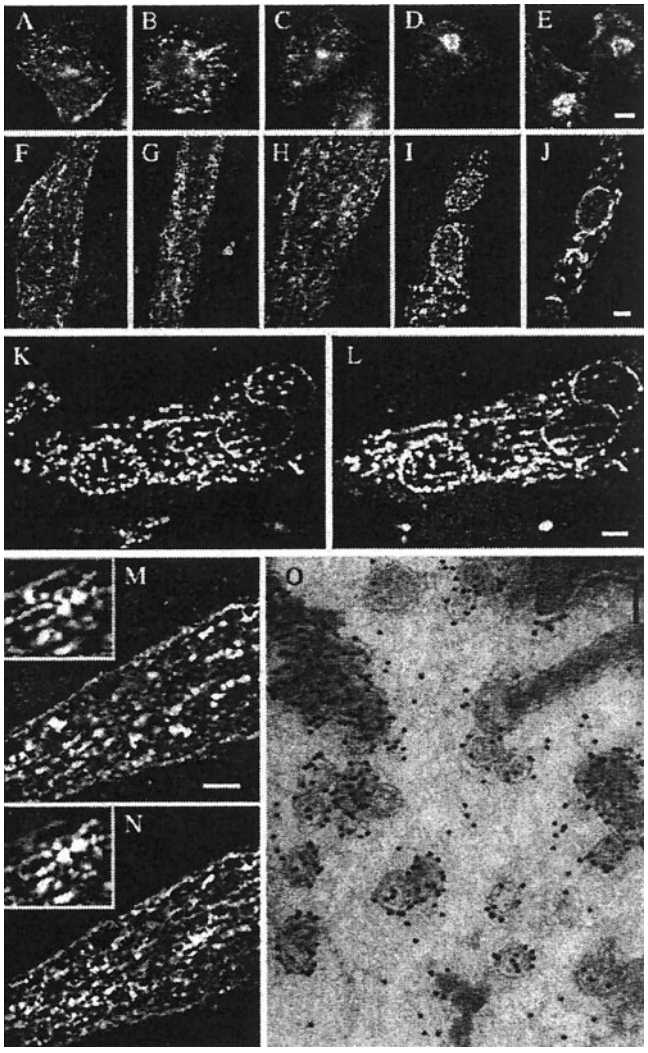

FIG. 3. **Localization of caveolin-3 mutants in BHK cells and C_2C_{12} cells.** HA-tagged caveolin mutants were transfected into BHK cells (*A–E*) or C_2C_{12} myotubes (*F–J*) as indicated: Cav3-WT (*A* and *F*), Cav3-G55S (*B* and *G*), Cav3-C71W (*C* and *H*), Cav3-P104L (*D* and *I*), Cav3-P28H/C71W (*E* and *J*). In both cell types note the similar staining patterns with Cav3-WT and both Cav3-C71W and Cav3-G55S, which contrasts with the perinuclear labeling of the Cav3-P104L mutant. *K* and *L* show colocalization between Cav3-P104L (*L*) and the Golgi marker p23 (*K*). *M* and *N* indicate labeling for the inhibitory mutant Cav3-C71W (*M*) and Cav3-WT-YFP (*N*). *O* shows electron microscopic localization of Cav3-C71W on plasma membrane sheets using anti-HA antibodies followed by 10-nm protein A-gold. Bars: *A–N*, 10 μm; *O*, 100 nm.

