Src Tyrosine Kinases, Gα Subunits, and H-Ras Share a Common Membrane-anchored Scaffolding Protein, Caveolin

CAVEOLIN BINDING NEGATIVELY REGULATES THE AUTO-ACTIVATION OF Src TYROSINE KINASES

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Caveolae are plasma membrane specializations present in most cell types. Caveolin, a 22-kDa integral membrane protein, is a principal structural and regulatory component of caveolae membranes. Previous studies have demonstrated that caveolin co-purifies with lipid modified signaling molecules, including Gα subunits, H-Ras, c-Src, and other related Src family tyrosine kinases. In addition, it has been shown that caveolin interacts directly with Gα subunits and H-Ras, preferentially recognizing the inactive conformation of these molecules. However, it is not known whether caveolin interacts directly or indirectly with Src family tyrosine kinases. Here, we examine the structural and functional interaction of caveolin with Src family tyrosine kinases. Caveolin was recombinantly expressed as a glutathione S-transferase fusion. Using an established in vitro binding assay, we find that caveolin interacts with wild-type Src (c-Src) but does not form a stable complex with mutagenically activated Src (v-Src). Thus, it appears that caveolin prefers the inactive conformation of Src. Deletion mutagenesis indicates that the Src-interacting domain of caveolin is located within residues 82–101, a cytosolic membrane-proximal region of caveolin. A caveolin peptide derived from this region (residues 82–101) functionally suppressed the auto-activation of purified recombinant c-Src tyrosine kinase and Fyn, a related Src family tyrosine kinase. We further analyzed the effect of caveolin on c-Src activity in vivo by transiently co-expressing full-length caveolin and c-Src tyrosine kinase in 293T cells. Co-expression with caveolin dramatically suppressed the tyrosine kinase activity of c-Src as measured via an immune complex kinase assay. Thus, it appears that caveolin structurally and functionally interacts with wild-type c-Src via caveolin residues 82–101. Besides interacting with Src family kinases, this cytosolic caveolin domain (residues 82–101) has the following unique features. First, it is required to form multivalent homo-oligomers of caveolin. Second, it interacts with G-protein α-subunits and down-regulates their GTPase activity. Third, it binds to wild-type H-Ras. Fourth, it is membrane-proximal, suggesting that it may be involved in other potential protein-protein interactions. Thus, we have termed this 20-amino acid stretch of caveolin residues the caveolin scaffolding domain.

Caveolae are small bulb-shaped invaginations located at or near the cell surface (1). They represent a micro-domain of the plasma membrane (1, 2). Although caveolae are present in most cells, they are most abundant in terminally differentiated cell types: endothelia, adipocytes, muscle cells (skeletal, cardiac, and smooth), and type I pneumocytes (reviewed in Refs. 3 and 4). For example, in adipocytes they may occupy up to 20% of the total plasma membrane surface area (5). In striking contrast, caveolin and caveolae are reduced or absent in fibroblasts transformed by certain activated oncogenes (such as v-Abl or H-Ras (G12V)) (6).

Caveolin, a 21–24-kDa integral membrane protein (7, 8), is a major structural and regulatory component of caveolae membranes (7, 9–11). As such, caveolin has served as a marker protein for the organelle (12–14). Using either Triton-based methods (12–17) or detergent-free methods (18–20), it has been demonstrated that caveolin co-purifies with cytoplasmic lipid-modified signaling molecules. These caveolin-associated proteins include G-proteins (α and β subunits), H-Ras, c-Src, and Src family tyrosine kinases. In direct support of these findings, caveolin was first identified as a major transformation-dependent v-Src substrate in Rous sarcoma virus-transformed cells (21). Based on these observations, we have proposed a “caveolae signaling hypothesis,” which states that caveolar localization of certain lipid modified signaling molecules could provide a compartmental basis for organizing a subset of signal transducing molecules (3, 12).

