p42/44 MAP Kinase-dependent and -independent Signaling Pathways Regulate Caveolin-1 Gene Expression

ACTIVATION OF RAS-MAP KINASE AND PROTEIN KINASE A SIGNALING CASCADES TRANSCRIPTIONALLY DOWN-REGULATES CAVEOLIN-1 PROMOTER ACTIVITY*

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Caveolin-1 is a principal component of caveolae membranes in vivo. Caveolin-1 mRNA and protein expression are down-regulated in NIH 3T3 cells in response to transformation by activated oncogenes, such as H-Ras(G12V) and v-Abl. The mechanisms governing this down-regulation event remain unknown. Here, we show that caveolin-1 gene expression is directly regulated by activation of the Ras-p42/44 MAP kinase cascade. Down regulation of caveolin-1 protein expression by Ras is independent of (i) the type of activating mutation (G12V versus Q61L) and (ii) the form of activated Ras transfected (H-Ras versus K-Ras versus N-Ras). Treatment of Ras or Raf-transformed NIH 3T3 cells with a well characterized MEK inhibitor (PD 98059) restores caveolin-1 protein expression. In contrast, treatment of v-Src and v-Abl transformed NIH 3T3 cells with PD 98059 does not restore caveolin-1 expression. Thus, there must be at least two pathways for down-regulating caveolin-1 expression: one that is p42/44 MAP kinase-dependent and another that is p42/44 MAP kinase-independent. We focused our efforts on the p42/44 MAP kinase-dependent pathway. The activity of a panel of caveolin-1 promoter constructs was evaluated using transient expression in H-Ras(G12V) transformed NIH 3T3 cells. We show that caveolin-1 promoter activity is up-regulated ∼5-fold by inhibition of the p42/44 MAP kinase cascade. Using electrophoretic mobility shift assays we provide evidence that the caveolin-1 promoter (from −156 to −561) is differentially bound by transcription factors in normal and H-Ras(G12V)-transformed cells. We also show that activation of protein kinase A (PKA) signaling is sufficient to down-regulate caveolin-1 protein expression and promoter activity. Thus, we have identified two signaling pathways (Ras-p42/44 MAP kinase and PKA) that transcriptionally down-regulate caveolin-1 gene expression.

The subcellular distribution of several signaling molecules is restricted and regulated by association with scaffolding proteins (Ste5p, AKAPs (protein kinase A anchor proteins), and 14-3-3) (1, 2), forming a signaling pathway or module. Accumulating evidence suggests that caveolins possess all the qualities of scaffolding proteins. We and other investigators have proposed the "caveolae signaling hypothesis," which states that caveolar localization of certain inactive signaling molecules could provide a compartmental basis for their regulated activation and explain cross-talk between different signaling pathways (3). In support of this idea, caveolin-1 binding can functionally suppress the GTPase activity of heterotrimeric G-proteins and inhibit the kinase activity of Src family tyrosine kinase through a common caveolin domain, termed the caveolin-scaling domain (4). Thus, we have suggested that caveolin may function as a negative regulator of many different classes of signaling molecules through the recognition of specific caveolin-binding motifs (4).

Caveolins form multivalent homo- and heter-oligomers and each caveolin-interacting protein binds to the same cytosolic membrane-proximal region of caveolin (5, 6). Domain-mapping studies have revealed that the interaction of caveolin-1 with signaling molecules is mediated via a membrane proximal region of caveolin, termed the caveolin-scaling domain (residues 82–101). Through this domain, caveolin-1 interacts with G-protein α subunits, H-Ras, Src family tyrosine kinases, protein kinase C isoforms, epidermal growth factor receptor, Neu, and eNOS (see Ref. 7; reviewed in Ref. 8). In many cases, it has been shown that mutational activation of these signaling molecules (G-proteins, H-Ras, or Src family kinases) prevents regulated interaction with the caveolin-scaling domain. These activating mutations include H-Ras(G12V) and Gαo (Q227L) that are found in human cancers.

The caveolin-scaling domain recognizes a well defined caveolin-binding motif that includes several crucial aromatic amino acid residues (4, 9, 10). This motif was identified by using the caveolin-scaling domain to select random peptide ligands from phage display libraries (4, 9, 10). The relevance of the motif we identified was stringently evaluated using a well characterized caveolin-binding protein, namely a G-protein α subunit (Gαq). Since the identification of the caveolin-scaling domain (6) and caveolin-binding sequence motifs (4, 9, 10), these observations have been extended to other caveolin-interacting proteins. Functional caveolin-binding motifs have been

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deduced in both tyrosine and serine/threonine kinases, as well as eNOS (reviewed in Ref. 8). In all cases examined, the caveolin-binding motif is located within the catalytic domain of a given signaling molecule. For example, in the case of tyrosine and serine/threonine kinases, a kinase domain consists of 11 conserved subdomains (I-XI), and the caveolin-binding motif occurs within subdomain IX (4, 9, 10). Caveolin-binding via the scaffolding domain is sufficient to inhibit the enzymatic activity of these kinases in vitro. Indeed, in many cases, a synthetic peptide corresponding to this caveolin domain is the most potent peptide inhibitor known for these enzymes. Agents that mimic the interaction with caveolins are potentially useful as general kinase inhibitors, and possibly as anti-tumor drugs.

