Caveolin-mediated regulation of signaling along the p42/44 MAP kinase cascade in vivo

A role for the caveolin-scaffolding domain

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Abstract The p42/44 mitogen-activated protein (MAP)-kinase cascade is a well-established signal transduction pathway that is initiated at the cell surface and terminates within the nucleus. More specifically, receptor tyrosine kinases can indirectly activate Raf, which in turn leads to activation of MEK and ERK and ultimately phosphorylation of Elk, a nuclear transcription factor. Recent reports have suggested that some members of p42/44 MAP kinase cascade can be sequestered within plasmalemmal caveolae in vivo. For example, morphological studies have directly shown that ERK-1/2 is concentrated in plasma membrane caveolae in vivo using immunoelectron microscopy. In addition, constitutive activation of the p42/44 MAP kinase cascade is sufficient to reversibly down-regulate caveolin-1 mRNA and protein expression. However, the functional relationship between the p42/44 MAP kinase cascade and caveolins remains unknown. Here, we examine the in vivo role of caveolins in regulating signaling along the MAP kinase cascade. We find that co-expression with caveolin 1 dramatically inhibits signaling from EGF-R, Raf, MEK-1 and ERK-2 to the nucleus. Using a variety of caveolin-1 deletion mutants, we mapped this in vivo inhibitory activity to caveolin-1 residues 32–95. Peptides derived from this region of caveolin 1 also inhibit the in vitro kinase activity of purified MEK-1 and ERK-2. Thus, we show here that caveolin-1 expression can inhibit signal transduction from the p42/44 MAP kinase cascade both in vitro and in vivo. Taken together with previous data, our results also suggest that a novel form of reciprocal negative regulation exists between p42/44 MAP kinase activation and caveolin-1 protein expression, i.e. up-regulation of caveolin-1 protein expression down-modulates p42/44 MAP kinase activity (this report) and up-regulation of p42/44 MAP kinase activity down-regulates caveolin-1 mRNA and protein expression.

Key words: Caveolin; p42/44 mitogen-activated protein kinase cascade; Protein phosphorylation; Kinase activity; Reciprocal regulation

1. Introduction

Caveolae are 50–100-nm vesicular invaginations of the plasma membrane [1,2]. They represent a sub-compartment of the plasma membrane. It has been proposed that caveolae participate in vesicular trafficking events and signal transduction processes. Caveolae are most abundant in terminally differentiated cells, such as adipocytes, endothelial cells and muscle cells [3–10]. Conversely, caveolae are down-regulated in response to activated oncogenes, such as v-Abl and H-Ras [11,12].

Caveolin, a 21–24-kDa integral membrane protein, is a principal component of caveolae membranes in vivo. [13–17]. Caveolin is only the first member of a new gene family; as a consequence, caveolin has been re-termed caveolin 1 [5]. Caveolin 2 shows the same tissue distribution as caveolin 1, co-localizes with caveolin 1 and forms a hetero-oligomeric complex with caveolin 1 in vivo [18]. In contrast, caveolin 3 is a muscle specific caveolin-related protein that is only expressed in striated muscle cell types (cardiac and skeletal) [19–21].

It has been proposed that caveolin family members function as scaffolding proteins [22] to organize and concentrate specific lipids (cholesterol and glycosphingolipids; [23–25]) and lipid-modified signaling molecules (Src-like kinases, H-Ras, eNOS and G-proteins; [23,26–30]) within caveolae membranes. These and other signaling molecules appear to be tightly associated as a discrete complex with caveolin 1 as shown using a poly-histidine tagged form of caveolin for detergent-free affinity purification of caveolae membranes [27].

Based on these and other observations, we and others 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 [31–36]. In support of this idea, caveolin-1 binding can functionally suppress the GTPase activity of hetero-trimeric G-proteins and inhibit the kinase activity of Src-family tyrosine kinases, the EGF-receptor kinase, and protein kinase C through a common caveolin domain, termed the caveolin-scaffolding domain [26–28,37–39]. 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 [37,40].

Recently, caveolae have also been implicated in signaling through the p42/44 MAP kinase pathway. Evidence has been presented suggesting that receptor tyrosine kinases (EGF-R; PDGF-R; Ins-R) [41–45], as well as other compo-

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nents of the p42/44 MAP kinase cascade are localized within caveolae membranes. These include H-Ras [27,42], Raf kinase [42], 14-3-3 proteins [43], ERK [33,43], Shc [43], Grb-2 [43], mSos-1 [43], and Nck [43]. In addition, morphological studies have directly shown that ERK-1/2 is concentrated in plasma membrane caveolae in vivo using immunoelectron microscopy [44]. The functional significance of the caveolar localization of the p42/44 MAP kinase cascade remains unknown.