In this regard, several independent lines of evidence suggest that caveolin may function as a scaffolding protein within caveolae membranes. (i) Both the N-terminal and C-terminal domains of caveolin face the cytoplasm, allowing them to freely interact with cytosolic molecules (22–24). In accordance with this membrane topology, caveolin remains inaccessible to biotinylation probes that have been used to efficiently label proteins that face the extracellular environment (25). (ii) Caveolin undergoes two stages of oligomerization. First, caveolin monomers assemble into discrete multivalent oligomers containing ~14–16 monomers per oligomer (25, 26). 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) (25). (iii) A cytosolic membrane proximal domain of caveolin (residues 82–101) interacts directly with Gα subunits and H-Ras (19, 27). This caveolin region preferentially recognizes the inactive conformation of these molecules, because mutagenically activated Gα subunits (GαQ227L) and H-Ras (G12V) fail to interact with caveolin (19, 27). (iv) Interaction of caveolin with purified heterotrimeric G-proteins functionally suppresses their GTPase activity, holding the G-pro-
tein in the inactive conformation (27). Thus, caveolin may organize the formation of caveolae microdomains and orchestrate caveolae-related signaling events.

Here, we examine the structural and functional interaction of caveolin with Src family tyrosine kinases. We find that caveolin interacts directly with wild-type Src (c-Src) but fails to stably interact with a mutationally activated form of Src (v-Src). The Src-interacting domain of caveolin was localized to caveolin residues 82–101, and a peptide encoding this sequence dose-dependently suppressed the auto-activation of purified Src kinases (c-Src and Fyn). Furthermore, transient co-expression of c-Src with the full-length caveolin in 293T cells inhibited the kinase activity of c-Src.

Thus, it appears that there are at least three distinct classes of lipid-modified signaling molecules (Gα subunits (27), H-Ras (19), and c-Src (this report)) that normally co-purify with caveolin all recognize the same cytosolic membrane-proximal region of caveolin (residues 82–101). In addition, caveolin prevents the inactive conformation of these molecules, and caveolin binding can hold these molecules in the inactive conformation. As such, caveolin may function as a common membrane-anchored scaffolding protein for these and other cytoplasmic signaling molecules.

Our current observations are analogous to another family of scaffolding proteins (28), the AKAPs (A-kinase anchor proteins), which preferentially recognize the inactive conformation of protein kinase A, protein phosphatase 2B (calcineurin), and protein kinase C (α- and β-isozymes) (29, 30). As we find with caveolin, each of these enzymes is inhibited when bound to AKAP-79 (29, 30).

**Experimental Procedures**

**Materials**—The cDNA for canine caveolin was as described previously (12). Antibodies and their sources were as follows: anti-caveolin mouse IgG (mAb 2297) and rabbit polyclonal antibody (gifts of Dr. John R. Glennie, Transduction Labs) and anti-Src monoclonal antibody (Oncogene Sciences, Inc.). A variety of reagents were purchased commercially: fetal bovine serum (JRH Biosciences); penicillin-streptomycin (BioFluids); Ex-cell 400 medium (JRH Biosciences); glutamine (Life Technologies, Inc.); γ-[32P]ATP (DuPont NEN); purified recombinant c-Src and Fyn tyrosine kinases (Upstate Biotechnologies, Inc.); glutathione-agarose beads and reduced glutathione (Sigma); protein A-Sepharose (Pharmacia, Inc.). Peptide synthesis was performed by the Biopolymers Facility at MIT (Massachusetts Institute of Technology). All other reagents were as detailed previously (27, 31).

**Construction and Purification of Glutathione S-Transferase-Caveolin Fusion Proteins**—The construction, expression, and purification of GST-caveolin1 fusion proteins were as described previously (24, 25, 27). Briefly, full-length caveolin (residues 1–178) the C-terminal domain of caveolin (residues 135–178), and regions of the N-terminal domain of caveolin (residues 1–21, 1–41, 1–61, 1–81, 1–101, and 61–101) were subcloned into the vector pGEX-4T-1. After expression in *Escherichia coli* (BL21 strain; Novagen, Inc.), GST-caveolin fusion proteins were affinity purified by affinity chromatography on glutathione-agarose beads (32).

**Cell Culture**—Insect SF21 cells were provided by Dr. Takashi Okamoto (Massachusetts General Hospital/Harvard Medical School). SF21 cells were grown in Ex-cell 400 medium, 10% fetal bovine serum, and 1% penicillin-streptomycin at 27 °C. For double transfection experiments, 293T cells (gift of Dr. Kunxin Luo, Whitehead Institute, and Dr. Anthony J. Kolesar, MIT) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, glutamine, and antibiotics, as described.