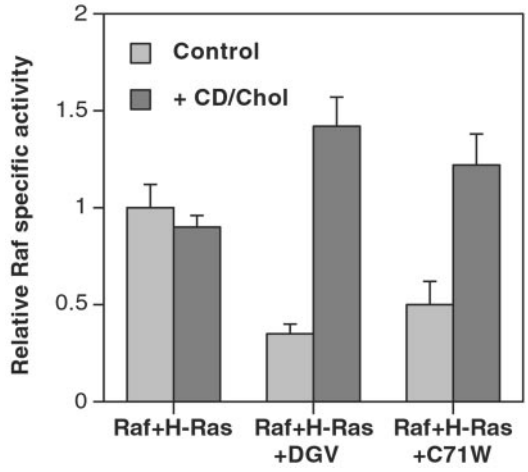

FIG. 4. **Inhibition of H-Ras signaling by caveolin-3 mutants is reversed by cholesterol addition.** BHK cells were transfected with Raf-1 and activated H-Ras (G12V) in combination with the caveolin constructs indicated. They were then treated with a cyclodextrin/cholesterol (*CD/Chol*) mixture for 1 h or left untreated as indicated. The cells were then fractionated, and 20 μg of each membrane (P100) fraction were immunoblotted for Ras and Raf-1. P100 fractions were then normalized for Raf content and assayed for Raf specific activity using a coupled MEK/ERK assay as described in Fig. 1. The results show relative Raf specific activity ± S.E. from three to five independent experiments for each *bar*. Expression of ectopically expressed caveolin constructs was verified by Western blotting (not shown).

Therefore Cav3-C71W behaves in a manner similar to the Cav3-DGV mutant in terms of its effect on H-Ras signaling but does not localize to the same compartment; in fact the Cav3-C71W mutant traffics in a manner similar to the wild type protein. The results also show for the first time that the Cav3-P104L mutant is strongly retained in the Golgi complex of muscle cells, consistent with results in fibroblasts (40). The results also show that accumulation of a caveolin mutant intracellularly is not sufficient to inhibit H-Ras signaling, emphasizing the specificity of the inhibition mediated by the Cav3-DGV mutant.

Inhibition by the Caveolin-3-C71W Mutant Is Reversed by Cholesterol Addition and Accompanied by Changes in Density of Raft Domains—In view of the different location of the inhibitory mutants Cav3-DGV and Cav3-C71W, we examined whether the Cav3-C71W mutant was inhibiting H-Ras signaling through an effect on cholesterol as demonstrated previously for the Cav3-DGV mutant. A cyclodextrin/cholesterol mixture was used to supplement the cholesterol content of the plasma membrane of cells expressing Cav3-C71W or Cav3-DGV. Strikingly a 1-h incubation with cyclodextrin/cholesterol completely restored Raf activity in cells expressing Raf, H-Ras, and Cav3-C71W (Fig. 4), a similar effect to that seen in cells expressing the Cav3-DGV mutant. This suggests that the Cav3-C71W mutant perturbs cholesterol-dependent surface signaling events. We therefore investigated whether Cav3-C71W caused gross changes in free cholesterol distribution. No significant changes were observed by filipin staining (results not shown). We also examined whether Cav3-C71W showed different detergent insolubility characteristics or oligomerization properties as compared with expressed wild type Cav3. In both cases the properties of the Cav3-C71W mutant were indistinguishable from the wild type protein (results not shown).

We next examined whether expression of the Cav3-C71W mutant affected raft domains. We used a modified flotation method in which carbonate-treated membranes are separated on a linear sucrose gradient to separate raft and non-raft domains (31). Cav3-WT, Cav3-C71W, or Cav3-DGV were transfected with GFP-tH, a raft marker comprising the H-Ras minimal membrane-targeting domain attached to the C terminus of GFP. Both Cav3-C71W and Cav3-DGV, but not Cav3-WT, caused a significant shift of the raft marker GFP-tH to denser fractions (Fig. 5) consistent with the postulated effect of these proteins on plasma membrane raft domains. Interestingly, however, it was noted that the extent of the shift was different for the two mutants with the Cav3-C71W mutant causing a more modest shift of a portion of the GFP-tH from its normal position on the density gradient. It is assumed that the increase in density of the GFP-tH-containing rafts is due to a decrease in cholesterol in raft domains, consistent with rescue of H-Ras inhibition by cholesterol addition. Note that there was no difference in the density of fractions containing Cav3-WT and Cav3-C71W on the gradients (Fig. 5), further supporting the localization data (Fig. 3). The flotation of endogenous Cav1 was also unaffected by co-expression of Cav3-C71W (data not shown), highlighting the significance of the reproducible shift