Here, we present evidence that caveolin can regulate signaling via the p42/44 MAP kinase cascade. We show that co-expression with caveolin 1 dramatically inhibits signaling from EGF-R, Raf, MEK-1 and ERK-2 to the nucleus in vivo. In addition, we localize this caveolin-dependent inhibitory activity to caveolin-1 residues 32–95 using an in vivo assay and show that peptides from this region of caveolin 1 directly inhibit the kinase activities of purified MEK-1 and ERK-2 in vitro. Taken together with previous data, our results also suggest that a novel form of reciprocal negative regulation exists between p42/44 MAP kinase activation and caveolin-1 protein expression, i.e. up-regulation of caveolin-1 protein expression down-modulates p42/44 MAP kinase activity (this report) and up-regulation of p42/44 MAP kinase activity down-regulates caveolin-1 mRNA and protein expression [12].

2. Materials and methods

2.1. Materials

The cDNAs encoding caveolins 1, 2, and 3, as well as a panel of caveolin-1 deletion mutants, were as we described previously [5,20,31]. Antibodies and their sources were as follows: anti-EGFR (mAb 13; Transduction Laboratories); anti-myc (mAb 9E10; Santa Cruz Biotech). A variety of other reagents were purchased commercially: fetal bovine serum (FBS; JRH Biosciences); pre-stained protein markers (Gibco-BRL). The PathDetect Elk-1 in vivo trans-reporting system was purchased from Stratagene. CHO cells (GRC^+ LR-73) were the generous gift of Dr. Jeffrey Pollard and were as described previously [46]. Constructs encoding EGF-R (wt); EGF-R (kd, K721M); Raf-1 (ca, BXB); Raf-1 (kd, K375W); HA-ERK-2; and MEK-1 (ca, S218/222E-Δ32-51), were as previously described [47–49]. MEK (PD 98059) and p38 MAP kinase (SB203580) inhibitors were purchased from Calbiochem., Inc.

2.2. Cell culture

CHO cells were propagated in t75 tissue-culture flasks in DME supplemented with antibiotics and 10% serum, as described previously [46].

2.3. Assay for in vivo signal transduction

To measure in vivo signal transduction, we employed the PathDetect Elk *trans*-Reporting System (Stratagene, Inc). This assay employs a fusion protein that contains the DNA binding domain of GAL4 and the transactivation domain of Elk to induce expression of a luciferase reporter driven by an artificial promoter containing five GAL4 binding sites. When MAPK (p42/44 ERK-1/2) is specifically activated, it phosphorylates the transactivation domain of Elk which in turn activates transcription of the luciferase gene from the reporter plasmid. Experiments testing a plasmid encoding only the GAL4 DNA binding domain demonstrated that luciferase expression is specifically dependent on activation of the Elk transactivation domain (data not shown). Transient transfections were performed using calcium phosphate precipitation. Briefly, 300 000 CHO cells were seeded in 6-well plates 12–24 h before the transfection. Each point was transfected with 1 μg of a plasmid encoding a form of either EGF-R, Raf-1, ERK-2 or MEK-2, 1 μg of pFR-Luc, 50 ng of pFA-Elk (as described by the manufacturer, Stratagene, Inc.) and 1 μg of the indicated caveolin or empty vector. Twelve hours post-transfection, the cells were rinsed twice with PBS and incubated in 1% FBS for another 24–36 h. The cells were then lysed in 200 μl of extraction buffer, 50 μl of which was used to measure luciferase activity, as described [50]. Each experimental value represented graphically is the average of two separate transfections performed in parallel; error bars represent the observed standard deviation. All experiments were performed at least three times independently and yielded virtually identical results. These assays were made possible through the use of a special CHO-derived cell line, called GRC^+ LR-73. Unlike parental CHO cells, GRC^+ LR-73 cells are a non-transformed growth control revertant that has 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 [46]. Also, these cells have a much higher transfection efficiency (~10-fold) than parental CHO cells.

2.4. Immunoblotting

Samples were separated by SDS-PAGE 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), except that we supplemented our blocking solution with both 1% BSA and 2% non-fat dry milk (Carnation).