**Construction of Recombinant Baculovirus-expressed c-Src and v-Src**—Baculovirus constructs encoding either c-Src or v-Src (from chicken) were the generous gifts of Drs. Zhou Songyang and Lewis Cantley (Beth Israel Hospital/Harvard Medical School) and were constructed as described previously (33). Briefly, the cDNA encoding pp60v-src was subcloned into a transfer plasmid vector pAC373 (34) using the BamHI site. A mixture of 2 μg of this recombinant plasmid pAC373(c-Src) DNA and 1 μg of purified wild-type baculoviral DNA was transfected into insect Sf9 cells. Four days later, culture supernatants were removed and centrifuged at 1,000 rpm for 10 min. Clarified supernatants containing wild-type and recombinant viruses were plaque assayed on a monolayer of Sf9 cells. Occlusion negative plaques were picked and seeded onto 2.5 × 106 cells. After 3 days of incubation, cells and culture supernatants were removed and centrifuged at 1,000 rpm for 10 min. The cell pellets were analyzed by both in vitro kinase assay and immunoblotting using anti-Src antibody. Those plaques testing positive for the presence of kinase-active pp60v-src were selected for three rounds of plaque purification (35). The selected plaques were used as virus stock for producing c-Src protein by infecting insect SF21 cells. Similarly, as a recombinant baculovirus, v-Src was constructed using a recombinant baculovirus expressing v-srck1, which is a wild-type c-Src kinase (36).

**Interaction of Recombinant Baculovirus-expressed Src with GST-Caveolin Fusion Proteins**—The interaction of GST-caveolin fusion proteins with baculovirus-expressed Src was evaluated essentially as we described for the interaction of caveolin with baculovirus-expressed heterotrimeric G protein α subunits (27) and H-Ras (19). Briefly, GST or GST-caveolin fusion proteins bound on glutathione-agarose beads were washed three times washed first with 20 mM Hepes, pH 7.5, 100 mM NaCl, 0.5% NP40, EDTA, and protease inhibitors and then eluted with 50 mM Tris, pH 8.0, 1 mM EDTA, 1% Triton X-100, 10 mM reduced glutathione, and protease eluters. The eluates were mixed 1:1 with 2× sample buffer and subjected to SDS-PAGE (10% acrylamide). After transfer to nitrocellulose, Western blot analysis was performed with an anti-Src mAb probe (1:10,000 dilution, Oncogene Sciences). Horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution, Abersham Corp.) were used to visualize bound primary antibodies by an enhanced chemiluminescence assay (ECL) (Abersham Corp.).

**Caveolin-derived Synthetic Peptides**—Caveolin peptides were designed based on the protein sequence of the N-terminal domain of canine caveolin. Peptides were synthesized using standard methodology and subjected to amino acid analysis and mass spectrometry (Massachusetts Institute of Technology Biopolymers Laboratory) to confirm their composition. The following four peptides were utilized: peptide 1 (NRPDPKHLNDVVKIDFVIAEPEGTHSF), caveolin residues 53–81; peptide 2 (DIKIWASPTTFTVTKYFWYR), caveolin residues 82–101; peptide 3 (WIKASPFTTF, caveolin residues 84–92); and peptide 4 (TIVTRKDWFRY, caveolin residues 92–101). Note that peptides 3 and 4 correspond to the N-terminal and C-terminal halves of peptide 2, respectively.

**In Vitro Auto-phosphorylation of Src Family Tyrosine Kinases**—Two to six units of either purified recombinant c-Src or Fyn (44–132 nmol; Upstate Biotechnology, Inc.) were incubated with caveolin peptides at concentrations of 0.3, 1, 3, and 10 μM in the kinase reaction buffer. Reactions were performed in a total volume of 50 μl of kinase reaction buffer (20 mM Hepes, pH 7.4, 5 mM MgCl2, 1 mM MnCl2). The reaction was initiated by addition of 15 μl of [γ-32P]ATP. After incubation for 15 min at 25 °C, the reaction was stopped by addition of 2× SDS-PAGE sample buffer and boiling for 2 min. Phosphorylated proteins were visualized by autoradiography using an intensifying screen. Control samples omitting either either γ-[32P]ATP or Src family tyrosine kinases showed no activity. The activity of purified c-Src and Fyn was measured by the manufacturer (UBI, Inc.) as follows: in kinase assays that use only γ-[32P]ATP one enzyme unit transfers 0.1 nmol of 32P/min to a peptide substrate, i.e. cd2 (residues 6–20). Also, purified c-Src had a specific activity of 900,000 units/mg.