Modification and/or inactivation of caveolin-1 appears to be a common feature of the transformed phenotype. Historically, caveolin was first identified as a v-Src substrate (11). Thus, caveolin may represent a critical target during cell transformation (11). In support of this notion, caveolin-1 mRNA and protein expression are reduced or absent in NIH 3T3 cells transformed by a variety of activated oncogenes (v-Ab1, Bcr-Ab1, H-Ras(G12V)), and caveolae are missing from these transformed cells (12); caveolin-2 protein is not down-regulated in response to oncogenic transformation (7, 13). In addition, caveolin-1 expression levels correlated inversely with the ability of these cells to grow in soft agar, i.e. cells expressing the smallest amount of caveolin-1 and lacking detectable caveolae formed the largest colonies in soft agar. Furthermore, our laboratory and other investigators have demonstrated that recombinant expression of caveolin-1 in transformed NIH 3T3 cells and mammary carcinoma cell lines abrogates their growth in soft agar (14, 15). These results suggest that down-regulation of caveolin-1 protein expression and caveolae organelles may be critical to maintaining the transformed phenotype.

The mechanisms that govern caveolin-1 down-regulation remain largely unknown. Here, we have identified two signaling pathways (Ras-p42/44 MAP kinase1 and PKA) that can transcriptionally down-regulate caveolin-1 promoter activity.

**EXPERIMENTAL PROCEDURES**

**Materials—**Anti-caveolin-1 IgG (monoclonal antibody 2297 (16)) and anti-caveolin-2 IgG (monoclonal antibody 65 (13)) were the gifts of Dr. Roberto Campos-Gonzalez, Transduction Labs. Antibodies against GDP-dissociation inhibitor were the generous gift of Dr. Perry Bickel, Washington University, St. Louis, MO. Other reagents were purchased commercially: anti-activated ERK-1/2 IgG (p42/44 MAP kinase; New England Biolabs, Inc.), fetal bovine serum (JRH Biosciences), and pre-stained protein markers (Life Technologies, Inc.). PD 98059 was purchased from Calbiochem and dissolved in dimethyl sulfoxide at a concentration of 50 μM and used at a final concentration of 50 μM. Forskolin and IBMX were purchased from Sigma and used at final concentrations of 10 and 500 μM, respectively.

**Cell Lines—**H-Ras(G12V), v-Ab1, and v-Src-transformed NIH 3T3 cells and normal non-transformed NIH 3T3 cells were as previously described (7). N-Ras(Q61K)-transformed NIH 3T3 cells (17) were the gift of Dr. Angel Pellicer (New York University Medical Center). H-Ras(Q61L)- and K-Ras(G12V)-transformed NIH 3T3 cells were obtained with permission from Dr. Channing J. Der, The Lineberger Comprehensive Cancer Center, UNC Chapel Hill, NC, via Dr. Shama Kajiji at Pfizer, Inc., Groton, CT. Ras(G12V) IPTG-inducible NIH 3T3 cells were obtained with permission from Dr. Yoshito Kozuro at the Tokyo Institute of Technology, Japan, via Drs. Mark Hamilton and Alan Wolfman at the Cleveland Clinic Foundation, OH. v-Raf-transformed NIH 3T3 cells were donated by Dr. D. Stave Kohtz at the Mt. Sinai School of Medicine, NY. CHO cells (GBC′/LR-73) were the generous gift of Dr. Jeffrey Pollard and were as described previously (18).

1 The abbreviations used are: MAP kinase, mitogen-activated protein kinase; PKA, protein kinase A; IBMX, isobutylmethylxanthine; IPTG, isopropyl-1-thio-β-D-galactopyranoside; CHO, Chinese hamster ovary; kb, kilobase(s); PCR, polymerase chain reaction; bp, base pair(s); EMSA, electrophoretic mobility shift assay.

**Luciferase Assays—**Transient transfections (using calcium phosphate precipitation) and luciferase assays were performed essentially as described previously (7, 21). Briefly, 300,000 cells (NIH 3T3 cells or CHO cells as specified) were seeded in six-well plates 12–24 h before the transfection. Each point was transfected with either 2 μg of reporter and 1 μg of each plasmid for experiments in which only one plasmid was transfected; or 1 μg of each plasmid for experiments in which two plasmids were co-transfected. 12 h post-transfection, the cells were rinsed twice with phosphate-buffered saline and incubated in fetal bovine serum for another 24–36 h. This incubation was done in the presence of 50 μM PD 98059 or 1 mM IPTG when applicable. The cells were then lysed in 200 μl of extraction buffer, 75 μl of which was used to measure luciferase activity, as described (22). For experiments assessing promoter activity in response to the p42/44 MAP kinase activators and PKA, after washing with phosphate-buffered saline, the cells were incubated in the presence of 1% fetal bovine serum for 24–36 h as described previously (21). These assays were made possible through the use of a special CHO-derived cell line, called GBC′/LR-73. Unlike parental CHO cells, GBC′/LR-73 cells are normally transformed growth-arrested cells that have normal fibroblastic morphology, does not grow in suspension, requires high serum concentrations for growth, and undergoes synchronized growth arrest in low concentrations of serum (1–2%) without a loss of viability (18). Also, these cells have a much higher transfection efficiency (~10-fold) than parental CHO cells.

