Interaction of a Receptor Tyrosine Kinase, EGF-R, with Caveolins

CAVEOLIN BINDING NEGATIVELY REGULATES TYROSINE AND SERINE/THREONINE KINASE ACTIVITIES

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Caveolin, a 21–24-kDa integral membrane protein, is a principal component of caveolae membranes. We and others have suggested that caveolin functions as a scaffolding protein to organize and concentrate certain caveolin-interacting signaling molecules within caveolae membranes. In this regard, it has been shown that a 20-amino acid membrane-proximal region of the cytosolic NH2-terminal domain of caveolin is sufficient to mediate the interaction of caveolin with signaling proteins, namely G-proteins, Src-like kinases, eNOS, and H-Ras. This caveolin-derived protein domain has been termed the caveolin-scaffolding domain. Binding of the caveolin-scaffolding domain functionally suppresses the activity of G-protein α subunits, eNOS, and Src-like kinases, suggesting that caveolin binding may also play a negative regulatory role in signal transduction.

Here, we report the direct interaction of caveolin with a growth factor receptor, EGF-R, a known caveolin-associated receptor tyrosine kinase. Two consensus caveolin binding motifs have been previously defined using phage display technology. One of these motifs is present within the conserved kinase domains of most known receptor tyrosine kinases (termed region IX). We now show that this caveolin binding motif within the kinase domain of the EGF-R can mediate the interaction of the EGF-R with the scaffolding domains of caveolins 1 and 3 but not with caveolin 2. In addition, the scaffolding domains of caveolins 1 and 3 both functionally inhibit the autophosphorylation of the EGF-R kinase in vitro. Importantly, this caveolin-mediated inhibition of the EGF-R kinase could be prevented by the addition of an EGF-R-derived peptide that (i) contains a well-conserved caveolin binding motif and (ii) is located within the kinase domain of the EGF-R and most known receptor tyrosine kinases. Similar results were obtained with protein kinase C, a serine/threonine kinase, suggesting that caveolin may function as a general kinase inhibitor. The implications of our results are discussed within the context of caveolae-mediated signal transduction. In this regard, caveolae-coupled signaling might explain how linear signaling pathways can branch and interconnect extendively, forming a signaling module or network.

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Caveolae are vesicular organelles that represent a subdivision of the plasma membrane. They are most abundant in terminally differentiated cell types, i.e. adipocytes, endothelial cells, and muscle cells (skeletal, cardiac, and smooth), although they are found in most cells (1–3). It has been suggested that caveolae may function as subcellular compartments to (i) store inactive signaling molecules for regulated activation and (ii) to facilitate cross-talk between distinct signaling cascades (1–4).

Caveolin, a 21–24-kDa integral membrane protein, is a major structural component of caveolae. Several independent lines of evidence indicate that caveolin may function as a scaffolding protein within caveolae membranes. (i) Both the NH2-terminal and COOH-terminal domains of caveolin face the cytoplasm, allowing them to freely interact with cytosolic molecules (5–7). (ii) Caveolin undergoes two stages of oligomerization. First, caveolin monomers assemble into discrete multivalent oligomers containing ~14–16 monomers per oligomer (8, 9). Subsequently, these individual caveolin homo-oligomers (4–6-nm particles) can interact with each other to form caveolae-like structures in vitro (25–50-nm clusters) (8). (iii) Interaction of caveolin with purified heterotrimeric G-proteins and c-Src functionally suppresses their enzymatic activity, holding these proteins in an inactive conformation (10, 11). Thus, caveolin may organize the formation of caveolae microdomains and regulate caveolae-related signaling events.

Using a variety of domain mapping approaches (deletion mutagenesis, GST fusion proteins, synthetic peptides), a region within caveolin has been defined that mediates the interaction of caveolin with itself and other proteins. This cytoplasmic 41-amino acid membrane-proximal region of caveolin is sufficient to mediate the formation of caveolin homo-oligomers (8), and the carboxyl-terminal half of this region (20 amino acids, residues 82–101) mediates the interaction of caveolin with G-protein α subunits and Src-family tyrosine kinases (10, 11). This caveolin region preferentially recognizes the inactive conformation of these molecules, as mutationally activated Gα subunits (Gαs, Q227L), v-Src, and H-Ras (G12V) fail to interact with caveolin (10–12). As this caveolin domain (residues 82–101) is critical for caveolin homo-oligomerization and the interaction of caveolin with certain caveolae-associated proteins (G-proteins, H-Ras, eNOS, and Src-family kinases), we have previously termed this protein domain the caveolin scaffolding domain (11, 13, 14).

What is the molecular basis of these interactions? Perhaps the caveolin scaffolding domain recognizes a common sequence motif within caveolin binding signaling molecules. To investigate this possibility, we have used the caveolin scaffolding domain as a receptor to select caveolin binding peptide ligands from random peptide sequences displayed at the surface of bacteriophage (phage display libraries). Two related caveolin binding motifs (ΦΦΟXXXΦΦ and ΦXXXΦΦΟΦ, Φ being aro-
Caveolin binding motifs are present within most Gₛ subunits and within the kinase domains of many distinct classes of protein kinases: serine-threonine kinases (PKCa),¹ dual specificity kinases (mitogen-activated protein kinase), nonreceptor tyrosine kinases (Src-family kinases), and receptor tyrosine kinases (EGF-R, Ins-R, PDGF-R). With the exception of Src-family kinases, however, it remains unknown whether caveolin interacts directly with these kinases or can functionally regulate their activity.