**Co-expression of c-Src and Caveolin in Mammalian 293T Cells**—Untagged caveolin and c-Src were co-expressed in 293T cells by cotransfection using a modified calcium phosphate precipitation procedure as described previously (19). Briefly, 293T cells were plated in 10-cm culture dishes at ∼1 × 105 cell/dish in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (complete medium) and cultured until cells reached ∼70% confluency. Just prior to transfection, the medium was replaced with 1 ml of the fresh complete medium. For each 10-cm dish, the transfection mixture was prepared by adding 6 μg of either c-Src and caveolin plasmid DNA (10 μg of DNA for single transfection) to sterile H2O such that the final volume is 438 μl. After addition of 62 μl of 2 M CaCl2 to the DNA/H2O solution, 500 μl of 2× Heps-buffered saline (pH 7.65) was added.
c-Src and v-Src were expressed well in baculovirus-infected expressed caveolin fusion proteins. As shown in Fig. 1, both virus-expressed Src proteins (c-Src and v-Src) and (ii) bacterially anti-rabbit antibody (5 μg/ml). The first incubation was 30 min, and the second and third incubations were 10 min each time. For double labeling, the cells were then successively incubated with (i) 50 μg/ml of normal goat and donkey IgGs; (ii) a 1:400 dilution of mAb anti-c-Src and 40 μg/ml anti-caveolin polyclonal IgG; and (iii) LRSC (lissamine rhodamine B sulfonyl chloride)-conjugated goat anti-mouse antibody (5 μg/ml) and fluorescein isothiocyanate-conjugated donkey anti-rabbit antibody (5 μg/ml). The first incubation was 30 min, whereas primary and secondary antibody reactions were 60 min each. Cells were washed three times with PBS between incubations. Slides were mounted with Slow-Fade anti-fade reagent and observed under a Bio-Rad MR600 confocal fluorescence microscope.

RESULTS

Interaction of Src Tyrosine Kinases with a Cytosolic Domain of Caveolin—Caveolin has been found associated with a number of cytoplasmic lipid-modified signal transducers using both Triton-based and detergent-free methods (12–20). These caveolin-associated proteins include G-protein α-subunits, H-Ras, c-Src, and Src family tyrosine kinases (12–20). Further functional analyses have indicated that a cytosolic domain of caveolin interacts directly with G-protein α-subunits and H-Ras (19, 27). These experiments suggest that caveolin may also interact with Src family tyrosine kinases.

To test this hypothesis, we examined the interaction of caveolin with Src family tyrosine kinases in vitro using (i) baculovirus-expressed Src proteins (c-Src and v-Src) and (ii) bacterially expressed caveolin fusion proteins. As shown in Fig. 1, both c-Src and v-Src were expressed well in baculovirus-infected insect SF21 cells using baculovirus-based expression vectors (see “Experimental Procedures”). After infection, insect cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane. The expression of Src tyrosine kinases was monitored by immunoblot analysis with a mAb that recognizes the conserved extreme N-terminal of Src. This region is identical in c-Src and v-Src. Note that both c-Src and v-Src migrate at 60 kDa as indicated. B, GST-FL-Cav (bound to glutathione-agarose beads) was incubated with detergent extracts of insect cells recombinantly expressing c-Src or v-Src as shown in A (derived from two 25-ml flasks containing ~1 × 10⁷ cells each). GST-FL-Cav represents full-length caveolin (residues 1–178) expressed as a glutathione S-transferase (GST) fusion protein. After binding, extensive washing, and elution with reduced glutathione, bound Src was visualized by immunoblot analysis. Equivalent amounts of GST and GST-FL-Cav were used in these binding experiments. Note that c-Src specifically binds to GST-FL-Cav but not to GST alone. In striking contrast, v-Src failed to bind to either GST-FL-Cav or GST alone. These results with v-Src further demonstrate the specificity of the interaction between c-Src and caveolin.

SF21 cells, and both migrated as a ~60-kDa band as expected. It should be noted that this band was not detectable in uninfected cells or in cells infected with vector alone (not shown). GST-caveolin fusion proteins were expressed and purified as described previously (24, 25, 27).