2.5. Purification of recombinant MEK-1, ERK-2 and Elk-1

The cDNA clones for rabbit MEK1 and constitutively active MEK1 (MEKmu, S217E/S221E; [51]) were ligated into pGEX-3X [52] and expressed in *E. coli* strain BL-21 as described by Catling et al. [53]. Cells were suspended in buffer (20 mM PIPES, 100 mM NaCl, 0.1% Triton X-100, 5% glycerol, 10 μg/ml aprotinin, 10 μg/ml bacitracin, 1 mM benzamidine, and 20 μg/ml leupeptin), sonicated, and lysates were clarified by centrifugation. Recombinant MEK1 was isolated by glutathione affinity chromatography in 20 mM PIPES, 100 mM NaCl. Mouse ERK2 and inactive ERK2 (ERKmu, K52R; [54]) were ligated into pMal-CR1 (New England Biolabs) and expressed in *E. coli* strain TB-1. Cells were sonicated in sonication buffer, and recombinant ERK2 was isolated by amylose resin chromatography in 20 mM PIPES, 100 mM NaCl. Active recombinant ERK2 was prepared by incubating recombinant ERK2 bound to amylose resin with purified recombinant MEKmu in kinase buffer (20 mM PIPES, 100 mM NaCl, 20 mM $MgCl_2$, and 1 mM ATP). Amylose-bound activated ERK2 was washed with high salt buffer (20 mM PIPES, 500 mM NaCl) to remove MEKmu, and eluted with 5 mM maltose. Residues 307–428 of Elk-1 were expressed as a fusion protein from pGEX-30X [55] and isolated by glutathione affinity chromatography in 20 mM PIPES, 100 mM NaCl. Note that GST-MEK-1 constructs were obtained from Dr. C. Marshall (Chester Beatty Laboratories, Institute of Cancer Research, University of London) through Dr. R. Krauss (Mount Sinai School of Medicine); MBP-ERK2 constructs were donated by Dr. M. Weber (University of Virginia); and the GST-ElkC construct was provided by Dr. R. Treisman (Transcription Laboratory, Imperial Cancer Research Fund).

2.6. In vitro kinase assays

Kinase reactions were performed in 20 mM PIPES, 100 mM NaCl, 50 μM ATP, 20 mM $MgCl_2$, and 1 μCi [γ-^{32}P]-ATP. Reactions typically contained 0.5 μg of enzyme and 1 μg of substrate in a final volume of 20 μl. Kinase reactions were allowed to proceed for 5 min at room temperature, and stopped by the addition of EDTA to 50 mM final concentration. Samples were resolved by SDS-PAGE and detected by autoradiography. Quantitation was performed by phosphor-imager analysis.

3. Results

3.1. Recombinant expression of caveolin 1 suppresses EGF-R mediated signal transduction in vivo

EGF-R is thought to signal through the activation of the p42/44 MAP kinase cascade. It is well known that transient over-expression of EGF-R is sufficient to cause receptor dimerization and signal transduction. Thus, we employed a MAP kinase reporter system (from Stratagene, Inc.) to measure EGF-R mediated signal transduction in vivo. This assay employs a fusion protein that contains the DNA binding domain of GAL4 and the transactivation domain of Elk to induce expression of a luciferase reporter driven by an artifi-

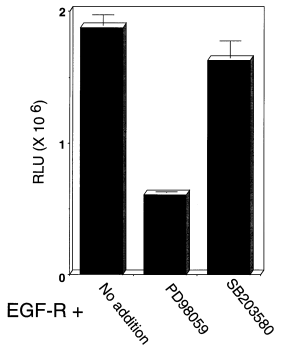

Fig. 1. Overexpression of EGF-R activates the p42/44 MAP kinase cascade, but not the p38 MAP kinase cascade. Transient co-expression of EGF-R with a luciferase reporter plasmid allows us to measure the transactivation of the transcription factor Elk. Transactivation of Elk can occur via phosphorylation by either the p42/44 MAP kinase (ERK) or via phosphorylation by p38 MAP kinases. To establish which MAP kinase pathway is activated by EGF-R overexpression, we used two well-characterized inhibitors: PD 98059 (100 μM) and SB 203580 (20 μM). Note that treatment with PD 98059 inhibited EGF-R mediated signaling to Elk by ~65-70%, while SB 203580 had little or no effect. These results indicate that EGF-R mediated transcriptional activation of Elk occurs primarily through the classical p42/44 MAP kinase cascade. RLU, relative light units. Each experimental value represented graphically is the average of two separate transfections performed in parallel; error bars represent the observed standard deviation. These experiments were performed at least three times independently and yielded virtually identical results.

cial promoter containing five GAL4 binding sites. When MAPK (p42/44 ERK-1/2) is specifically activated, it phosphorylates the transactivation domain of Elk which in turn activates transcription of the luciferase gene from the reporter plasmid. Experiments using a plasmid encoding only the GAL4 DNA binding domain demonstrated that luciferase expression is specifically dependent on activation of the Elk transactivation domain (data not shown).

These assays were made possible through the use of a special CHO-derived cell line, called GRC$^+$ LR-73. Unlike parental CHO cells, GRC$^+$ LR-73 cells are a non-transformed growth control revertant that has 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 [46]. Also, these cells have a much higher transfection efficiency (~10-fold) than parental CHO cells.

