

Phosphorylation of Caveolin by Src Tyrosine Kinases

THE α -ISOFORM OF CAVEOLIN IS SELECTIVELY PHOSPHORYLATED BY v-Src *IN VIVO**

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Caveolae are flask-shaped plasma membrane specializations that are thought to exist in most cell types. A 22-kDa protein, caveolin, is an integral membrane component of caveolae membranes *in vivo*. Previous studies have demonstrated that caveolin is phosphorylated on tyrosine by oncogenic viral Src (v-Src) and that caveolin is physically associated as a hetero-oligomeric complex with normal cellular Src (c-Src) and other Src family tyrosine kinases. Caveolin contains eight conserved tyrosine residues that may serve as potential substrates for Src. Here, we have begun to study the phosphorylation of caveolin by Src family tyrosine kinases both *in vitro* and *in vivo*. Using purified recombinant components, we first reconstituted the phosphorylation of caveolin by Src kinase *in vitro*. Microsequencing of Src-phosphorylated caveolin revealed that phosphorylation occurs within the extreme N-terminal region of full-length caveolin between residues 6 and 26. This region contains three tyrosine residues at positions 6, 14, and 25. Deletion mutagenesis demonstrates that caveolin residues 1–21 are sufficient to support this phosphorylation event, implicating tyrosine 6 and/or 14. *In vitro* phosphorylation of caveolin-derived synthetic peptides and site-directed mutagenesis directly show that tyrosine 14 is the principal substrate for Src kinase. In support of these observations, tyrosine 14 is the only tyrosine residue within caveolin that bears any resemblance to the known recognition motifs for Src family tyrosine kinases. In order to confirm or refute the relevance of these *in vitro* studies, we next analyzed the tyrosine phosphorylation of endogenous caveolin in v-Src transformed NIH 3T3 cells. *In vivo*, two isoforms of caveolin are known to exist: α -caveolin contains residues 1–178 and β -caveolin contains residues 32–178. Only α -caveolin underwent tyrosine phosphorylation in v-Src transformed NIH 3T3 cells, although β -caveolin is well expressed in these cells. As β -caveolin lacks residues 1–31 (and therefore tyrosine 14), these *in vivo* studies directly demonstrate the validity of our *in vitro* studies. Because α - and β -caveolin are known to assume a distinct but overlapping subcellular distribution within a single cell, v-Src phosphorylation of α -caveolin may only affect a subpopulation of caveolae that contain α -caveolin.

Caveolae are specialized domains of the plasma membrane (1, 2). They are most numerous in endothelial cells, fibroblasts, adipocytes, and smooth muscle cells, although they appear to exist in most cells (3). Their exact function remains unknown, although they have recently been implicated in certain transmembrane signaling events, including G protein-coupled signaling (1, 4–13).

Caveolin, a 21–24-kDa integral membrane protein, is a principal component of caveolae membranes *in vivo* (14). Several independent lines of evidence suggest that caveolin may act as a scaffolding protein within caveolae membranes. In support of this assertion: (i) both the N-terminal and C-terminal domains of caveolin face the cytoplasm (15); (ii) caveolin exists within caveolae membranes as a high molecular mass homo-oligomer (16, 17); (iii) these caveolin homo-oligomers have the capacity to self-associate *in vitro* to form larger structures that resemble caveolae (16); (iv) caveolin co-purifies with cytoplasmic signaling molecules including trimeric G proteins, Src family tyrosine kinases, and Ras-related GTPases (4–6); (v) recombinant caveolin interacts directly with hetero-trimeric G proteins (10); and (vi) caveolin binding can functionally suppress the GTPase activity and GTP binding of purified trimeric G proteins, holding the G protein in the inactive conformation (10). Thus, caveolin may serve as an oligomeric docking site for organizing and concentrating inactive signaling molecules within caveolae membranes (16).

Modification and/or inactivation of caveolin may be a common feature of the transformed phenotype (11). Historically, caveolin was first identified as a major v-Src substrate in Rous sarcoma virus-transformed cells (18). Caveolin is one of only a few known transformation-dependent v-Src substrates; both cell transformation and tyrosine phosphorylation of caveolin by v-Src are critically dependent on membrane attachment of Src via N-terminal myristoylation (19). Based on these observations, Glenney and co-workers have proposed that caveolin may represent a critical target during cellular transformation. In direct support of this notion, caveolin expression and caveolae are dramatically reduced or absent in cells transformed by activated oncogenes other than v-Src (v-Abl, Bcr-Abl, and activated Ras) (11).