As a first step, we examined the interaction of Src-tyrosine kinases with full-length caveolin affixed to glutathione-agarose beads (GST-FL-Cav, residues 1–178); GST alone served as a control for nonspecific binding. After binding, extensive washing, and elution with reduced glutathione, bound Src was visualized by immunoblot analysis. Fig. 1B shows that c-Src bound specifically to full-length caveolin but not to GST alone. In addition, v-Src failed to form a stable complex with caveolin under these binding conditions. This may reflect differences in the protein sequences of these two Src kinases, which diverge most extensively at their extreme C termini, resulting in the constitutive auto-activation of v-Src.

In order to define a minimal region of caveolin that can functionally support an association with c-Src, GST-caveolin fusion proteins encoding distinct portions of the N- or C-terminal domains of caveolin were used as the substrate for c-Src binding. Fig. 2 shows that only the GST fusion bearing caveolin residues 61–101 retained c-Src binding activity. However, similar fusion containing caveolin residues 1–81 did not show any interaction. This suggests that caveolin residues 82–101 are most critical for interaction with c-Src. This 20-amino acid stretch of caveolin residues is located within a membrane-proximal region of the cytosolic N-terminal domain of caveolin. Since Src family tyrosine kinases (residues 82–101) has been previously implicated in the direct interaction of caveolin with Gα subunits and H-Ras (19, 27).

Caveolin Residues 82–101 Functionally Inhibit the Auto-ac-
tivation of c-Src and Fyn Tyrosine Kinases—To examine the functional consequences of the interaction of caveolin with Src tyrosine kinases, we generated a panel of caveolin peptides that span the 61–101 region of caveolin. Caveolin peptides 1–4 contain caveolin residues 53–81, 82–101, 84–92, or 93–101, respectively (Table I). Using purified recombinant c-Src tyrosine kinase, we evaluated the effects of these caveolin peptides on the functional auto-activation of c-Src kinase in vitro.

Auto-activation of c-Src occurs through auto phosphorylation of tyrosine 416. Tyrosine 416 is located within a conserved “activation loop” that is located within the kinase domain of many distinct families of protein kinases (37, 38). Tyrosine 416 is not phosphorylated when c-Src is inactive and is phosphorylated when c-Src is active (38, 39). In addition, the Y416F mutation in activated forms of c-Src is sufficient to inhibit their transforming ability (40–43).

Tyrosine 416 is also the major site of auto-phosphorylation within c-Src in vitro (39, 44). Thus, in vitro auto-phosphorylation of c-Src serves as a measure of its auto-activation. Fig. 3 illustrates the effect of these caveolin peptides on the functional auto-activation of purified recombinant c-Src kinase. Peptide 1 (Fig. 3A) had no effect at any concentration, whereas peptide 2 (Fig. 3B) dose-dependently suppressed the auto-activation of c-Src kinase. Approximately 85% inhibition was observed at a peptide concentration of 300 nm and was completely abolished at 3 μM. It should be noted that peptide 2 corresponds to the caveolin region (residues 82–101) that we have implicated in the direct interaction of caveolin with c-Src using caveolin expressed as a GST fusion protein (see Fig. 2 above).

Interestingly, when peptide 2 was divided into two peptides (peptides 3 and 4; Fig. 3C), no inhibition of c-Src auto-activation was observed, even at a peptide concentration of 10 μM. Quantitation of these experiments (Fig. 3, A–C) is presented in Fig. 3D. These observations suggest that the intact domain (caveolin residues 82–101) is required for both structural and functional interaction with c-Src tyrosine kinase.

To test the generality of this phenomenon, we next assessed the effect of caveolin peptides on the auto-phosphorylation of a related Src family tyrosine kinase, Fyn. Fig. 4 shows that similar results were obtained with Fyn kinase. The inhibitory effect of peptide 2 was slightly less potent on Fyn tyrosine kinase. Approximately 90% inhibition was observed at a peptide concentration of 3 μM; this is about 10-fold less potent than the effect we observed with c-Src. As with c-Src, peptide 1 had no effect on Fyn auto-phosphorylation, even at a peptide concentration of 10 μM. The observed differences in the potency of peptide 2 with different Src family tyrosine kinases suggests that (i) the interaction of caveolin with Src family tyrosine kinases is highly specific and (ii) that caveolin may preferentially interact with a select subset of Src family members in vivo.