**Immunoblotting—**Samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. After transfer, nitrocellulose sheets were stained with Ponceau S to visualize protein bands and subjected to immunoblotting. For immunoblotting, incubation conditions were as described by the manufacturer (Amersham Pharmacia Biotech), except we supplemented our blocking solution with 2% non-fat dry milk (Carnation). Bound antibodies were visualized using ECL (Amersham Pharmacia Biotech), except we supplemented our blocking solution with phosphate-buffered saline, the cells were incubated in the presence of 1% fetal bovine serum for 24–36 h as described previously (21). These assays were made possible through the use of a special CHO-derived cell line, called GBC′/LR-73. Unlike parental CHO cells, GBC′/LR-73 cells are normally transformed growth-arrested cells that have normal fibroblastic morphology, does not grow in suspension, requires high serum concentrations for growth, and undergoes synchronized growth arrest in low concentrations of serum (1–2%) without a loss of viability (18). Also, these cells have a much higher transfection efficiency (~10-fold) than parental CHO cells.

**Immunoblotting with Phospho-specific Antibody Probes—**To investigate the activation state of p42/44 MAP kinase, we employed a phospho-specific antibody probe that has been generated against the activated form of ERK-1/2 (New England Biolabs, Inc.). It has been shown that this antibody can be used to selectively detect activated p42/44 MAP kinase by Western blotting. Cells were lysed in boiling sample buffer, as suggested by the manufacturer of phospho-specific antibody probes (New England Biolabs, Inc.). Samples were then collected and boiled for a total of 5 min. Samples were homogenized using a 26-g needle and a 1-ml syringe. After SDS-polyacrylamide gel elec-
trophoresis and transfer to nitrocellulose, blots were probed with primary antibodies (dilution of 1:500; New England Biolabs, Inc.) and the same horseradish peroxidase-conjugated secondary antibody (dilution of 1:5000; Transduction Laboratories). Bound antibodies were visualized using ECL (Amersham Pharmacia Biotech).

Northern Analysis—Total RNA was extracted and purified according to the manufacturer’s instructions (QIagen, Inc.). Ten micrograms of total cellular RNA was denatured with formaldehyde and subjected to Northern blot analysis with \(^{32}P\)-labeled probes for the mouse caveolin-1 mRNA (2.4 kb). The 28 S and 18 S rRNA were visualized by ethidium bromide staining.

Electrophoretic Mobility Shift Assay (EMSA)—Electrophoretic mobility shift assays were performed as described (23, 24), with minor modifications. Briefly, nuclear extracts were prepared by the method described by Schreiber et al. (25). Extracts were isolated from ~10^6 cells, aliquoted, and frozen immediately. Concentrations were determined using the BCA Protein Assay Reagent (Pierce Chemical Co.). DNA probes for the EMSA were constructed by PCR using the mouse caveolin-1 genomic clone described above. The four overlapping probes were amplified with 5’-AGCTGGAGTCCAC GTATTTGCCC-3’; 5’-CCTCCACCCCTGCTGAGATGATG-3’; 5’-AGACCCGGCGCAGAG-3’; and 5’-TTAAATCACAGCCCAGGGAAACCTCCTCAGAGCCTGCAGCCAGCC-3’ primers; and Probe B was amplified with 5’-GGTTCGCCAGCATCGCTTGATATCTC-3’ and 5’-AACCTAGAGGAGCCTAGG-3’ primers; and Probe D was amplified with 5’-CTTCTGTAACGAGTTGAACCTC-3’ and 5’-TCTGTCTCCGTTCTCACAGAG-3’ primers. Approximately 200 ng of purified PCR product was end-labeled with \(\gamma\)-\(^{32}P\)ATP (NEN Life Science Products Inc.). EMSA was performed by the method of Singh et al. (23, 24), with minor modifications. Briefly, 15 \(\mu\)g of nuclear extracts was incubated with 5 \(\mu\)g of poly(dI-dC) (Amersham Pharmacia Biotech) in binding buffer (12% glycerol, 12 mM HEPES, pH 7.9], 4 mM Tris, pH 8.0, 50 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol) on ice for 15 min. Approximately 40,000 cpm of end-labeled probe was added and incubated for an additional 30 min on ice. Protein-DNA complexes were separated on a 5% polyacrylamide gel in 1 x TBE at 20 mA. The gels were dried and complexes were visualized by autoradiography.

Determination of the Transcription Start Site—The mouse transcriptional start site was determined by 5’-rapid amplification of cDNA ends analysis using a previously described 3T3-L1 adipocyte library cloned into pCDNA1 (26). Briefly, PCR products were amplified using an anchor primer from pCDNA1 and an oligonucleotide primer that is antisense to nucleotides 9–26 of murine caveolin-1. PCR products were cloned into pCR-Blunt (Invitrogen). The transcriptional start site was determined to be at ~63 by direct sequencing of the subcloned inserts. Thus, sequence of the 5’-untranslated region is: CAGTTCTCTTAAAACTCACGAGAGAAACCCTAGCTACTGACTGACGCCAGGCGCCAGCC.