Transactivation of Elk can occur via phosphorylation by either the p42/44 MAP kinase (ERK) or via phosphorylation by p38 MAP kinases. To establish which MAP kinase pathway is activated by EGF-R over-expression, we used two well-characterized inhibitors. PD 98059 is a specific inhibitor of MEK-1/2 that is just upstream of ERK and SB 203580 is a specific inhibitor of p38 MAP kinases. Fig. 1 shows that treatment with PD 98059 inhibited EGF-R mediated signaling to Elk by ~65–70%, while SB 203580 had little or no effect. These results indicate that EGF-R mediated transcriptional activation of Elk occurs primarily through the classical p42/44 MAP kinase cascade.

We next used this assay system to test the hypothesis that caveolin-1 expression inhibits EGF-R mediated signaling in vivo. Although these cells endogenously express low levels of both caveolins 1 and 2, much higher levels of caveolin expression were achieved by transient expression of recombinant caveolins using constitutive expression driven by CMV-based vectors. As shown in Fig. 2A, co-expression with caveolin 1 dramatically inhibited EGF-R-mediated signal transduction by approximately 20-fold. In contrast, co-transfection with β-galactosidase or with the empty vector used to express caveolin 1 (pCB7) had no effect on EGF-R mediated signaling. As expected, a kinase dead (kd) form of EGF-R did not show any signaling capacity in this assay system.

To rule out the possibility that co-expression with caveolin 1 would block or reduce expression of EGF-R, cell lysates were prepared after co-transfection and the levels of EGF-R expression were assessed by immunoblot analysis. Fig. 2B shows that EGF-R expression is unaffected by co-transfection

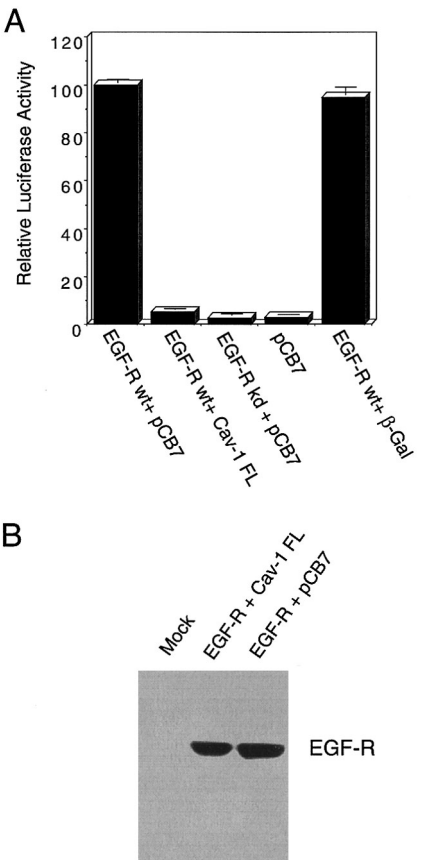

Fig. 2. Caveolin-1 expression blocks EGF-R mediated signal transduction to the nucleus in vivo. A: Transient co-expression of EGF-R with a luciferase reporter plasmid allowed us to measure the transactivation of the transcription factor Elk that is a substrate for MAP kinases (see Section 2). Note that co-expression with caveolin 1 dramatically inhibited EGF-R-mediated signal transduction evoked by overexpression of wild-type (wt) EGF-R by approximately ~10–20-fold. Importantly, co-transfection with the empty vector used to express caveolin 1 (pCB7) or with β-galactosidase had no effect on EGF-R mediated signaling. The activity of kinase dead (kd) EGF-R was evaluated in parallel as a negative control. Each experimental value represented graphically is the average of two separate transfections performed in parallel; error bars represent the observed standard deviation. These experiments were performed at least three times independently and yielded virtually identical results. B: Co-expression with caveolin 1 does not affect the expression levels of transfected EGF-R. CHO cells were transiently transfected with EGF-R plus caveolin 1, EGF-R plus empty vector (pCB7), or mock-transfected. Lysates were prepared and subjected to immunoblot analysis with an anti-EGF-R specific mAb probe. Note that EGF-R expression is unaffected by co-transfection with caveolin 1.