Here, we present evidence that the EGF-R kinase interacts directly with the caveolin scaffolding domain via a conserved caveolin binding motif (WXXWLVW; SYGVTWV) that is localized within the kinase domain of most receptor tyrosine kinases. We also show that caveolin binding can functionally regulate the kinase activity of EGF-R in vitro. Thus, EGF-R is the first signaling receptor to be shown to interact directly with caveolin. In support of our current observations, many receptor tyrosine kinases as well as their signaling cascades have been previously localized to caveolae or caveola-related domains, including EGF-R, PDGF-R, fibroblast growth factor receptor, Ins-R, and nerve growth factor receptor (trk) (16–20).

**EXPERIMENTAL PROCEDURES**

**Materials—**Antibodies and their sources were as follows: anti-caveolin 1 polyclonal Ig (gifts of Dr. John R. Glenney, Transduction Laboratories); anti-EGF-R, anti-activated-EGF-R (recognizes only the autophosphorylated form of EGF-R; gifts of Dr. John R. Glenney, Transduction Laboratories); anti-EGF-R, antiphosphotyrosine antibodies (4G10, 2E7), anti-EGF-R mAb probe (1:1,000 dilution). Secondary IgGs (1:5,000 dilution, Amersham) were used to visualize material that was eluted with sample buffer. After SDS-PAGE and transfer to nitrocellulose, immunoblot analysis was performed with an anti-EGF-R mAb probe (1:1,000 dilution). Horseradish peroxidase-conjugated secondary IgGs (1:5,000 dilution, Amersham) were used to visualize bound primary antibodies by ECL (Amersham).

**Construction and Purification of GST-EGF-R Fusion Proteins—**The construction, expression, and purification of GST fusion proteins was as we described previously (7). Briefly, portions of the cytoplasmic tail of the EGF-R were amplified by polymerase chain reaction using specific primers containing engineered restriction sites and subcloned into the vector pGEX-4T-1. After expression in Escherichia coli (BL21 strain; Novagen, Inc.), GST-EGF-R fusion proteins were purified by affinity chromatography on glutathione-agarose beads (21). Fusion proteins were analyzed by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose. After transfer, nitrocellulose sheets were stained with Ponceau S to visualize protein bands and subjected to immunoblot analysis with anti-GST IgG (1:1,000) (Santa Cruz Biotechnologies). ELISAs—We used 96-well plates (Nunc Maxisorp) to perform all ELISAs. The ELISA were carried out essentially as described previously (15). Briefly, 500 pmol of peptide in 100 mM sodium bicarbonate, pH 8.0, was used per well for coating the plate for 16–20 h at 4 °C or for 2 h at room temperature. Plates were saturated for 1 h at room temperature with TBST (10 mM Tris, pH 8.0, 0.15 M NaCl, 0.05% Tween 20) containing 0.2% bovine serum albumin. Purified GST-EGF-R fusion proteins (200 ng/well) (22) or biotinylated peptides (100 pmol) in TBST were then added to the wells. After 2 h of incubation, the cells were washed (3–5 times) with TBST. Binding of GST fusion proteins was detected using an anti-GST antibody conjugated to horseradish peroxidase (diluted 1:1,000) followed by horseradish peroxidase-conjugated anti-mouse secondary antibody (diluted 1:2,000). Binding of biotinylated peptides was observed using horseradish peroxidase-conjugated streptavidin (diluted 1:2,000). The color reaction was initiated by the addition of ABTS (2,2’-azino-di(3-ethylbenzthiazoline-6-sulfonate)) (Boehringer Mannheim). Optical density was measured on a microplate reader at 410 nm.

Peptide Competition of GST Fusion Protein Binding—To the coated well (182–101) peptide-coated well, a mixture of the GST fusion protein (3 pmol) and increasing amounts of competing peptide were added. The mixture was assembled just before addition to the well. The procedure that followed was as described above. We also used biotinylated peptides in several experiments, and binding was revealed using horseradish peroxidase-conjugated streptavidin (diluted 1:2,000).

**In Vitro Phosphorylation of the EGF-R Kinase—**100 ng of purified recombinant EGF-R kinase (Stratagene) was incubated with caveolin peptides at concentrations of 0, 1, 3, and 10 μM in kinase reaction buffer. Reactions were performed in a total volume of 40 μl of kinase reaction buffer (20 mM Heps, pH 7.4, 40 mM MnCl₂, 200 μM Na₃VO₄). When caveolin or EGF-R peptides were included in the reaction, the mixture was pre-incubated for 1 h at 4 °C. The reaction was initiated by the addition of 10 μCi of [γ-32p]ATP (6,000 Ci/mmol). After incubation for 15 min at 25 °C, the reaction was halted by the addition of 2× SDS-PAGE sample buffer and boiling for 2 min. Phosphorylated proteins were visualized by autoradiography. Control samples omitting either [γ-32p]ATP or EGF-R showed no activity.