RESULTS

Down-regulation of Caveolin-1 Protein Expression by p42/44 MAP Kinase-dependent and -Independent Signaling Pathways—Expression of caveolin-1 mRNA and protein are down-regulated in H-Ras(G12V) and v-Abl transformed NIH 3T3 cells (12, 14). In contrast, expression of the caveolin-2 protein is largely unaffected in these cells (13). The mechanism by which these transforming oncogenes down-regulate caveolin-1 expression remains unknown. One possibility is that caveolin-1 gene expression is negatively regulated by constitutive activation of the Ras-p42/44 MAP kinase cascade.

To investigate this hypothesis further, we examined the expression of caveolin-1 protein in a number of other Ras-transformed NIH 3T3 cells. Fig. 1A shows that caveolin-1 protein expression was down-regulated in NIH 3T3 cells transformed by H-Ras(G12V), K-Ras(G12V), N-Ras(G12V), and v-Raf. These results indicate that Ras-induced down-regulation of caveolin-1 is (i) independent of the type activating mutation (G12V versus Q61L), (ii) independent of the type of Ras transformed (H-Ras versus K-Ras versus N-Ras), and (iii) also occurs if transformation is mediated by an element of the Ras-MAP kinase pathway that is directly downstream of Ras itself (v-Raf). The expression of caveolin-2 was unaffected by these activated oncogenes. The expression of both caveolin-1 and -2 in v-Abl and v-Src transformed NIH 3T3 cells is shown for comparison. Down-regulation of caveolin-1 protein expression by v-Src is also secondary to down-regulation of the caveolin-1 mRNA, as seen by Northern analysis (Fig. 1B). In accordance with these observations, we have shown that caveolin-1 mRNA levels are also dramatically down-regulated in Ras- and v-Abl transformed NIH 3T3 cells (12, 14).

We previously demonstrated that treatment of H-Ras (G12V)-transformed NIH 3T3 cells with a well characterized MEK inhibitor (PD 98059) is sufficient to restore expression of the caveolin-1 protein product in these cells (14). Fig. 2A shows that treatment with PD 98059 restores the expression of the caveolin-1 protein in H-Ras(Q61L), K-Ras(G12V), N-Ras(Q61K), and v-Raf-transformed NIH 3T3 cells. In contrast, PD 98059 has no significant effect on caveolin-1 protein expression levels in normal NIH 3T3 cells. These results are consistent with the
hypothesis that the down-regulation of caveolin-1 expression in these cells is due to constitutive activation of the p42/44 MAP kinase cascade. Interestingly, treatment of v-Src and v-Abl transformed NIH 3T3 cells with PD 98059 did not restore caveolin-1 expression. Thus, there must be at least two or three independent pathways for down-regulating caveolin-1 expression: one that is p42/44 MAP kinase-dependent and others that are p42/44 MAP kinase-independent (i.e., activated by v-Abl or v-Src). In accordance with the observation that caveolin-1 protein expression is restored by treatment of H-Ras(G12V) transformed cells with PD 98059, we also observed that the same treatment up-regulated expression of the caveolin-1 mRNA by ~5-fold (Fig. 2B).

Fig. 3 shows the effects of various concentrations of PD 98059 on caveolin-1 protein expression and p42/44 MAP kinase activation in H-Ras(G12V)-transformed NIH 3T3 cells. We evaluated the effect of various concentrations of PD 98059 (5, 10, 25, and 50 μM) on caveolin-1 expression and p42/44 MAP kinase activation by Western blot analysis. Each lane contains equal amounts of total protein. Note that as little as 5–10 μM PD 98059 is sufficient to up-regulate caveolin-1 expression in H-Ras(G12V)-transformed NIH 3T3 cells and that p42/44 MAP kinase activation is progressively inhibited. In contrast, caveolin-2 protein levels remain relatively constant and are shown for comparison. To detect activated p42/44 MAP kinase, we employed a phospho-specific antibody probe that has been generated against the activated form of ERK-1/2, this antibody can be used to selectively detect activated p42/44 MAP kinase by Western blotting (Ref. 27; New England Biolabs, Inc.).

Activation of the p42/44 MAP Kinase Cascade Transcriptionally Down-regulates Caveolin-1 Promoter Activity—As the down-regulation of caveolin-1 protein expression is strictly correlated with a loss of caveolin-1 mRNA expression (12), this event may be governed by transcriptional control. To test this hypothesis directly, we identified and cloned the murine caveolin-1 gene. The DNA sequence of the murine caveolin-1 promoter region has been deposited in GenBank under accession number AF124227. The murine transcriptional start site was determined to be at −63 (see “Experimental Procedures”).

We next used this murine genomic clone to generate three caveolin-1 promoter constructs that use luciferase expression as the reporter. These three constructs are illustrated schematically in Fig. 4A. Briefly, the first construct contains the 750 bp of sequence upstream of the caveolin-1 ATG (Pr-750bp), the second construct contains ~3 kb upstream of the caveolin-1
The activity of these caveolin promoter constructs was next evaluated using transient expression in H-Ras(G12V)-transformed NIH 3T3 cells. In addition, these cells were treated with PD 98059 or left untreated. Our results indicate that all three promoter constructs were stimulated by treatment with PD 98059, but that Pr-750 behaved similarly, while Pr-3kb and Int 1 had about twice the promoter activity. These results indicate that additively co-transfected CHO cells with either vector alone, a constitutively active form of Raf, or ERK-2 itself and the caveolin-1 promoter (Pr-3kb and Int 1). Under these conditions, co-transfection with either constitutively active Raf or ERK-2 was sufficient to down-regulate caveolin-1 promoter activity (Fig. 7). These results clearly indicate that ERK itself can down-regulate caveolin-1 promoter activity. Conversely, we have previously shown that these Raf and ERK-2 constructs dramatically up-regulate a p42/44 MAP kinase-sensitive Elk-reporter (21).