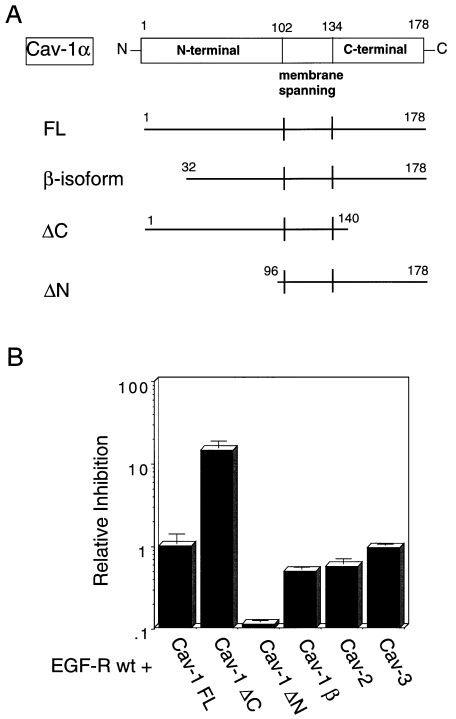

Fig. 3. Defining a region of caveolin 1 that blocks EGF-R mediated signal transduction in vivo. A: Diagram summarizing wild-type caveolin 1 and a series of caveolin-1 deletion mutants. Note that the full-length caveolin-1 molecule contains residues 1–178 (α-isoform), the β-isoform lacks residues 1–31, ΔC lacks residues 141–178 of the C-terminal domain, and ΔN lacks residues 1–95 of the N-terminal domain. B: Effects of transiently expressing caveolin-1 deletion mutants and caveolins 2 and 3 on EGF-R-mediated signal transduction. Differences in inhibitory activity were best visualized by representing the data on a log scale. The actual average relative light units (RLU) obtained in this experiment were: 3 658 423 – EGFR+-vector alone (not displayed); 273 506 – EGFR+caveolin 1 FL; 18 503 – EGFR+caveolin 1 ΔC; 2 506 019 – EFGR+caveolin 1 ΔN; 558 207 – EFGR+caveolin-1 β-isoform; 487 431 – EGFR+caveolin 2; 287 238 – EGFR+caveolin 3. Each experimental value represented graphically is the average of two separate transfections performed in parallel; error bars represent the observed standard deviation. These experiments were performed at least three times independently and yielded virtually identical results.

with caveolin 1, as compared to co-transfection with the empty pCB7 vector. This result also underscores that the transfection efficiencies were quite comparable. In addition, all luciferase assay experiments were repeated several times independently and invariably yielded similar results.

3.2. Deletion mutagenesis defines a region of caveolin 1 that inhibits EGF-R-mediated signal transduction

We next employed this assay system and a variety of caveolin-1 deletion mutants to map this caveolin-1 dependent inhibitory activity to a given region of the caveolin-1 molecule. The caveolin-1 deletion mutants that were used are shown schematically in Fig. 3A. Note that the full-length caveolin-1 molecule contains residues 1–178 (α-isoform), the β-isoform lacks residues 1–31, ΔC lacks residues 141–178 of the C-terminal domain, and ΔN lacks residues 1–95 of the N-terminal domain. These constructs have all been previously characterized and are expressed to equivalent levels in transfected cells [56].

Fig. 3B shows the results of a typical experiment analyzing these different caveolin mutants. The graph is depicted on a log scale so one can best appreciate the true differences in inhibitory activity displayed by these mutants. Values are represented as inhibition relative to Caveolin-1 FL. Relative to wild-type full-length caveolin 1 (FL; α-isoform), ΔC was ~10–20-fold more potent, and ΔN was ~10-fold less potent; the β-isoform was almost as potent as wild-type full-length caveolin 1. In addition, full-length caveolins 1, 2, and 3 all showed similar inhibitory activities. From this mutational analysis, we can conclude that the in vivo inhibitory activity of caveolin 1 is contained within the N-terminal domain, and to a first approximation, within caveolin-1 residues 32–95.

3.3. Recombinant expression of caveolin 1 suppresses activation of the MAP kinase cascade in vivo

We next tested the effects of caveolin co-expression on the in vivo activities of Raf, MEK and ERK kinases. Figs. 4 and 5 show the results of these experiments. In the case of Raf, co-expression with caveolin 1 dramatically inhibited signaling mediated by a constitutively active (ca) form of Raf-1 kinase by ~75%. In contrast, co-expression with β-galactosidase had no effect. In addition, a kinase dead (kd; K375W) form of Raf-1 did not show any signaling in this assay system as expected (Fig. 4A).

Similar results were obtained for MEK and ERK. Caveolin-1 co-expression inhibited signaling mediated by a constitutively active form of MEK-1 (Δ32-51, S 218/222 E) by ~20-