The functional consequences of the phosphorylation of caveolin on tyrosine are not yet known. At steady state, caveolin is not phosphorylated on tyrosine (12, 20). This is in contrast to v-Src-transformed cells where caveolin is constitutively phosphorylated on tyrosine (19). However, caveolin tyrosine phosphorylation also occurs in normal cells but in a tightly regulated fashion (12). If 3T3-L1 adipocytes are serum-starved and rapidly stimulated with insulin, caveolin transiently undergoes phosphorylation on tyrosine in a time- and dose-dependent manner (12). It has been suggested that insulin-stimulated tyrosine phosphorylation of caveolin occurs indirectly via an endogenous Src family tyrosine kinase, rather than directly via

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the insulin receptor. In support of this idea, caveolin can be purified as part of a hetero-oligomeric complex that contains c-Src and other Src family tyrosine kinases (4, 5).

Here, we have begun to study the tyrosine phosphorylation of caveolin by Src family tyrosine kinases both *in vitro* and *in vivo*. We first reconstituted the tyrosine phosphorylation of caveolin by c-Src *in vitro*. Our results indicate that tyrosine 14 of caveolin is the principal site that is recognized by the Src kinase *in vitro*. In addition, we also demonstrated the *in vivo* relevance of these *in vitro* studies using v-Src-transformed NIH 3T3 cells that endogenously express caveolin.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal and mouse monoclonal antibodies directed against caveolin were provided by John R. Glenney (Transduction Laboratories). The cDNA for canine caveolin was obtained as we described previously (4). NIH 3T3 cells expressing v-Src were provided by David Shalloway (Cornell University, Ithaca, NY). A series of overlapping caveolin peptides coupled via their C-terminal amino acid to a membranous support were from Research Genetics. A variety of other reagents were purchased from commercial suppliers: purified recombinant c-Src tyrosine kinase (Upstate Biotechnologies, Inc); glutathione-agarose (Sigma); polyadenylic acid (Boehringer Mannheim); anti-phosphotyrosine IgG (4G10; Upstate Biotechnologies, Inc.); phosphatase inhibitors (Sigma); pre-stained protein markers (Life Technologies, Inc); fetal bovine serum (JRH Biosciences, Inc); and protein A-Sepharose (Pharmacia Biotech Inc.).

GST-Caveolin Fusion Proteins—The construction, expression, and purification of GST-caveolin fusion proteins were as we described previously (10, 16, 21). Briefly, full-length caveolin (residues 1–178) or regions of the caveolin N-terminal domain (residues 1–21, 1–41, 1–81, 1–101, and 61–101) were subcloned into the vector pGEX-4T-1, expressed in *E. coli* (BL21 strain; Novagen, Inc), and affinity purified by chromatography on glutathione-agarose (22). A single tyrosine residue at position 14 was mutated to phenylalanine by changing TAC to TTC. Single base mutations were introduced by polymerase chain reaction site-directed mutagenesis (23).

In Vitro Phosphorylation—GST or GST-caveolin fusion proteins (1–5 μ g) were incubated with 2 units of purified c-Src in 20 μ l of kinase reaction buffer (20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM $MnCl_2$), and the reaction was initiated by addition of 10 μ Ci of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. After 15 min at 25 °C, the reaction was stopped by addition of 2 \times SDS-PAGE¹ sample buffer and boiling for 2 min. Phosphorylated proteins were visualized by autoradiography using an intensifying screen.

Microsequence Analysis—*In vitro* phosphorylated GST-caveolin was separated by SDS-PAGE, transferred to nitrocellulose, and visualized by Ponceau S staining. The GST-caveolin band was excised and enzymatically digested with the endoproteinase Lys-C in the presence of 1% hydrogenated Triton X-100/10% acetonitrile/100 mM Tris, pH 8.0, for 24 h at 37 °C (5, 21). Eluted peptides were isolated by HPLC (~80 column fractions) and dot blotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Inc.), and radioactive fractions were identified by autoradiography using an intensifying screen. Fractions containing the phosphorylated peptide were subjected to N-terminal sequencing using a pulsed liquid protein sequencer (model 447A/120A; Applied Biosystems).

Synthesis of Immobilized Caveolin-derived Peptides—Caveolin-derived polypeptides were synthesized directly onto an activated polymeric membrane by Research Genetics. The peptide chemistry was standard Fmoc with coupling mediated through HOBt/DIC and Fmoc removal with piperidine/*N,N*-dimethylformamide (1:3). For final peptide protecting group removal, the membrane was placed in a bath of DCM/trifluoroacetic acid/thioanisole/EDTA/anisole (50:47.5:2.5:1.5:1) for 1 h and finally washed and dried.