To evaluate if the p42/44 MAP kinase cascade is directly involved in controlling caveolin-1 promoter activity, we transiently co-transfected CHO cells with either vector alone, a constitutively active form of Raf, or ERK-2 itself and the caveolin-1 promoter (Pr-3kb and Int 1). Under these conditions, co-transfection with either constitutively active Raf or ERK-2 was sufficient to down-regulate caveolin-1 promoter activity (Fig. 7). These results clearly indicate that ERK itself can down-regulate caveolin-1 promoter activity. Conversely, we have previously shown that these Raf and ERK-2 constructs dramatically up-regulate a p42/44 MAP kinase-sensitive Elk-reporter (21).

The Caveolin-1 Promoter Region from 156 to 561 Is Differentially Bound by Transcription Factors in Normal and H-Ras(G12V) Transformed Cells—As caveolin-1 mRNA is down-regulated in response to activation of the p42/44 MAP kinase cascade, it is likely that the caveolin-1 promoter region is differentially bound by transcription factors in normal and H-Ras(G12V) transformed cells. To test this, we performed chromatin immunoprecipitation (ChIP) experiments using antibodies against Elk-1, Sp1, and c-jun. As shown in Fig. 8, Elk-1 and Sp1 binding is significantly increased in H-Ras(G12V) transformed cells compared to normal NIH 3T3 cells. In contrast, c-jun binding is decreased, consistent with the down-regulation of caveolin-1 mRNA in these cells. Altogether, these results provide evidence that the caveolin-1 promoter is differentially bound by transcription factors in normal and H-Ras(G12V) transformed cells.
kinase pathway, we wished to determine if different complexes are formed on the caveolin-1 promoter in normal NIH 3T3 cells as compared with Ras(G12V)-transformed NIH 3T3 cells. To this end, we conducted a series of EMSAs covering the smallest promoter region that was responsive for regulation by activation of the p42/44 MAP kinase cascade. Four overlapping fragments (staggered by 50 bp) were end-labeled and incubated with nuclear extracts from either normal NIH 3T3 cells or H-Ras(G12V)-transformed NIH 3T3 cells. After incubation, samples were run on nondenaturing acrylamide gels and subjected to autoradiography. Fig. 8 shows that no differences were observed with fragment A (exon 1 plus 2197). In contrast, dramatic differences were noted with fragments B (2156 to 2401) and C (2345 to 2561). No differences were observed with fragment D (2509 to 2736; data not shown).

Thus, our results provide evidence that the caveolin-1 promoter region from 2156 to 2561 is differentially bound by transcription factors in normal and H-Ras(G12V)-transformed cells. Four overlapping fragments (staggered by 50 bp) were end-labeled and incubated with nuclear extracts from either normal NIH 3T3 cells or H-Ras(G12V)-transformed NIH 3T3 cells. A minus (−) indicates that no nuclear extract was added as a negative control. Samples were run on non-denaturing acrylamide gels and subjected to autoradiography. Note that no differences were observed with fragment A (exon 1 plus −1 to −197). However, dramatic differences were noted with fragments B (−156 to −401) and C (−344 to −561). No differences were observed with fragment D (−509 to −736; data not shown). Thus, our results provide evidence that the caveolin-1 promoter region from −156 to −561 is differentially bound by transcription factors in normal and H-Ras(G12V)-transformed cells.

Down-regulation of Caveolin-1 Protein and Promoter Activity by Activation of PKA—As caveolin-1 expression can be down-regulated by constitutive activation of the p42/44 MAP kinase cascade through transcriptional regulation, we next evaluated the effects of another well established signaling cascade on caveolin-1 promoter activity. We transiently co-transfected CHO cells with either vector alone or the catalytic subunit of protein kinase A (PKA) and the caveolin-1 promoter (Pr-3kb and Int 1). Our results indicate that overexpression of PKA was sufficient to down-regulate caveolin-1 promoter activity (Fig. 9A). In support of these observations, treatment of CHO cells with agents that elevate cellular cAMP and activate the PKA pathway (either IBMX (a PDE inhibitor) or forskolin (an activator of adenylyl cyclase)) dramatically down-regulates caveolin-1 protein expression (Fig. 9B). Similarly, treatment with IBMX or forskolin also dramatically down-regulated caveolin-2...
The expression of another cellular protein (GDP-dissociation inhibitor) is shown as an additional control for equal protein loading.

Fig. 9C shows that the effects of PKA activation and p42/44 MAP kinase activation on caveolin-1 expression are independent. Note that addition of the MEK inhibitor (PD 98059) to forskolin or IBMX-treated fibroblasts does not restore caveolin-1 expression (upper panel), although we show that PD 98059 effectively inhibits the activation of p42/44 MAP kinase under these conditions (lower panel).