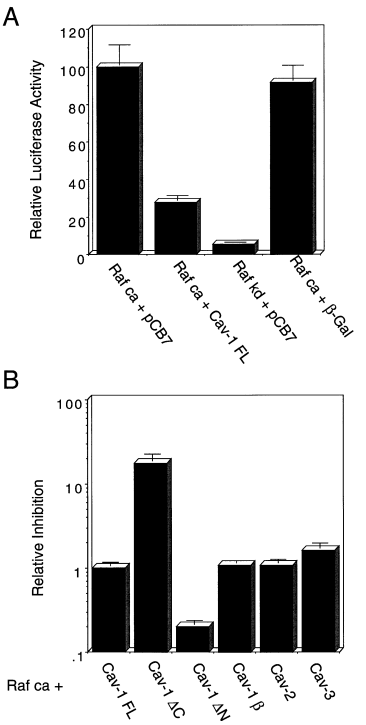

Fig. 4. Overexpression of caveolin 1 blocks Raf-mediated signal transduction in vivo. A: Effects of full-length caveolin 1 on Raf-mediated signaling. Note that co-expression with empty vector (pCB7) or β-galactosidase has no effect on signaling from constitutively active (ca) Raf. The activity of kinase dead (kd) Raf was evaluated in parallel as a negative control. B: Effects of caveolin-1 deletion mutants, caveolin 2, and caveolin 3 on Raf-mediated signaling. Transactivation of Elk was measured as luciferase activity as described in the legend of Fig. 1. These experiments were performed at least three times independently and yielded virtually identical results.

fold, while caveolin-1 co-expression inhibited signaling mediated by ERK-2 by ~10-fold in the same assay system (Fig. 5A,B).

The in vivo effects of caveolin-1 deletion mutants, caveolin 2 and caveolin 3 on the activities of Raf, MEK and ERK were next evaluated (Figs. 4B and 5C,D). Relative to wild-type full-length caveolin 1 (FL; α-isoform), ΔC was ~10–30-fold more potent, and ΔN was ~4–10-fold less potent; the β-isoform was almost as potent as wild-type full-length caveolin 1. Also, full-length caveolins 1, 2, and 3 all showed similar inhibitory activities toward Raf-1, MEK-1 and ERK-2.

This mutational analysis implicates caveolin-1 residues 32–95 in the inhibition of in vivo signaling by Raf-1, MEK-1 and ERK-2. Interestingly, the caveolin-1 scaffolding domain (residues 82–101) lies within this inhibitory region defined by in vivo mutational analysis, suggesting that this in vivo inhibitory activity may be due to direct interactions of MEK and ERK with the caveolin-1 scaffolding domain.

3.4. *Caveolin-derived peptides directly inhibit the kinase activity of MEK-1 and ERK-2 in vitro*

As our above data suggested that caveolin 1 may interact directly with MEK-1 or ERK-2 in vivo, we decided to test this hypothesis by reconstituting individual steps within the MAP-kinase cascade in vitro using purified recombinant components (MEK-1, ERK-2 and Elk) and synthetic peptides (derived from caveolins 1, 2, and 3).

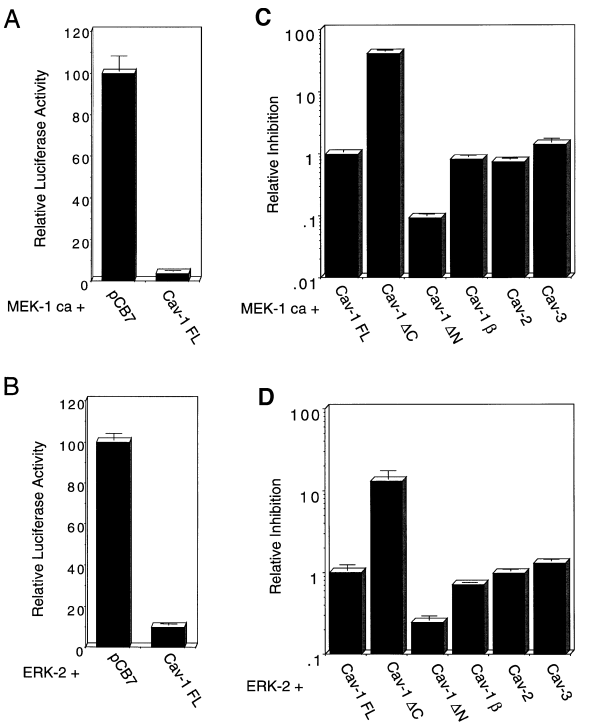

Fig. 5. Overexpression of caveolin 1 blocks MEK and ERK-mediated signal transduction in vivo. A: Effects of full-length caveolin 1 on MEK1-mediated signaling. B: Effects of full-length caveolin 1 on ERK2-mediated signaling. C: Effects of caveolin-1 deletion mutants, caveolin 2, and caveolin 3 on MEK1-mediated signaling. D: Effects of caveolin-1 deletion mutants, caveolin 2, and caveolin 3 on ERK2-mediated signaling. Transactivation of Elk was measured as luciferase activity as described in the legend of Fig. 1. These experiments were performed at least three times independently and yielded virtually identical results.