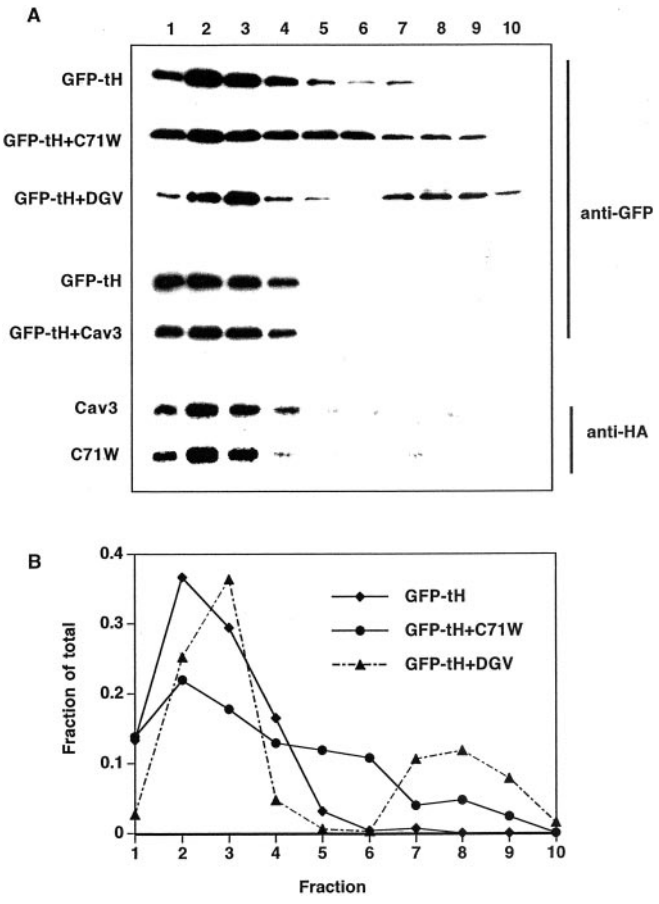

FIG. 5. **Caveolin-3 mutants increase density of raft-containing membranes.** BHK cells were transfected with GFP-tH alone or in combination with Cav3-WT, Cav3-C71W, or Cav3-DGV as indicated. Whole cell lysates prepared in 45% sucrose, 250 mM Na_2CO_3 were centrifuged under a sucrose gradient. Each fraction, numbered from the top of the gradient, was then collected, diluted into MES-buffered saline, pelleted, and immunoblotted. *A*, expression of Cav3-C71W and Cav3-DGV alters the distribution of GFP-tH, whereas expression of Cav3-WT does not. The *lower two profiles* show blotting for HA-tagged Cav3-WT or Cav3-C71W in the same gradients. Cav3 shows an identical distribution to Cav3-C71W in the sucrose gradient. *B*, the *top three immunoblots* of *A* were then quantified by phosphorimaging. These experiments show that Cav3-DGV and Cav3-C71W expression cause a significant increase in the density of GFP-tH-containing microdomains. Similar results were obtained in three independent experiments.

observed for GFP-tH and suggesting that caveolae domains are not significantly affected by mutant expression.

Amino Acid Substitution at Position 71 or Reduced Plasma Membrane Localization of Caveolin-3-C71W Ablates the Inhibitory Effects of the Mutant Protein—Finally we explored the relevant features of the Cav3-C71W mutant that make it inhibitory to H-Ras function. We first generated a new HA-tagged Cav3 mutant, Cav3-C71A, in which the amino acid at position 71 was changed from a cysteine to an alanine. This mutant was used to determine whether the impact of the mutation in Cav3-C71W is due to the loss of the cysteine residue, which is conserved within Cav3 from different species, or to the presence of the substituted bulky hydrophobic tryptophan. Cav3-C71A localized in BHK cells in a manner indistinguishable from that observed for the Cav3-WT or Cav3-C71W mutant (data not shown). Unlike the Cav3-C71W mutant, however, the Cav3-C71A mutant was unable to inhibit H-Ras-mediated Raf activation (Fig. 1) and did not inhibit activated H-Ras-mediated neurite outgrowth in the PC12 assay (Fig. 2). This would argue that it is the acquisition of the tryptophan at position 71 that alters Cav3 function.

In addition we investigated whether plasma membrane localization was essential for the inhibition of H-Ras signaling by Cav3-C71W. The N terminus of Cav3 has been shown to be required for caveolar localization of Cav3 (32). Consistent with this, we have found that the introduction of point mutants in conserved amino acids in this region, such as the arginine at position 26 (a naturally occurring dystrophy mutant) and the highly conserved proline at position 28, resulted in the intracellular retention of the resultant mutants in the C_2C_{12} system (data not shown). As shown in Fig. 3, introducing a proline to histidine change in Cav3-C71W at position 28 caused the mutant protein (Cav3-P28H/C71W) to accumulate intracellularly in BHK and C_2C_{12} cells. The mutant protein was unable to inhibit H-Ras-mediated Raf activation (Fig. 1). The lack of inhibitory effect of the double mutant was confirmed using the PC12 neurite outgrowth assay (Fig. 2).