In the case of PKA activation, both caveolin-1 and caveolin-2 protein are down-regulated (Fig. 9B), while in the case of v-Raf and various forms of Ras, caveolin-1 levels are down-regulated and caveolin-2 levels are relatively unaffected (Figs. 1 and 2). Thus, these data independently support the idea that the effects of PKA and p42/44 MAP kinase activation on caveolin-1 protein expression are separate and independent.

**DISCUSSION**

Down-regulation of the caveolin-1 protein is a direct consequence of the oncogenic stimulus as it can be reversed by employing a temperature-sensitive form of v-Abl or by treating Ras(G12V)-transformed 3T3 cells with an inhibitor of the p42/44 MAP kinase pathway (PD 98059) (14). Re-introduction of caveolin-1 under control of an inducible expression system is sufficient to block the anchorage-independent growth of these transformed cells in soft agar and restore the formation of morphologically detectable caveolae (14). Consistent with its antagonism of Ras-mediated cell transformation, caveolin-1 expression dramatically inhibited both Ras/MAPK-mediated and basal transcriptional activation of a mitogen-sensitive promoter (14). Taken together, these results indicate that down-regulation of caveolin-1 expression and caveolae organelles may be critical to maintaining the transformed phenotype in certain cell populations (14).

Recently, we have employed an antisense approach to derive stable NIH 3T3 cell lines that contain normal amounts of caveolin-2, but express dramatically reduced levels of caveolin-1 (27). NIH 3T3 cells harboring antisense caveolin-1 spontaneously formed foci, exhibited anchorage-independent growth in soft agar, formed tumors in immunodeficient mice, and appeared morphologically transformed as seen by scanning electron microscopy (27). Biochemically, these cells also showed increased levels of activated MEK and ERK (27). Taken together, these results suggest that down-regulation of caveolin-1 expression is sufficient to drive oncogenic transformation and constitutively activate the p42/44 MAP kinase cascade (27). Importantly, cell transformation induced by targeted down-regulation of caveolin-1 expression was completely reversed when caveolin-1 protein levels were restored to normal by loss of the caveolin-1 antisense vector (27). Thus, caveolin-1 and activate the PKA pathway (either IBMX (a PDE inhibitor) or forskolin (an activator of adenylyl cyclase)). Note that both pharmacological agents dramatically down-regulate caveolin-1 protein expression. Similarly, treatment with IBMX or forskolin also dramatically down-regulated caveolin-2 protein expression. Each lane contains equal amounts of total protein. The expression of another cellular protein (GDI, GDP-dissociation inhibitor) is shown as an additional control for equal protein loading. C, PKA versus p42/44 MAP kinase activation. As in panel B, except CHO cells were also incubated in the absence (−) or presence (+) of the MEK inhibitor (PD 98059; 50 μM), as indicated. Note that addition of PD 98059 to forskolin or IBMX-treated CHO fibroblasts does not restore caveolin-1 expression (upper panel), although PD 98059 effectively inhibits the activation of p42/44 MAP kinase under these conditions (lower panel). These results indicate that the effects of PKA activation and p42/44 MAP kinase activation on caveolin-1 protein expression are clearly independent. Each lane contains equal amounts of total protein.
behaves as would be expected for a tumor suppressor.

Here, we have examined the signaling pathways that govern caveolin-1 gene expression. We show that caveolin-1 gene expression is directly regulated by activation of the p42/44 MAP kinase cascade. Treatment of Ras(H-, K-, and N-Ras) or v-Raf-transformed NIH 3T3 cells with a well characterized MEK inhibitor (PD 98059) restores the expression of the caveolin-1 protein. However, treatment of v-Src and v-Abl transformed NIH 3T3 cells with PD 98059 has no effect on caveolin-1 expression. Thus, there are at least two or three pathways for down-regulating caveolin-1 expression: one that is p42/44 MAP kinase dependent and others that are p42/44 MAP kinase independent and depend on the activation of non-receptor tyrosine kinases (such as Src or Abl).

The activity of caveolin-1 promoter constructs was evaluated using expression in H-Ras(G12V)-transformed NIH 3T3 cells. Caveolin-1 promoter activity was up-regulated by ~5-fold through inhibition of the p42/44 MAP kinase cascade with PD 98059. In addition, transient transfection of CHO cells with ERK-2 dramatically down-regulates caveolin-1 promoter activity. To determine if different complexes form on the caveolin-1 promoter in normal and Ras(G12V)-transformed NIH 3T3 cells, we performed electromobility shift assays. Our results provide evidence that the caveolin-1 promoter from –156 to –561 is differentially bound by transcription factors in normal and H-Ras(G12V)-transformed cells.

We also evaluated the effects of the PKA pathway on caveolin-1 gene expression. Activation of the PKA pathway by pharmacological agents (IBMX and forskolin) or by overexpression of the PKA catalytic subunit was sufficient to down-regulate caveolin-1 promoter activity and caveolin-1 protein expression. Thus, there may be three “independent” signaling pathways (Ras-p42/44 MAP kinase, NRTKs, and PKA) that can transcriptionally down-regulate caveolin-1 gene expression.