**Interaction of EGF-R with Caveolin—**A near confluent 150-mm dish of A431 cells was washed three times with phosphate-buffered saline, and incubated in lysis buffer (50 mM Heps, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% CHAPS, and protease inhibitors) for 30 min at 4 °C. Lysates were clarified by centrifugation at 15,000 × g for 15 min and pre-cleared by incubation with protein G-Sepharose for 6 h at 4 °C. After pre-clearing, supernatants were transferred to 1.5-ml microfuge tubes containing anti-caveolin or anti-EGF-R IgGs pre-bound to protein G-Sepharose. After incubation rotating overnight at 4 °C, immunoprecipitates were washed 3 × with lysis buffer and subjected to immunoblot analysis with an anti-EGF-R mAb probe.

**Interaction of Cellular EGF-R with a Biotinylated Caveolin 1 Peptide—**One ml of a pre-cleared A431 cell lysate was incubated overnight at 4 °C with streptavidin-agarose alone or with streptavidin-agarose containing a pre-bound biotinylated caveolin 1 peptide (residues 61–101). The beads were washed (6–8 times) with lysis buffer, and bound material eluted with sample buffer. After SDS-PAGE and transfer to nitrocellulose, immunoblot analysis was performed with an anti-EGF-R mAb probe (1:1,000 dilution). Horseradish peroxidase-conjugated secondary IgGs (1:5,000 dilution, Amersham) were used to visualize bound primary antibodies by ECL (Amersham).

**Cell Culture—**A431 cells and CHO cells were cultured in Dulbecco’s modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Cells were serum-starved for more than 6 h before performing experiments. Cells were washed twice with phosphate-buffered saline, and EGF treatment (50 ng/ml in Dulbecco’s modified Eagle’s medium) was performed for 5 min.

**Detergent-free Purification of Caveolin-enriched Membrane Fractions—**Caveolin-enriched membrane fractions were prepared as described previously (12). EGF-treated A431 cells (three 150-mm dishes) were washed twice in phosphate-buffered saline and scraped into 2 ml of 500 mM sodium carbonate, pH 11.0. The cell suspension was homogenized using a loose-fitting Dounce homogenizer (10 strokes), sheared in a Polytron tissue grinder (three 10-s bursts, Kinematica GmbH, Brinkmann Instruments, Westbury, NY), and subjected to sonication using an ultrasonicator (three 20-s bursts; Branson Sonifier 250, Branson Ultrasonic Corp., Danbury, CT). The homogenate was then adjusted to 45% sucrose by the addition of 2 ml of 90% sucrose prepared in MBS (25 mM Mes, pH 6.5, 0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient was formed above (4 ml of 5% sucrose, 4 ml of 35% sucrose, both in MBS containing 250 mM sodium carbonate) and centrifuged at 39,000 rpm for 16–20 h in an SW41 rotor (Beckman Instruments). Twelve-to-thirteen 1-ml fractions were collected and analyzed by SDS-PAGE (7.5 or 15% acrylamide). After transfer to nitrocellulose, Western blot analysis was performed with various antibody probes. Horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution; Amersham Life Science, Inc.) were used to visualize bound primary antibodies by enhanced chemiluminescence assay (ECL) (Amersham). Protein concentrations were determined using the BCA assay (Pierce).

**Communoprecipitation of EGF-R with Caveolin—**A near confluent 150-mm dish of A431 cells was washed three times with phosphate-buffered saline, and incubated in lysis buffer (50 mM Heps, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% CHAPS, and protease inhibitors) for 30 min at 4 °C. Lysates were clarified by centrifugation at 15,000 × g for 15 min and pre-cleared by incubation with protein G-Sepharose for 6 h at 4 °C. After pre-clearing, supernatants were transferred to 1.5-ml microfuge tubes containing anti-caveolin or anti-EGF-R IgGs pre-bound to protein G-Sepharose. After incubation rotating overnight at 4 °C, immunoprecipitates were washed 3 × with lysis buffer and subjected to immunoblot analysis with an anti-EGF-R mAb probe.

**Lysates**

1 The abbreviations used are: PKCα, protein kinase C; -R, receptor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; Ins, insulin; Mes, 4-morpholinolinesulfonic acid; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonic acid; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary; wt, wild type; -R, receptor peptides; 1-R, insulin receptor peptide; KD, kinase domain; mAb, monoclonal antibody; CR, cytoplasmic region.
from untransfected CHO cells, CHO-EGF-R wild type (wt), and CHO kinase dead EGF-R cells were prepared as described above for A431 cells in the following buffer (phosphate-buffered saline, pH 8.0, 1 mM EDTA, 0.5% CHAPS, and protease/phosphatase inhibitors). For EGF-treated samples, EGF (100 ng/ml) was present at all steps during the experiment. GST and GST-caveolin 1 (residues 61–101) were prebound to agarose beads and prewashed with phosphate-buffered saline and lysis buffer extensively. These beads contained ~100 pmol/100 μl of packed volume. Approximately 50 μl of this material was incubated with 1.5 ml of lysate. After binding, the beads were extensively washed with lysis buffer and eluted with elution buffer (lysis buffer containing 10 mM reduced glutathione). The eluate was then analyzed by SDS-PAGE/immunoblotting using antibodies directed against EGF-R.