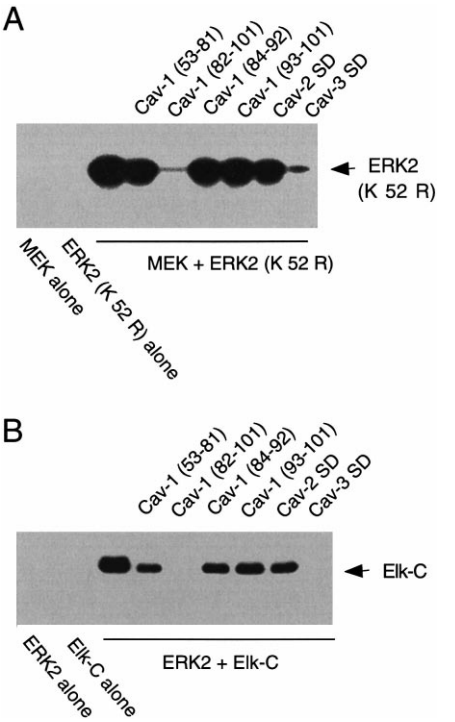

Fig. 6. Caveolin-derived peptides directly inhibit the kinase activity of MEK-1 and ERK-2 in vitro. A: The activity of purified MEK-1 was assessed using purified kinase dead ERK-2 (K52R) as the substrate. B: The activity of purified ERK-2 was assessed using purified Elk C-terminus as the substrate. Note that only peptides corresponding to the scaffolding domains of caveolin 1 (residues 82–101) and caveolin 3 demonstrated inhibitory activity towards MEK-1 and ERK-2 in vitro. Other caveolin-derived peptides employed showed no inhibitory activity. SD, scaffolding domain. All peptides were added to a final concentration of 10 μM. These experiments were performed at least three times independently and yielded virtually identical results.

First, the potential inhibitory activities of caveolin-1 derived peptides were evaluated using an assay that measures the kinase activity of purified recombinant MEK-1. In this assay system, purified recombinant kinase dead ERK-2 (K52R) was used as the phospho-acceptor substrate for MEK-1 (Fig. 6A). Only the caveolin-1 peptide encoding caveolin-1 residues 82–101 showed significant inhibitory activity, while a peptide encoding an adjacent region of caveolin 1 (residues 53–81) had no effect at the same concentration. If the peptide encoding caveolin-1 residues 82–101 was divided into two shorter peptides (residues 84–92 and 93–101), its inhibitory activity was abolished.

As residues 82–101 correspond to the scaffolding domain in caveolin 1, we assessed the effects of analogous peptides encoding the scaffolding domains of caveolins 2 and 3. Note that only the scaffolding domain of caveolins 1 and 3 showed inhibitory activity; the scaffolding domain of caveolin 2 had no effect. This is consistent with previous reports demonstrating that only peptides encoding the scaffolding domains of caveolins 1 and 3 possess inhibitory activity toward G-proteins, Src-like kinases, eNOS, PKC and EGF-R; the peptide encoding caveolin 2 has been shown not to be an inhibitor of these signaling molecules [28,37–39,57].

Next, the potential inhibitory activities of caveolin-1 derived peptides were evaluated using an assay that measures the kinase activity of purified recombinant ERK-2 (Fig. 6B).

In this assay system, purified recombinant Elk-C-terminal domain was used as the phospho-acceptor substrate for ERK-2. Using this in vitro assay, virtually identical results were obtained for ERK-2 as with MEK-1.

In these in vitro kinase assays, the caveolin-1 scaffolding domain inhibited MEK-1 and ERK-2 with similar potencies in the micromolar range (IC_{50} (MEK-1) $\sim$1 μM; IC_{50} (ERK-2) $\sim$2.5 μM) (data not shown). These results suggest that the scaffolding domain of caveolin 1 recognizes MEK and ERK in a similar manner. These values correspond well to those observed previously for inhibition of other kinases involved in signal transduction [28,38,39].

4. Discussion

Many distinct classes of signaling molecules are required to interact sequentially to activate the p42/44 MAP kinase cascade. These include the peptide ligand, the corresponding receptor tyrosine kinase, adaptor molecules (Grb2, SHC, and 14-3-3), Raf-1, MEK-1/2 and ERK-1/2. Several independent laboratories have now shown that these components are concentrated within caveolae membranes, using both in vitro biochemical methods and in vivo immunolabeling techniques [27,41–45]. For example, morphological studies have directly shown that ERK-1/2 is concentrated in plasma membrane caveolae in vivo using immunoelectron microscopy [44]. However, the functional significance of the caveolar localization of the p42/44 MAP kinase cascade remains unknown.

Here, we have examined if a functional relationship exists between caveolins and components of the p42/44 MAP kinase cascade in vivo. Co-expression with caveolin 1 dramatically inhibited signaling from EGF-R, Raf, MEK-1 and ERK-2 to the nucleus, as measured using an assay that reflects the transcriptional activation of Elk, a direct substrate of ERK-1/2. Using this in vivo assay and a panel of caveolin-1 deletion mutants, we mapped this caveolin-1-mediated inhibitory activity to the N-terminal domain of caveolin, within residues 32–95. Peptides derived from this region of caveolin also inhibit the in vitro kinase activity of purified MEK-1 and ERK-2. Our data directly support the hypothesis that caveolar localization of MEK and ERK can negatively regulate their signal transduction to the nucleus.