Co-expression with Caveolin Suppresses the Auto-activation of c-Src in Vivo—The experiments described above indicate that caveolin residues 82–101 interact directly with c-Src and functionally suppress auto-activation of c-Src in vitro. However, it is not clear from our experiments whether full-length caveolin can perform this function in vivo. To test this idea under more physiological conditions, we transiently co-expressed c-Src with full-length caveolin in 293T cells. These cells express extremely low levels of endogenous caveolin; little or no immunoreactivity was observed with anti-caveolin IgG by Western blotting (not shown). After expression, c-Src kinase was isolated by immunoprecipitation and subjected to the immune complex kinase assay (Fig. 5). Although immunoblot analysis indicates that these immunoprecipitates contain equivalent amounts of c-Src kinase, little or no kinase activity was associated with c-Src when co-expressed with caveolin in vivo. Thus, co-expression of caveolin with c-Src can abolish its capacity for auto-phosphorylation, as predicted based on our experiments with caveolin peptides (see Fig. 3).

![Diagram](image)

**FIG. 2. Defining a region of caveolin that contains c-Src binding activity.** A, diagrammatic summary of each GST-caveolin fusion protein relative to a complete caveolin molecule. The **numbers** at end points reflect their exact amino acid position within caveolin. These fusion proteins correspond to the full-length caveolin protein (residues 1–178), the C-terminal caveolin domain (residues 135–178), and various portions of the N-terminal domain of caveolin (residues 1–21, 1–41, 1–61, 1–81, or 61–101). These GST-caveolin fusion proteins were constructed and characterized as we described previously (24, 25, 27). B, molecular mapping of a caveolin region that interacts with c-Src. Using a panel of GST-caveolin fusion proteins enumerated in A, we systematically identified a 41-amino acid region of caveolin (residues 61–101) that is functionally sufficient to interact with c-Src. After binding, extensive washing, and elution with reduced glutathione, bound Src was visualized by immunoblot analysis as in Fig. 1B. Detergent extracts of insect cells recombantly expressing c-Src were prepared from a total of four 125 flasks containing ~1 × 10^6 cells each. Equivalent amounts of GST and GST-caveolin fusion proteins were used in these binding experiments.

**Table I**

*Effect of caveolin peptides on the auto-phosphorylation of c-Src and Fyn tyrosine kinases*

| Peptide | Sequence | Caveolin residues | Inhibitory activity |
|---------|----------|-------------------|--------------------|
| 1       | NRDPKHLNDVVKIDFEDVIAEPEGTHSF | 53–81 | – |
| 2       | DG1QASFFTFVTXKWFYR | 82–101 | + |
| 3       | IWKASFTTF | 84–92 | – |
| 4       | TVTKYWFYR | 93–101 | – |
These results imply that the interaction of caveolin with Src family kinases may serve to negatively regulate their functional activity.

In addition, double labeling of 293T cells co-transfected with c-Src and caveolin revealed significant co-localization of these two distinct gene products (Fig. 6). This is consistent with results demonstrating co-immunoprecipitation of caveolin with c-Src using antibodies directed against c-Src (Fig. 5A, bottom panel).

Is Tyrosine Phosphorylation of Caveolin Required for the Caveolin-mediated Inhibition of Src Family Kinases?—We do not yet know the mechanism by which caveolin-derived peptides inhibit Src family kinases. 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 Src family kinases. In support of this possibility, peptide 2 (DGIWKASPTTVFY; 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 two mutated caveolin peptides in which both of these tyrosine residues were changed to alanine (DGIVKASFTVTKWFAR; termed Y$_3$A) or phenylalanine (DGIVKASFTVTKWFAR; termed Y$_3$F). Fig. 7 shows that both mutated caveolin peptides lacking tyrosine (Y$_3$A; Y$_3$F) were as effective or more effective than the wild-type peptide sequence in suppressing the auto-phosphorylation of c-Src. In addition, the mutant Y$_3$F peptide was approximately twice as potent as the wild-type caveolin peptide. These results demonstrate that tyrosine phosphorylation of this caveolin sequence (residues 82–101) is not required for its inhibitory activity toward c-Src.

In accordance with these results, the Src-binding region we have defined here within caveolin (caveolin residues 61–101) is not a substrate for tyrosine phosphorylation by c-Src, as we have published previously (see Fig. 2 within Ref. 45). Also, the site of caveolin phosphorylation by c-Src occurs at a single tyrosine residue (tyrosine 14; Ref. 45), and this residue is outside caveolin’s Src-binding region (caveolin residues 61–101). Thus, these experimental observations also argue against competitive substrate inhibition via tyrosine phosphorylation.