Interestingly, the caveolin-1 protein product can act as an inhibitor of many elements of these signaling cascades, such as Src (6), epidermal growth factor-receptor (10), Raf (21), MEK (21, 27), ERK (21, 27), G protein α subunits (28–30), adenyl cyclase (31), and PKA (32), by the recognition of a common caveolin-binding motif (4, 9, 10), and many of these proteins have been localized to caveolae membranes (8, 33). These observations suggest a general pattern of negative reciprocal regulation. In this sense, caveolin-1 is both upstream and downstream of these signaling pathways.

These findings may have relevance to human cancers. 1) Using differential display and subtractive hybridization techniques, Sager and co-workers (34) have identified a number of “candidate tumor suppressor genes”; these are genes whose mRNAs are down-regulated in human mammary carcinomas. In this screening approach, caveolin-1 was independently identified as one of 26 gene products down-regulated during human mammary tumorigenesis. In addition, caveolin-1 expression was absent in several transformed cell lines derived from human mammary carcinomas including: MT-1, MCF-7, ZR-75-1, T47D, MDA-MB-361, and MDA-MB-474 (34). In contrast, caveolin-1 mRNA was abundantly expressed in normal mammary epithelium.

2) Human tumor cytogenetic data are also consistent with this proposal. Loss of heterozygosity analysis implicates 7q31.1 in the pathogenesis of multiple types of cancer, including breast, ovarian, prostate, and colorectal carcinomas, as well as uterine sarcomas and leiomyomas. The locus of the presumed 7q31.1 tumor suppressor gene has been narrowed to a ~1 Mbp region that includes the highly polymorphic marker D7S 522. Zenklusen and colleagues (see references cited in Refs. 35 and 36) have shown that the D7S 522 locus is the most commonly deleted marker in primary breast cancers, and they note that loss of heterozygosity at this site is strongly associated with systemic progression and death in prostate cancers. D7S 522 also spans the aphidicolin-induced fragile site FRA7G at 7q31. Given the usefulness of 7q31.1 and D7S 522 loss of heterozygosity as markers for carcinogenesis, many laboratories are currently searching this chromosomal region for a novel tumor suppressor gene. Recently, we have shown that CAV1 and CAV2 map within 100 kb of D7S 522, in the middle of the 1 Mb smallest common deleted region for the presumed tumor suppressor gene (35, 36). Evidence that caveolin-1 can suppress cell transformation in murine fibroblasts and human breast cancer cell lines provides independent support for the model that CAV1 is the missing tumor suppressor gene (14, 15).

3) Neu, c-erbB2, is a proto-oncogene product that encodes an epidermal growth-factor receptor tyrosine kinase. Amplification of wild-type c-Neu and mutational activation of Neu (Neu T) have been implicated in oncogenic transformation of cultured fibroblasts and the pathogenesis of human breast cancers in vivo. Recently, we examined the relationship between Neu tyrosine kinase activity and caveolin-1 protein expression in vitro and in vivo. These studies demonstrated that mutational activation of c-Neu down-regulated caveolin-1 protein expression, but not caveolin-2, in cultured NIH 3T3 and Rat 1a cells (7). Conversely, recombinant overexpression of caveolin-1 blocked Neu-mediated signal transduction in vivo. These results indicate that a negative reciprocal relationship exists between c-Neu tyrosine kinase activity and caveolin-1 protein expression. In accordance with these in vitro studies, a 20-amino acid peptide derived from this region (the caveolin-1 scaffolding domain) was sufficient to inhibit Neu-autophosphorylation in an in vitro kinase assay (7). Based on these studies, caveolin-1 expression also inhibits the function of c-Neu, suggesting that caveolin-1 based mimetic peptides or drugs that up-regulate caveolin-1 gene expression would provide an independent and valid approach for the treatment of human breast cancers.

In conclusion, as caveolin-1 down-regulation appears to be involved in mammary and fibroblastic cell transformation (14, 15, 27), an understanding of the signaling pathways that control caveolin-1 expression may ultimately yield novel cancer treatments. For example, our results suggest that the caveolin-1 promoter may be useful in identifying compounds that reverse oncogenic transformation of Ras-transformed cells. We show here that PD 98059, a well characterized inhibitor of MEK, clearly up-regulates caveolin-1 promoter activity in Ras-transformed cells. This MEK inhibitor is also known to revert the phenotype of Ras-transformed cells (37). Thus, screening assays employing the caveolin-1 promoter could provide a general strategy to identify novel inhibitors of the p42/44 MAP kinase cascade, the PKA cascade, or other signaling cascades whose inhibition up-regulates caveolin-1 gene expression.