PKC Assay—PKC kinase activity was measured using histone H1 as the substrate, as suggested by the manufacturer (Calbiochem). Briefly, kinase reactions were performed using 4 ng of PKC-M and 3 pmol of histone H1 in kinase reaction buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 12.5 mM MgCl2). After 1 h preincubation with a given peptide at 4 °C, the reaction was initiated by the addition of 10 μCi of [γ-32P]ATP (6,000 Ci/mmol). After 10 min at 30 °C, the reaction was terminated by the addition of 2 × SDS-PAGE sample buffer and boiling for 2 min. Phosphorylated proteins were visualized by autoradiography. Control samples omitting either [γ-32P]ATP or PKC showed no activity.

RESULTS

Co-fractionation of Receptor Tyrosine Kinases, EGF-R and erbB2, with Caveolin 1 in A431 Cells—A431 cells are known to express both EGF-R (23) and caveolin 1 (5, 24). Thus, we used this cell line to examine the potential association of EGF-R with caveolin. A carbonate-based fractionation scheme was employed for the purification of caveolin-enriched membranes. Fig. 1 shows that EGF-R co-fractionates with caveolin 1 in A431 cells (Fig. 1, upper panel). Interestingly, erbB2 (also known as neu), a close relative to the EGF-R, also co-fractionates with caveolin 1. In contrast, β-adaptin, gelsolin, and greater than 99% total cellular proteins were excluded from these caveolin-rich membrane fractions (Fig. 1, lower panel). β-Adaptin has been implicated in the formation of plasma membrane-derived clathrin-coated vesicles, whereas gelsolin is a cytosolic actin binding protein.

Interaction of Full-length Cellular EGF-R with a Cytosolic Domain of Caveolin 1—Caveolin 1 has been shown to interact directly with a variety of cytoplasmic signal transducing molecules (i.e. G subunits, H-Ras, eNOS and c-Src) through a common membrane-proximal region of its NH2-terminal cytoplasmic domain. This 20-amino acid region (residues 82–101) has recently been termed the caveolin scaffolding domain. However, it is not known whether caveolin interacts directly with signaling receptors, such as EGF-R.

To evaluate the potential interaction of EGF-R with caveolin 1, we used two independent approaches: (i) coimmunoprecipitation and (ii) peptides encoding the caveolin 1 scaffolding domain coupled to agarose. Fig. 2A shows that EGF-R coimmunoprecipitates with caveolin 1 when either a polyclonal or monoclonal antibody directed against caveolin 1 is employed. However, EGF-R failed to coimmunoprecipitate when antibodies directed against caveolin-3 were utilized. As caveolin-3 is only expressed in muscle tissue types and is not expressed in A431 cells, this serves as an appropriate negative control for nonspecific coimmunoprecipitation.

To assess the role of the caveolin 1 scaffolding domain in this interaction, we next incubated A431 cell lysates with the caveolin 1 scaffolding domain coupled to agarose beads. Fig. 2B shows that this caveolin 1 peptide retained the EGF-R, whereas agarose beads alone showed no binding of EGF-R. These studies directly implicate the caveolin 1 scaffolding domain in this interaction.

A Caveolin Binding Motif within the Kinase Domain of EGF-R Mediates the Interaction of EGF-R with Caveolin 1—Using the caveolin 1 scaffolding domain as a receptor to select random peptide ligands, two similar caveolin binding motifs were recently detected (ΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦPhi
evaluated the ability of these three GST-EGF-R fusion proteins to interact with the caveolin 1 scaffolding domain. For this purpose, we used three different caveolin peptides encoding the scaffolding domains of caveolin 1 or caveolin 2 or an irrelevant caveolin 1 peptide (residues 53–81). As illustrated in Fig. 4, the KD and C-CR fusion proteins selectively interacted with the caveolin 1 scaffolding domain but not with the caveolin 2 scaffolding domain or with the irrelevant caveolin 1 peptide. In addition, the N-CR fusion did not show an interaction with any of these peptides. Taken together, these results indicate the caveolin binding region of EGF-R lies between EGF-R residues 889 and 979 (by comparison of the binding of N-CR and KD fusions). These mapping studies are as predicted based on the presence of a caveolin binding motif within EGF-R residues 894–907.

To evaluate if this EGF-R region was indeed implicated in caveolin binding, we examined the binding of a biotinylated version of the EGF-Rp peptide to the caveolin 1 scaffolding domain. Fig. 5A shows that the EGF-Rp peptide interacted with the caveolin 1 scaffolding domain; a homologous peptide from within the insulin receptor (I-Rp) also showed a strong interaction with the caveolin 1 scaffolding domain. It has been previously demonstrated that aromatic residues within the caveolin binding motif are essential for interaction with caveolin (15). In accordance with these observations, a mutated EGF-R peptide in which all aromatic residues were changed to glycine failed to interact with the caveolin 1 scaffolding domain (Fig. 5A).