What about the Functional Activity of Other Caveolin Family

Fig. 4. Effects of caveolin peptides on the auto-activation of Fyn, a closely related Src family tyrosine kinase. Caveolin peptides were examined for their effect on the autophosphorylation of purified recombinant Fyn kinase in vitro. The effects of peptide 1 (A) and peptide 2 (B) are shown. Note that peptide 1 had no effect, whereas peptide 2 dose-dependently suppressed the auto-activation of Fyn. Quantitation of these experiments is provided in C. Cumulative data are shown as the means ± S.D. These experiments were performed at least three times independently in duplicate.

Fig. 5. In vitro effect of the full-length caveolin molecule on the auto-activation of c-Src tyrosine kinase revealed by co-expression of caveolin and c-Src in 293T cells. A. 293T cells (1–2 × 10^6 cells/10-cm dish) were transfected with c-Src alone or co-transfected with c-Src plus caveolin. 48 h post-transfection, cells were washed and collected in lysis buffer. Cell lysates were immunoprecipitated with anti-Src IgG bound to protein A-Sepharose. These immunoprecipitates were subjected to immunoblot analysis with a Src mAb probe (top panel), an immune complex kinase assay to detect Src auto-phosphorylation (middle panel), and immunoblot analysis with a caveolin mAb probe (bottom panel). Note that although both immunoprecipitates contain equivalent amounts of c-Src (top panel), co-expression with caveolin prevents the auto-phosphorylation of c-Src (middle panel). Also, caveolin co-immunoprecipitates with c-Src when using antibodies directed against c-Src (bottom panel). One 10-cm dish was used per immunoprecipitation. B, quantitation of A (middle panel). The auto-phosphorylation of c-Src tyrosine kinase is expressed in arbitrary units.
Both classes of tyrosine kinases can function as transforming non-receptor tyrosine kinases must be activated indirectly. Binding of ligand to the extracellular protein domain, whereas receptor tyrosine kinases are activated by the membrane receptors and nonreceptor cytoplasmically oriented cellular growth control and differentiation. Two general classes of tyrosine kinases have been defined: transmembrane and non-receptor. c-Src is the prototype of a family of non-receptor tyrosine kinases. v-Src, the transforming component of the Rous sarcoma virus, is derived from c-Src, a normal cellular gene. Mutationally activated c-Src has been implicated in the pathogenesis of carcinomas, including breast tumors. In contrast, recombinant overexpression of wild-type c-Src does not result in efficient cell transformation or increased tyrosine phosphorylation, indicating that the tyrosine kinase activity of c-Src is normally repressed. However, the tightly regulated tyrosine kinase activity of c-Src can be activated transiently by a number of growth factors.

To date, a total of nine distinct members of the Src family of tyrosine kinases have been identified and cloned. c-Src and all other family members contain five conserved functional domains. i) The extreme N terminus contains a consensus sequence for lipid modification by addition of myristate, palmitate, or both fatty acyl moieties. ii) and (iii) The SH2 and SH3 domains function in protein-protein interactions. The SH2 domain recognizes specific phosphotyrosine based motifs, whereas the SH3 domain binds proline-rich peptide sequences with the consensus PXXP. (iv) The SH1 domain (or catalytic domain) functions in the recognition and tyrosine phosphorylation of specific substrates. (v) Finally, the extreme C terminus contains a site of negative regulation, Tyr527. As a consequence of C-terminal divergence, v-Src does not contain Tyr527, which when phosphorylated can act to functionally inactivate c-Src kinase.

It is now well established that tyrosine phosphorylation of c-Src itself plays a major role in controlling its intrinsic kinase activity. Autophosphorylation of Tyr416 located within the “activation domain” of the kinase domain results in activation of the enzyme, whereas phosphorylation of Tyr527 results in kinase inactivation. Phosphorylation of Tyr527 is thought to be mediated by a C-terminal Src kinase (Csk). After phosphorylation of Tyr527, c-Src is thought to “fold up” by the intramolecular interaction of Tyr527 with the SH2-domain, resulting in an inactive enzyme conformation.

Because elevated Src tyrosine kinase activity is associated with cellular transformation, many investigators have
searched for mechanisms to inactivate the kinase activity of c-Src. For example, Shoelson and colleagues have shown that high concentrations of peptide substrates inhibited the auto-phosphorylation of c-Src kinase in vitro. However, in both reports, the investigators used much higher concentrations of peptides, such as 0.24 mM or even 8.5 mM.