We have shown, therefore, that a single point mutation in Cav3, a cysteine to tryptophan substitution at amino acid position 71, can cause perturbation of cholesterol-dependent raft signaling domains using two independent Ras assays. The Ras assays provide powerful model systems to study raft-dependent and -independent signaling pathways as shown in this and previous studies (29, 31). Ras has been implicated in regulation of muscle genes during differentiation and in response to nerve activity, but the involvement of distinct Ras isoforms in muscle is unknown. Genetic ablation of H-Ras (and N-Ras) expression in mice had no discernible phenotype (41) in contrast to the embryonically lethal effects of K-Ras ablation (42), suggesting that H-Ras is not essential for muscle differentiation *in vivo*. While we have used Ras as an assay, it is likely that other signaling events utilizing raft domains may be affected in muscle cells expressing the Cav3-C71W protein *in vivo*. A possibly analogous situation was reported recently in neurons from mice with Niemann-Pick type C in which it was shown that the cholesterol imbalance associated with the disease caused defects in specific raft-dependent signaling pathways (43). Interestingly it has been reported that in Cav3 knockout mice the dystrophin complex no longer floats on a sucrose density gradient (19) implying that Cav3, via an ability to profoundly influence raft domain formation, may also impact on the performance of raft resident structural proteins.

We speculate that the Cav3-C71W-induced disruption of cholesterol-rich domains in muscle may underlie the mild dystrophic phenotype caused by the mutant protein. However, it is important to note that a recent study suggested that a number of the described Cav3 point mutations also occurred in nondystrophic individuals and may therefore represent polymorphisms (44). In the case of Cav3-C71W, of 100 apparently healthy individuals screened, one subject was found to carry that mutation. In view of the striking effects of the mutant protein described here, it is possible that the effects of Cav3-C71W may be balanced by compensatory mechanisms in some, but not all, genetic backgrounds. In support of this, it has recently been reported that the Cav3-P104L mutation, originally documented in patients with limb-girdle muscular dystrophy, is also found in patients with a separate muscle disorder known as hereditary rippling muscle disease (10), further verifying that both genetic and environmental backgrounds influence phenotypic outcome.

The effects of the Cav3-C71W mutant show similarities to those previously described for the Cav3-DGV mutant in the specificity for H-Ras-mediated signaling events, rescue by addition of cholesterol, and the effect on the density of raft-containing membranes (Ref. 29 and this study). However, unlike the Cav3-DGV mutant, Cav3-C71W reaches cell surface caveolae, and based on comparison with a double mutant im-

paired in transport to the surface, this surface localization may be required for its inhibitory effects. Caveolins have been implicated in cholesterol transport to the plasma membrane and may therefore be important in regulating the availability of plasma membrane cholesterol for raft domains. We have speculated that Cav3-DGV perturbs the distribution of free cholesterol in the cell, causing intracellular accumulation in late endosomes, and therefore reduces the availability of cholesterol required for functional surface raft domains (37). As the Cav3-C71W mutant has a similar effect and yet reaches the cell surface and has no apparent gross effects on free cholesterol distribution, the mechanisms involved are presumably distinct. It is possible that the C71W mutation increases the affinity of Cav3 for cholesterol and prevents release of cholesterol from caveolae to noncaveolar lipid rafts, but further work is required to test this hypothesis. Our results suggest that the addition of tryptophan rather than the loss of cysteine may be important. Interestingly the corresponding amino acid is highly conserved in mammalian Cav1 and is a phenylalanine. Thus, the tryptophan substitution might make Cav3 more like Cav1 in this region.

The identification of a single amino acid residue that changes the properties of the Cav3 protein so dramatically provides a valuable tool for studies of caveolins, cholesterol, and raft domains. Moreover, we have revealed a potential mechanism by which mutations in Cav3 can induce defects in specific signaling pathways that could lead to long term pathological changes.

Acknowledgment—We are grateful to Rob Luetterforst for excellent technical assistance.