Similarly, we evaluated the ability of these EGF-R and I-R peptides to compete with the EGF-R kinase domain for binding to the caveolin 1 scaffolding domain. In this assay, the EGF-R and I-R peptides were incubated in presence of the GST-EGF-R KD fusion protein to observe inhibition of the interaction of the kinase domain with the caveolin 1 scaffolding domain. Fig. 5B
Based peptide (predicted, EGF-Rp and I-Rp were as effective as a G-protein-binding domain of Gi2 folding domain. A peptide designated GP, encoding the caveolin I-Rp) to compete for G-protein binding to the caveolin 1 scaffolding domain. Affinity-purified GST-Gi2 fusion protein (120 ng, Ref. 15) was allowed to interact with the scaffolding domain of caveolin 1 in the absence or presence of increasing amounts of competing peptide. Two different peptides containing caveolin binding motifs (the EGF-R peptide and the I-Rp peptide) competitively inhibited the binding of the GST-Gi2 fusion protein to caveolin 1. In contrast, two mutant EGF-R peptides were unable to inhibit this interaction. A peptide designated GP encoding the caveolin binding domain of Gi2 was included as a positive control. All results are expressed as the mean ± S.E. of triplicate determinations.

FIG. 5. EGF-R and I-R peptides interact with the caveolin 1 scaffolding domain and competitively inhibit the binding of a GST-EGF-R fusion protein. A, biotinylated EGF-R and I-R peptides were evaluated for their ability to interact with the scaffolding domain of caveolin 1. Binding of a mutated EGF-R peptide was also evaluated in parallel. In this mutant EGF-R peptide, all aromatic residues were changed to glycine (Φ→G). Binding to an irrelevant region of caveolin 1 (residues 53–81) was included as a negative control (not shown). Note that the wild-type EGF-R peptide and I-R peptide interact with the scaffolding domain of caveolin 1. In contrast, the mutant EGF-R peptide showed little or no interaction with the caveolin 1 scaffolding domain. B, the GST-EGF-R fusion protein encoding the kinase domain (termed KD, 120 ng) was allowed to interact with the scaffolding domain of caveolin 1 in the absence or presence of competing peptide (70 μM). Note that two different peptides containing caveolin binding motifs (the wild-type EGF-R peptide and the I-R peptide) competitively inhibited the binding of the GST-EGF-R fusion protein to caveolin 1. In contrast, the mutant EGF-R peptide was unable to inhibit this interaction. All results are expressed as means ± S.E. of triplicate determinations.

shows that EGF-Rp and I-Rp were able to effectively compete for binding to caveolin 1, whereas the mutated EGF-Rp lacking aromatic residues had no effect.

G-protein α subunits represent a well defined class of caveolin-interacting proteins. Our current findings suggest that the EGF-R kinase domain interacts with caveolin 1 in a similar fashion as observed for G-proteins. Thus, we next tested the ability of these receptor tyrosine kinases (EGF-Rp and I-Rp) to compete for G-protein binding to the caveolin 1 scaffolding domain. A peptide designated GP, encoding the caveolin binding domain of Gi2, was included as a positive control. As predicted, EGF-Rp and I-Rp were as effective as a G-protein-based peptide (GP) in competing for the binding of a Gi2 fusion protein (Fig. 6). This competition was strictly dependent on the presence of aromatic residues within the EGF-R peptide, as two mutated EGF-R peptides lacking aromatic residues failed to compete with the Gi2 fusion protein (Fig. 6).

Binding of the Caveolin 1 Scaffolding Domain Functionally Inhibits the Autophosphorylation of the EGF-R Kinase—To examine the functional consequences of the interaction of caveolin 1 with EGF-R, we tested the effect of the caveolin 1 scaffolding domain on the autophosphorylation of a purified recombinant form of the EGF-R kinase in vitro. Fig. 7A illustrates that the caveolin 1 scaffolding domain dose-dependently suppressed the kinase activity of EGF-R. Approximately 85% inhibition was observed at a peptide concentration of 3 μM, and complete inhibition was observed at 10 μM.

How does the caveolin 1 scaffolding domain inhibit the EGF-R kinase? One possibility is that this caveolin-mediated inhibition is related to tyrosine phosphorylation of caveolin or caveolin-derived peptides, resulting in a form of competitive substrate inhibition of the EGF-R kinase. In support of this possibility, the caveolin 1 scaffolding domain (DGIWKASFTTVKFWYPF, residues 82–101) contains both inhibitory activity and two tyrosine residues.