How does caveolin binding negatively regulate c-Src auto-activation? The functional existence of such a negative regulator or suppressor of c-Src has been suggested by earlier studies (37). Because mutations in the extreme C terminus, the kinase domain, the SH2 domain, or the SH3 domain can constitutively activate c-Src, this activation may be mediated a conformational change in one of these regions (38). Such a conformational change could also be induced normally by unknown allosteric effectors. Putative allosteric activators could stabilize c-Src in the activated state, whereas putative allosteric inhibitors could hold c-Src in an inactive conformation (37). Regarding allosteric inhibitors, they could act by shifting the equilibrium in favor of the inactive conformation, perhaps by masking Tyr416 from kinases or Tyr527 from phosphatases (37, 38).

Recent studies have identified and cloned caveolin (28). Two caveolin-related proteins (caveolin-2 and caveolin-3) have been identified and cloned; caveolin has been retermed caveolin-1. As a consequence, we also evaluated the effects of peptides derived from caveolin-2 and -3 that correspond to the 82–101 region in caveolin-1; the sequences of these peptides are detailed in A. All three peptides contain two conserved tyrosine residues (marked by arrows) and are extremely homologous. The effects of these peptides are shown in B. All peptides were added at a concentration of 1 μM. Note that only peptides derived from caveolin-1 and -3 exhibited inhibitory effects; the caveolin-2-derived peptide had no inhibitory effect.

Because a discrete cytosolic domain of caveolin (residues 82–101) (i) directly interacts with wild-type Gα subunits, H-Ras and c-Src; (ii) fails to interact with mutationally activated Gα subunits (Gα1, Q227L), H-Ras (G12V), and Src (v-Src); (iii) functionally suppresses the activity of both G-proteins and Src family tyrosine kinases, holding them in the inactive conformation; and (iv) is involved in the formation of multivalent caveolin-homooligomers, we propose the term caveolin-scaffolding domain be used when describing this region of caveolin.

![Image of caveolin peptides](image-url)

**Fig. 7. Effects of mutated caveolin peptides on the auto-activation of c-Src tyrosine kinase**. Wild-type and mutant caveolin peptides detailed in A were examined for their effect on the auto-phosphorylation of purified recombinant c-Src kinase in vitro. The effects of these peptides are shown in B. All peptides were added at a concentration of 1 μM. Note that both mutated caveolin peptides lacking tyrosine residues (Y→A and Y→F) were as effective or more effective than the wild-type (WT) peptide 2 (residues 82–101) peptide sequence in suppressing the auto-phosphorylation of c-Src. The Y→F peptide was approximately twice as potent as the wild-type caveolin peptide.

**Fig. 8. Differential effect of peptides derived from caveolins-1, -2, and -3 on the auto-activation of c-Src tyrosine kinase**. Recently, two caveolin-related proteins (caveolin-2 and caveolin-3) have been identified and cloned; caveolin has been retermed caveolin-1. As a consequence, we also evaluated the effects of peptides derived from caveolin-2 and -3 that correspond to the 82–101 region in caveolin-1; the sequences of these peptides are detailed in A. All three peptides contain two conserved tyrosine residues (marked by arrows) and are extremely homologous. The effects of these peptides are shown in B. All peptides were added at a concentration of 1 μM. Note that only peptides derived from caveolin-1 and -3 exhibited inhibitory effects; the caveolin-2-derived peptide had no inhibitory effect.

Because caveolin preferentially interacts with inactive c-Src (but not v-Src) and a caveolin-derived peptide or caveolin co-expression can inhibit auto-activation of c-Src, caveolin could act as an allosteric inhibitor of c-Src and other Src family kinases.

The subcellular distribution of several signaling molecules is restricted by association with scaffolding proteins (28). These scaffolding proteins, Ste5p (73, 74), AKAP-79 (29, 30, 75, 76), and 14–3-3 (77–80) may simultaneously associate with distinct classes of signaling proteins to form a signaling pathway or module. So far, the accumulated evidence suggests that caveolin possesses all the qualities of a “classic” scaffolding protein because caveolin forms multivalent homo-oligomers and each caveolin-interacting protein binds to the same cytosolic membrane-proximal region of caveolin. As such, caveolin may provide a selective framework that segregates one group of signaling events from the next by preventing cross-talk between functionally unrelated signaling modules while facilitating cross-talk between related signaling modules.

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