REFERENCES

1. Way, M. & Parton, R. G. (1995) *FEBS Lett.* **376,** 108–112
2. Tang, Z., Scherer, P. E., Okamoto, T., Song, K., Chu, C., Kohtz, D. S., Nishimoto, I., Lodish, H. F. & Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 2255–2261
3. Fra, A. M., Williamson, E., Simons, K. & Parton, R. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8655–8659
4. Kurzchalia, T. V. & Parton, R. G. (1999) *Curr. Opin. Cell Biol.* **11,** 424–431
5. McNally, E. M., de Sa Moreira, E., Duggan, D. J., Bonnemann, C. G., Lisanti, M. P., Lidov, H. G., Vainzof, M., Passos-Bueno, M. R., Hoffman, E. P., Zatz, M. & Kunkel, L. M. (1998) *Hum. Mol. Genet.* **7,** 871–877
6. Minetti, C., Sotgia, F., Bruno, C., Scartezzini, P., Broda, P., Bado, M., Masetti, E., Mazzocco, M., Egeo, A., Donati, M. A., Volonte, D., Galbiati, F., Cordone, G., Bricarelli, F. D., Lisanti, M. P. & Zara, F. (1998) *Nat. Genet.* **18,** 365–368
7. Carbone, I., Bruno, C., Sotgia, F., Bado, M., Broda, P., Masetti, E., Panella, A., Zara, F., Bricarelli, F. D., Cordone, G., Lisanti, M. P. & Minetti, C. (2000) *Neurology* **54,** 1373–1376
8. Herrmann, R., Straub, V., Blank, M., Kutzick, C., Franke, N., Jacob, E. N., Lenard, H. G., Kroger, S. & Voit, T. (2000) *Hum. Mol. Genet.* **9,** 2335–2340
9. Sunada, Y., Ohi, H., Hase, A., Hosono, T., Arata, S., Higuchi, S., Matsumura, K. & Shimizu, T. (2001) *Hum. Mol. Genet.* **10,** 173–178
10. Betz, R. C., Schoser, B. G., Kasper, D., Ricker, K., Ramirez, A., Stein, V., Torbergsen, T., Lee, Y. A., Nothen, M. M., Wienker, T. F., Malin, J. P., Propping, P., Reis, A., Mortier, W., Jentsch, T. J., Vorgerd, M. & Kubisch, C. (2001) *Nat. Genet.* **28,** 218–219
11. Parton, R. G., Way, M., Zorzi, N. & Stang, E. (1997) *J. Cell Biol.* **136,** 137–154
12. Rahkila, P., Takala, T. E., Parton, R. G. & Metsikko, K. (2001) *Exp. Cell Res.* **267,** 61–72
13. Song, K. S., Scherer, P. E., Tang, Z., Okamoto, T., Li, S., Chafel, M., Chu, C., Kohtz, D. S. & Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 15160–15165
14. Crosbie, R. H., Yamada, H., Venzke, D. P., Lisanti, M. P. & Campbell, K. P. (1998) *FEBS Lett.* **427,** 279–282
15. Sotgia, F., Lee, J. K., Das, K., Bedford, M., Petrucci, T. C., Macioce, P., Sargiacomo, M., Bricarelli, F. D., Minetti, C., Sudol, M. & Lisanti, M. P. (2000) *J. Biol. Chem.* **275,** 38048–38058
16. Ralston, E. & Ploug, T. (1999) *Exp. Cell Res.* **246,** 510–515
17. Carozzi, A. J., Ikonen, E., Lindsay, M. R. & Parton, R. G. (2000) *Traffic* **1,** 326–341
18. Ishikawa, H. (1968) *J. Cell Biol.* **38,** 51–66
19. Galbiati, F., Engelman, J. A., Volonte, D., Zhang, X. L., Minetti, C., Li, M., Hou, H., Jr., Kneitz, B., Edelmann, W. & Lisanti, M. P. (2001) *J. Biol. Chem.* **276,** 21425–21433
20. Razani, B., Schlegel, A. & Lisanti, M. P. (2000) *J. Cell Sci.* **113,** 2103–2109
21. Schlegel, A., Schwab, R. B., Scherer, P. E. & Lisanti, M. P. (1999) *J. Biol. Chem.* **274,** 22660–22667
22. Arbuzova, A., Wang, L., Wang, J., Hangyas-Mihalyne, G., Murray, D., Honig, B. & McLaughlin, S. (2000) *Biochemistry* **39,** 10330–10339