To directly examine the possible requirement for tyrosine phosphorylation in this event, we generated a mutated caveolin 1 scaffolding domain in which both of these tyrosines were changed to phenylalanine (DGIWKASFTVTKFWEF, termed Y→F). Fig. 7B shows that the mutated caveolin 1 scaffolding domain lacking tyrosine (Y→F) was as effective as the wild-type (wt) peptide sequence in suppressing the auto-phosphorylation of EGF-R. In addition, the Y→F mutant was ~60% more potent than the wild-type sequence in binding to the EGF-R peptide (bottom panel). These results demonstrate that tyrosine phosphorylation of the caveolin 1 scaffolding do-
main (residues 82–101) is not required for its inhibitory activity toward EGF-R. Inhibition of the EGF-R kinase by the caveolin 1 scaffolding domain could be mediated by the direct interaction of caveolin with the kinase domain. In support of this notion, caveolin-mediated inhibition of the EGF-R kinase could be prevented by the addition of the EGF-R-derived peptide (residues 894–907) that (i) contains a well conserved caveolin binding motif and (ii) is located within the kinase domain of the EGF-R (Fig. 7C).

Caveolin 1 is only the first member of a multigene family of caveolin molecules. Thus, we next evaluated the inhibitory activity of the scaffolding domains of caveolins 1, 2, and 3 in parallel. Fig. 8A shows that the scaffolding domains of caveolins 1 and 3 both inhibit the EGF-R kinase autophosphorylation, whereas the caveolin 2 scaffolding domain has little or no effect. This caveolin-mediated inhibition appears to be due to a direct interaction with the EGF-R kinase domain, as the caveolin 2 scaffolding domain also fails to interact with the appropriate region of the EGF-R kinase domain (Fig. 8B); in contrast, the scaffolding domains of caveolins 1 and 3 show strong binding activity; however, the caveolin 2-derived peptide demonstrated little or no interaction. Data are shown as the means ± S.E. in triplicate.

Inhibition of the EGF-R kinase by the caveolin 1 scaffolding domain could be mediated by the direct interaction of caveolin with the kinase domain. In support of this notion, caveolin-mediated inhibition of the EGF-R kinase could be prevented by the addition of the EGF-R-derived peptide (residues 894–907) that (i) contains a well conserved caveolin binding motif and (ii) is located within the kinase domain of the EGF-R. Note that only the scaffolding domains of caveolins 1 and 3 show strong binding activity; however, the caveolin 2-derived peptide demonstrated little or no interaction. Data are shown as the means ± S.E. in triplicate.

**Fig. 7.** Effect of the caveolin 1 (Cav-1) scaffolding domain on the in vitro phosphorylation of the EGF-R kinase. A, a peptide encoding the scaffolding domain of caveolin 1 (residues 82–101) was evaluated for its dose-dependent effect on the autophosphorylation of the purified recombinant EGF-R kinase (EGF-R CR). A control reaction without the caveolin 1 peptide is indicated by the letter C. B, wild-type and mutant peptides encoding the caveolin 1 scaffolding domain (residues 82–101) were examined for their effect on the autophosphorylation of purified recombinant EGF-R kinase. All peptides were added at a concentration of 10 μM. Note that the mutated caveolin 1 peptide lacking tyrosine residues (Y→F) was as effective as the wild-type peptide in suppressing the autophosphorylation of the EGF-R kinase. The lower panel illustrates the relative binding of these caveolin 1 peptides to the EGF-R peptide. Data are shown as the means ± S.E. in triplicate. C, importantly, a peptide encoding the caveolin binding domain of EGF-R (EGF-Rp) can effectively abrogate the inhibition of the EGF-R kinase induced by the caveolin 1 scaffolding domain.

**Fig. 8.** Peptides encoding the scaffolding domains of caveolins (Cav) 1, 2, and 3 differentially effect the autophosphorylation of the EGF-R kinase. A, peptides encoding the scaffolding domains of caveolins 1, 2, and 3 were evaluated for their effect on the activity of the EGF-R kinase. The three peptides are extremely homologous, and each contains two conserved tyrosine residues. All peptides were added at a concentration of 10 μM. Note that only the scaffolding domains of caveolins 1 and 3 show strong inhibitory activity; in contrast, the caveolin 2-derived peptide had little or no inhibitory effect. B, peptides encoding the scaffolding domains of caveolins 1, 2, and 3 were also evaluated for their ability to interact with an EGF-R-derived peptide that (i) contains a well conserved caveolin binding motif and (ii) is located within the kinase domain of the EGF-R. Note that only the scaffolding domains of caveolins 1 and 3 show strong binding activity; however, the caveolin 2-derived peptide demonstrated little or no interaction. Data are shown as the means ± S.E. in triplicate.
Certain critical residues within the caveolin 1 scaffolding domain are required for binding and inhibition of the EGF-R kinase. Deletion mutant peptides encoding the caveolin 1 scaffolding domain were constructed and tested for their effect on the EGF-R kinase (panel A) and their ability to bind the EGF-R peptide (panel B). Note that a minimal caveolin 1 domain of 18 amino acids (residues 84–101) was required to mediate inhibition of the kinase, whereas a minimal domain of 16 amino acids (residues 86–101) was required for recognition of the EGF-R peptide. In panel B, results are expressed relative to the binding of the complete caveolin 1 scaffolding domain (residues 82–101) as the mean ± S.E. of three determinations.