We observed here that co-expression of caveolins with either EGF-R, Raf-1, MEK-1, or ERK-2 blocked their ability to stimulate the transactivation of Elk in vivo. One possibility is that caveolins may inhibit multiple kinases or 'nodes' along this cascade. Alternatively, caveolins might only inhibit ERK-2 (the last kinase in the cascade) and still block EGF-R, Raf-1, and MEK-1 induced transactivation of Elk. We favor the former possibility, as we have previously shown that caveolin 1 can form a stable hetero-oligomeric complex with EGF-R as revealed by a series of co-immunoprecipiation studies, and caveolin-derived peptides will directly inhibit the kinase activity of the purified recombinant EGF-R tyrosine kinase in vitro [38]. In addition, we show here through a series of complementary in vivo and in vitro domain-mapping studies that these studies both implicate the same domain of caveolin in the in vivo and in vitro inhibition of MEK-1 and ERK-2.

How do caveolins negatively regulate these signaling events? Recent evidence suggests that caveolins function as kinase inhibitors by recognizing a conserved caveolin-binding motif that is present within the catalytic domain of many known kinases. Since the identification of the caveolin-scaffolding domain and caveolin-binding sequence motifs these observations have been extended to other caveolin-interacting proteins. Functional caveolin-binding motifs have been deduced in both tyrosine and serine/threonine kinases, as well as in eNOS [28,37–39,57]. In all cases examined, the caveolin binding motif is located within the enzymatically active 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) [38,39]. The caveolin binding motif is located within conserved kinase subdomain number IX, suggesting that caveolin could function as a general kinase inhibitor [38,39]. We suspect that caveolin inhibits signal transduction from MEK and ERK by a similar mechanism. This hypothesis is supported by the observation that the caveolin scaffolding domain inhibits EGF-R, PKC, MEK and ERK with similar potency. Thus, peptide mimetics based on the caveolin-scaffolding domain could be used potentially as anti-tumor agents.

Interestingly, we find that although co-expression of caveolin 2 with EGF-R, Raf-1, MEK-1 or ERK-2 blocks transcriptional activation of Elk in vivo, a peptide derived from the caveolin-2 scaffolding domain did not block the activity of MEK or ERK in vitro. One possible explanation for this difference between in vivo and in vitro results is that there may be another region within caveolin 2, other than the scaffolding domain, that contains inhibitory activity. In support of this idea, recent experimental evidence suggests that caveolins may also contain a second inhibitory domain within the C-terminus [58]. Alternatively, as caveolins 1 and 2 can form a stable hetero-oligomeric complex in vivo [18], the in vivo effect of caveolin 2 may be mediated by the caveolin-1/-2 hetero-oligomer.

In a recent report, Anderson and colleagues have shown that the pool of ERK-1 that localizes to caveolae is initially inactive and can be activated by stimulation with growth factor ligands, such as PDGF [44]. After such stimulation, activated ERK-1 translocates to the cytosol, suggesting that ERK-1 is activated as it leaves the caveolar membrane [44]. These results are consistent with our current findings that caveolin-1 expression and direct interaction with caveolin-1 peptides can block signaling from ERK-2 both in vivo and in vitro, perhaps by sequestering ERK-1/2 and other components of the p42/44 MAP kinase cascade in the inactive conformation – poised for activation by the appropriate cell surface stimulus.

Our results also suggest that a novel reciprocal relationship may exist between caveolin-1 protein expression and activation of the p42/44 MAP kinase pathway. We show here that caveolin 1 can inhibit signal transduction from the p42/44 MAP kinase cascade both in vitro and in vivo. Conversely, we have previously shown that transformation of NIH 3T3 cells with activated H-Ras (G12V) results in down-regulation of caveolin-1 protein expression; such down-regulation is reversed by treatment of these cells with the MEK inhibitor, PD 98059 [12]. Thus, it appears that constitutive activation of the p42/44 MAP kinase cascade is sufficient to down-regulate caveolin-1 mRNA and protein expression.

Recently, we have identified a family with an autosomal dominant form of limb girdle muscular dystrophy. In this family, a critical region of the caveolin-scaffolding domain is deleted within caveolin 3 [59]. Caveolin 3 with this deletion

acts as a dominant-negative to reduce the total amount of skeletal muscle caveolin 3 by ~95% and results in the classical symptoms associated with muscular dystrophies [59]. This finding provides the first genetic evidence that the caveolin-scaffolding domain is critical for caveolin functioning in vivo.

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