23. Murata, M., Peranen, J., Schreiner, R., Wieland, F., Kurzchalia, T. V. & Simons, K. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10339–10343
24. Trigatti, B. L., Anderson, R. G. & Gerber, G. E. (1999) *Biochem. Biophys. Res. Commun.* **255,** 34–39
25. Hailstones, D., Sleer, L. S., Parton, R. G. & Stanley, K. K. (1998) *J. Lipid Res.* **39,** 369–379
26. Bist, A., Fielding, P. E. & Fielding, C. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 10693–10698
27. Fielding, C. J., Bist, A. & Fielding, P. E. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3753–3758
28. Smart, E. J., Ying, Y., Donzell, W. C. & Anderson, R. G. (1996) *J. Biol. Chem.* **271,** 29427–29435
29. Roy, S., Luetterforst, R., Harding, A., Apolloni, A., Etheridge, M., Stang, E., Rolls, B., Hancock, J. F. & Parton, R. G. (1999) *Nat. Cell Biol.* **1,** 98–105
30. Rojo, M., Pepperkok, R., Emery, G., Kellner, R., Stang, E., Parton, R. G. & Gruenberg, J. (1997) *J. Cell Biol.* **139,** 1119–1135
31. Prior, I. A., Harding, A., Yan, J., Sluimer, J., Parton, R. G. & Hancock, J. F. (2001) *Nat. Cell Biol.* **3,** 368–375
32. Luetterforst, R., Stang, E., Zorzi, N., Carozzi, A., Way, M. & Parton, R. G. (1999) *J. Cell Biol.* **145,** 1443–1459
33. Roy, S., Lane, A., Yan, J., McPherson, R. & Hancock, J. F. (1997) *J. Biol. Chem.* **272,** 20139–20145
34. Furuchi, T. & Anderson, R. G. (1998) *J. Biol. Chem.* **273,** 21099–21104
35. Parton, R. G. & Hancock, J. F. (2001) *Methods Enzymol.* **333,** 172–183
36. Pol, A., Calvo, M., Lu, A. & Enrich, C. (1999) *Hepatology* **29,** 1848–1857
37. Pol, A., Luetterforst, R., Lindsay, M., Heino, S., Ikonen, E. & Parton, R. G. (2001) *J. Cell Biol.* **152,** 1057–1070
38. Kolch, W. (2000) *Biochem. J.* **351,** 289–305
39. Cowley, S., Paterson, H., Kemp, P. & Marshall, C. J. (1994) *Cell* **77,** 841–852
40. Galbiati, F., Volonte, D., Minetti, C., Chu, J. B. & Lisanti, M. P. (1999) *J. Biol. Chem.* **274,** 25632–25641
41. Esteban, L. M., Vicario-Abejon, C., Fernandez-Salguero, P., Fernandez-Medarde, A., Swaminathan, N., Yienger, K., Lopez, E., Malumbres, M., McKay, R., Ward, J. M., Pellicer, A. & Santos, E. (2001) *Mol. Cell. Biol.* **21,** 1444–1452
42. Johnson, L., Greenbaum, D., Cichowski, K., Mercer, K., Murphy, E., Schmitt, E., Bronson, R. T., Umanoff, H., Edelmann, W., Kucherlapati, R. & Jacks, T. (1997) *Genes Dev.* **11,** 2468–2481
43. Henderson, L. P., Lin, L., Prasad, A., Paul, C. A., Chang, T. Y. & Maue, R. A. (2000) *J. Biol. Chem.* **275,** 20179–20187
44. de Paula, F., Vainzof, M., Bernardino, A. L., McNally, E., Kunkel, L. M. & Zatz, M. (2001) *Am. J. Med. Genet.* **99,** 303–307

Inhibition of Lipid Raft-dependent Signaling by a Dystrophy-associated Mutant of Caveolin-3

Amanda J. Carozzi, Sandrine Roy, Isabel C. Morrow, Albert Pol, Bruce Wyse, Jodi Clyde-Smith, Ian A. Prior, Susan J. Nixon, John F. Hancock and Robert G. Parton

J. Biol. Chem. 2002, 277:17944-17949.
doi: 10.1074/jbc.M110879200 originally published online March 7, 2002