First, deletion mutagenesis of the 82–101 region indicated that a minimal length of 18 amino acids is required (residues 84–101) for inhibition of EGF-R kinase activity (Fig. 9A), whereas only a minimal length of 16 amino acids (residues 86–101) is required for binding (Fig. 9B). These results are consistent with the idea that binding to the kinase domain is required to mediate inhibition of the EGF-R kinase, but that binding alone is not necessarily sufficient to mediate inhibition. In addition, these findings are the first suggestion that these two activities (binding and kinase inhibition) can be separated.

Second, alanine-scanning mutagenesis was then performed using the minimal 16-amino acid caveolin 1 scaffolding domain. Fig. 10 shows that a central core of four amino acids (Phe92-Thr-Val-Thr95) is strictly required for interaction of the caveolin 1 scaffolding domain with both the EGF-R peptide and the corresponding region of EGF-R (kinase domain KD). As the PTFTV sequence appears as FEIS in caveolin 2 and FTVS in caveolin 3, this may explain why the EGF-R kinase domain is recognized by the scaffolding domains of caveolins 1 and 3 but not by caveolin 2. Importantly, this PTFTV region has also been shown to be critical for the interaction of caveolin 1 with another class of caveolin-interacting proteins, namely G-protein α subunits.

Activated EGF-R Is Present within Caveolin-rich Membrane Domains and Stably Interacts with Caveolin 1—Fig. 11 shows that in EGF-treated A431 cells the EGF-R and caveolin 1 cofractionate. Does this represent the activated or inactive pool of the receptor? To address this issue, we used an antibody that specifically recognizes only the activated tyrosine-phosphorylated form of EGF-R. Interestingly, activated EGF-R is also targeted to the same low density fractions containing caveolin-enriched domains. These results suggest that activated EGF-R may also have the capacity to interact with caveolin.

To further address this issue, we next evaluated whether kinase activity is required for EGF-R to interact with caveolin. For this purpose, we utilized CHO cells stably transfected with either wild-type EGF-R or with a mutated kinase dead form of EGF-R; untransfected CHO cells served as a negative control. Lysates from these cell lines were prepared and used as the substrate for binding to the caveolin 1 scaffolding domain encoded by a GST-caveolin 1 fusion protein. Fig. 11B shows that both wild-type and kinase dead EGF-R stably interact with the GST fusion protein encoding the caveolin scaffolding domain but not with GST alone. Interestingly, binding of EGF-R to caveolin also occurred with lysates prepared from EGF-stimulated cells, indicating that both inactive and activated forms of EGF-R have the capacity to stably interact with caveolin (Fig. 11C). These results suggest that the EGF-R–caveolin interaction is independent of receptor tyrosine autophosphorylation and receptor kinase activity. In support of our current observations, Anderson and co-workers (17) have shown that EGF-R remains within caveolae for up to 15 min after EGF stimulation in normal human fibroblasts.

Can the Caveolin Scaffolding Domain Also Recognize Serine/Threonine Kinases?—In this work, we have demonstrated that the caveolin scaffolding domain can inhibit the autophosphorylation of a receptor tyrosine kinase, EGF-R. Previously, we have shown that caveolin binding both in vitro and in vivo inhibits that activity of nonreceptor tyrosine kinases such as c-Src and Fyn (11). However, it remains unknown whether caveolin can interact with serine/threonine kinases. To evaluate this possibility, we examined the effect of the caveolin scaffolding domain on protein kinase C, a well characterized
serine/threonine kinase. We chose PKC because (i) most members of the PKC family contain a caveolin binding motif (EEXXX) within their kinase domain that is analogous to the caveolin binding motif found in tyrosine kinases (13). The PKC isoforms have been previously localized to caveolae by cell fractionation and immunogold labeling of intact cell plasma membranes (25, 26).

For these experiments, we used the catalytic subunit of PKC purified from rat brain, termed PKC-M. PKCs isolated from this tissue consist mainly of α, β, and δ subtypes. PKC-M is generated by limited proteolysis of full-length PKC with trypsin and, therefore, does not require calcium or phosphatidylserine for its activity. PKC kinase activity was measured using histone H1 from calf thymus as the substrate.

Fig. 12 shows that a peptide encoding the caveolin 1 scaffolding domain effectively inhibits the serine/threonine kinase activity of purified PKC. This inhibition is near maximal at concentrations of ~10 μM with an IC₅₀ of ~0.3 μM; this potency is comparable to the activity of other well characterized peptide-based inhibitors of PKC (27). As observed for the autophosphorylation of EGF-R, the scaffolding domain of caveolin 2 had no effect, whereas the caveolin 3 scaffolding domain is a potent inhibitor. In addition, it appears that the complete 20-amino acid caveolin 1 scaffolding domain (residues 82–101) is required for potent inhibition of PKC, as the 18-mer peptide deletion mutant retains only ~50% of its inhibitory activity; smaller deletion mutants showed no inhibitory activity.