REFERENCES
1. Rubin, C. S. (1994) Biochim. Biophys. Acta 1224, 467–479
2. Faux, M. C., and Scott, J. D. (1996) Cell 85, 9–12
3. Lisanti, M. P., Scherer, P., Tang, Z.-L., and Sargiacomo, M. (1994) Trends Cell Biol. 4, 231–235
4. Couet, J., Li, S., Okamoto, T., Ikezu, T., and Lisanti, M. P. (1997) J. Biol. Chem. 272, 6525–6533
5. Sargiacomo, M., Scherer, P. E., Tang, Z.-L., Kabuler, E., Song, K. S., Sanders, M. C., and Lisanti, M. P. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 9407–9411
6. Li, S., Couet, J., and Lisanti, M. P. (1996) J. Biol. Chem. 271, 29182–29190
7. Engelman, J. A., Lee, R. J., Karnaizas, A., Bearss, D. J., Webster, M., Siegel, P., Muller, W. J., Windle, J. J., Pestell, R. G., and Lisanti, M. P. (1998) J. Biol. Chem. 273, 20448–20455
8. Okamoto, T., Schlegel, A., Scherer, P. E., and Lisanti, M. P. (1998) J. Biol. Chem. 273, 5419–5422
9. Couet, J., Li, S., Okamoto, T., Scherer, P. S., and Lisanti, M. P. (1997) Trends Cardiovasc. Med. 7, 103–110
10. Couet, J., Sargiacomo, M., and Lisanti, M. P. (1997) J. Biol. Chem. 272, 0000.
11. Glenney, J. R., and Soppet, D. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10517–10521
12. Koleske, A. J., Baltimore, D., and Lisanti, M. P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 1381–1385
13. Scherer, P. E., Lewis, R. Y., Volonte, D., Engelman, J. A., Galbiati, F., Couet, J., Kohtz, D. S., van Donselaar, E., Peters, P., and Lisanti, M. P. (1997) *J. Biol. Chem.* **272**, 29337–29346
14. Engelman, J. A., Wycoff, C. C., Yasuhara, S., Song, K. S., Okamoto, T., and Lisanti, M. P. (1997) *J. Biol. Chem.* **272**, 16374–16381
15. Lee, S. W., Reimer, C. L., Oh, P., Campbell, D. B., and Schnitzer, J. E. (1998) *Oncogene* **16**, 1391–1397
16. Scherer, P. E., Tang, Z.-L., Chun, M. C., Sargiacomo, M., Lodish, H. F., and Lisanti, M. P. (1995) *J. Biol. Chem.* **270**, 16395–16401
17. Guerrero, I., Villasante, A., Corces, V., and Pellicer, A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 7810–7814
18. Pollard, J. W., and Stanners, C. P. (1979) *J. Cell. Physiol.* **98**, 571–585
19. Wood, W. M., Kao, M. Y., Gordon, D. F., and Ridgway, E. C. (1989) *J. Biol. Chem.* **264**, 14840–14847
20. Maxwell, L. H., Harrison, G. S., Wood, W. M., and Maxwell, F. (1989) *BioTechniques* **7**, 276–280
21. Engelman, J. A., Chu, C., Lin, A., Jo, H., Ikezu, T., Okamoto, T., Kohtz, D. S., and Lisanti, M. P. (1998) *FEBS Lett.* **428**, 205–211
22. Pestell, R. G., Hollenberg, A., Albanese, C., and Jameson, J. L. (1994) *J. Biol. Chem.* **269**, 31090–31096
23. Singh, M., and Birshtein, B. K. (1993) *Mol. Cell. Biol.* **13**, 3611–3622
24. Singh, H., Sen, R., Baltimore, D., and Sharp, P. A. (1986) *Nature* **319**, 154–158
25. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419
26. Baldini, G., Hohl, T., Lin, H., and Lodish, H. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 5049–5052
27. Galbiati, F., Volonte, D., Engelman, J. A., Watanabe, G., Burk, R., Pestell, R., and Lisanti, M. P. (1998) *EMBO J.* **17**, 6633–6648
28. Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J. E., Hansen, S. H., Nishimoto, I., and Lisanti, M. P. (1995) *J. Biol. Chem.* **270**, 15693–15701
29. Scherer, P. E., Okamoto, T., Chun, M., Nishimoto, I., Lodish, H. F., and Lisanti, M. P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 131–135
30. Tang, Z.-L., Scherer, P. E., Okamoto, T., Song, K., Chu, C., Kohtz, D. S., Nishimoto, I., Lodish, H. F., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271**, 2255–2261
31. Tota, Y., Schwemcke, C., Couet, J., Lisanti, M. P., and Ishikawa, Y. (1998) *Endocrinology* **139**, 2025–2031
32. Razani, B., Rubin, C. S., and Lisanti, M. P. (1999) *J. Biol. Chem.* **274**, 26353–26360
33. Engelman, J. A., Zhang, X. L., Galbiati, F., Volonte, D., Setgja, F., Pestell, R. G., Minetti, C., Scherer, P. E., Okamoto, T., and Lisanti, M. P. (1998) *Am. J. Hum. Genet.* **63**, 1578–1587
34. Sager, R., Sheng, S., Anisowicz, A., Sotiropoulou, G., Zou, Z., Stenman, G., Swisshelm, K., Chen, Z., Hendrix, M. J. C., Pemberton, P., Rafidi, K., and Ryan, K. (1994) *Cold Spring Harbor Symp. Quant. Biol.* **LIX**, 537–546
35. Engelman, J. A., Zhang, X. L., Galbiati, F., and Lisanti, M. P. (1998) *FEBS Lett.* **439**, 310–316
36. Engelman, J. A., Zhang, X. L., and Lisanti, M. P. (1998) *FEBS Lett.* **436**, 403–410
37. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7686–7689