As the caveolin scaffolding domain can inhibit both tyrosine and serine/threonine kinases with similar potency, this caveolin-derived protein module may represent a novel class of general kinase inhibitor. In support of this notion, a recognizable caveolin binding motif (EEXXX) is present within a highly conserved region of the kinase domain of most known protein kinases (15). This region has been previously been termed region IX and is currently of unknown function.²

**DISCUSSION**

Protein kinases play an essential role in the regulation of cellular growth and differentiation. Two general classes of tyrosine kinases have been defined: transmembrane receptors and nonreceptor cytoplasmically oriented kinases (29, 30). Receptor tyrosine kinases, such as EGF-R, are activated by the binding of ligand to the extracellular protein domain, whereas nonreceptor tyrosine kinases must be activated indirectly. Both classes of tyrosine kinases can function as transforming oncoproteins when mutationally activated (30, 31).

In this study, we report the direct interaction of the EGF-R with caveolin. EGF-R is the first receptor to be shown to interact with caveolin. However, the characteristics of the EGF-R-caveolin interaction are strikingly similar to the interaction of caveolin with other caveolin-interacting proteins such as G-protein α subunits. This suggests that caveolin may interact with different classes of signaling molecules via a common mechanism. As with G-protein α subunits, the caveolin binding domain within EGF-R requires the presence of aromatic residues to recognize the scaffolding domain of caveolin 1. Interestingly, the caveolin binding region of G-protein α subunits is located near the active site of the enzyme. Similarly, the caveolin binding domain of EGF-R is located within a conserved region of the tyrosine kinase domain.

How does the caveolin scaffolding domain inhibit the activity of the EGF-R kinase? One possibility is that caveolin binding stabilizes the receptor kinase in an inactive conformation; alternatively, caveolin binding could prevent receptor dimerization and subsequent transphosphorylation as a consequence of steric hindrance. As caveolin also inhibits the activity of other signaling molecules that do not require dimerization for activation (such as G-protein α subunits), we favor the former possibility.

Several independent lines of evidence are consistent with a direct interaction between a receptor tyrosine kinase, EGF-R, and caveolin. (i) Nonreceptor tyrosine kinases (c-Src and Src-related kinases, i.e. Fyn) interact directly with caveolin, and the kinase domains of nonreceptor tyrosine kinases and receptor tyrosine kinase are extremely homologous (11, 32, 33). (ii) Four receptor tyrosine kinases (EGF-R, PDG-F-R, insulin-R, and nerve growth factor receptor (trk)) have now been shown to co-purify with caveolae or caveolae-like membrane domains (16, 17, 20, 25, 34), suggesting that a structural property that is common to this protein family directs caveolar localization.

A protein kinase domain consists of 11 major conserved subdomains that have been previously defined through exten-

²A protein kinase domain consists of 11 major conserved subdomains that have been previously defined and are designated by roman numerals I-XI (28).
sive sequence analysis and are designated by roman numerals I-XI (28). A recognizable caveolin binding motif (PXΦpXXXXΦ) is present within conserved region number IX, yet the function of this conserved region remains unknown. Perhaps caveolin functions as a general kinase inhibitor through recognition of this conserved caveolin binding motif. In support of this notion, we show here that the caveolin scaffolding domain can inhibit both tyrosine and serine/threonine kinases with similar potency. Thus, this caveolin-derived protein module may represent a novel class of general kinase inhibitor.

Interaction of signaling molecules with caveolin or their caveolar localization could explain the cross-talk observed between receptor tyrosine kinases and G-protein-coupled receptor pathways. For example, the role of EGF-R transactivation in signaling by G-protein-coupled receptors (especially the case of the endothelin receptor A) has been demonstrated in Rat-1 fibroblasts (35). Interestingly, the EGF-R signaling pathway is located in these cells in caveolae (17), and we have previously shown that endothelin and its receptor are colocalized with caveolin in transfected COS cells (36). Conversely, ligand binding to G-protein-coupled receptors is known to be sufficient to activate nonreceptor tyrosine kinases (37). In this regard, caveolea-coupled signaling might explain how linear signaling pathways can branch and interconnect extensively, forming a signaling module or network.

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**FIG. 12.** The caveolin scaffolding domain inhibits the serine/threonine kinase activity of the purified PKC catalytic domain in vitro. A, a peptide encoding the caveolin 1 scaffolding domain (residues 82–101) was evaluated for its dose-dependent effect on the phosphorylation of histone-H1 by the purified catalytic domain of PKC. B, histogram showing dose-dependent inhibition of PKC serine/threonine kinase activity by a peptide encoding the caveolin 1 scaffolding domain (residues 82–101). C, peptides encoding the scaffolding domains of caveolins (Cav) 1, 2, and 3 differentially effect the serine kinase activity of PKC in vitro. All peptides were added at a concentration of 1 μM. Note that only the scaffolding domains of caveolin 1 and 3 show strong inhibitory activity. In contrast, the scaffolding domain of caveolin 2 and a peptide encoding caveolin 1 residues 53–81 had no inhibitory effect. D, a series of deletion mutant peptides encoding the caveolin 1 scaffolding domain were constructed and tested for their effect on the kinase activity of PKC. Note that a minimal caveolin 1 domain of 18 amino acids (residues 84–101) was required to mediate inhibition of PKC.
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