Src Phosphorylation of Caveolin Peptides—Immobilized caveolin-derived peptides were first incubated overnight at 4 °C with kinase reaction buffer (defined above) containing purified GST (~1 mg/ml) and polyadenylic acid (0.1 mg/ml). After washing two times with kinase reaction buffer, peptides were incubated with 15 units of purified c-Src kinase in 2 ml of kinase reaction buffer; the reaction was initiated by the addition of 1 mM ATP. After 30 min at 25 °C, the reaction was

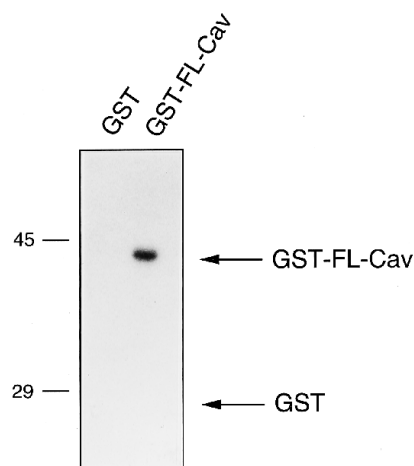


FIG. 1. ***In vitro* phosphorylation of recombinant full-length caveolin by Src kinase.** Purified full-length caveolin (GST-FL-Cav) expressed as a GST fusion protein or GST alone were subjected to *in vitro* phosphorylation with purified c-Src and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in kinase reaction buffer. After SDS-PAGE, phosphorylated proteins were visualized by autoradiography. Note that each reaction contained equivalent amounts of GST and purified full-length caveolin, although only caveolin undergoes phosphorylation. Phosphorylation of purified full-length caveolin was specifically dependent on addition of both c-Src and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Omission of either c-Src or $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ prevented phosphorylation (not shown).

stopped by removal of c-Src and extensive washing with 2 \times SSC/0.1% SDS (three washes of 20 min each). Samples were then subjected to immunoblotting with mAb 4G10 (1:500 dilution) to detect tyrosine-phosphorylated peptides.

Immunoprecipitation and Western Blotting of Caveolin—NIH 3T3 cells expressing v-Src were collected and solubilized with lysis buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1% Triton X-100, 60 mM octyl-glucoside, 1 mM phenylmethylsulfonyl fluoride) containing phosphatase inhibitors (30 mM sodium pyrophosphate, 50 mM NaF, and 100 μ M Na_3VO_4). After pre-clearing with protein A-Sepharose, samples were immunoprecipitated with rabbit polyclonal IgG (from Transduction Laboratories) that recognizes both caveolin isoforms and whose epitope maps to caveolin residues 38–49.² After immunoprecipitation, both α - and β -caveolin isoforms were visualized by immunoblotting with mAb 2297 (at a dilution of 1:500; Transduction Laboratories), and tyrosine-phosphorylated caveolin was visualized by immunoblotting with mAb 4G10 (at a dilution of 1:500; Upstate Biotechnologies, Inc.).

RESULTS

In order to understand the potential role of caveolin in v-Src transformation, we have begun to map the site or sites of caveolin tyrosine phosphorylation by Src. Caveolin contains eight tyrosine residues that are conserved across species and could serve as potential sites for tyrosine phosphorylation (Tyr⁶, Tyr¹⁴, Tyr²⁵, Tyr⁹⁷, Tyr¹⁰⁰, Tyr¹¹⁸, Tyr¹⁴⁸, and Tyr¹⁵¹; see Tang *et al.* for an alignment (24)). Note that tyrosine 42 is found only in canine caveolin.

In Vitro Phosphorylation of Caveolin by Purified Src Kinase—We reconstituted the tyrosine phosphorylation of caveolin by Src *in vitro*. Recombinant full-length caveolin, expressed as a GST-fusion protein, was incubated with purified c-Src kinase in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Under these conditions, only caveolin underwent Src-mediated phosphorylation. GST alone failed to be phosphorylated, despite the fact that it contains numerous tyrosine residues (Fig. 1). This result demonstrates the specificity of this phosphorylation event.

To identify a region of caveolin that is phosphorylated, we digested Src-phosphorylated caveolin with the endoproteinase

¹ The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; mAb, monoclonal antibody.

² M. P. Lisanti, S. Li, and R. Seitz, unpublished observations.

TABLE I
Caveolin peptides expected after digestion with Lys-C

Peptides containing tyrosine residues are bold, and individual tyrosine residues are underlined.

Peptide	Sequence	Caveolin residues
1	MSGGK	1-5
2	YVDSEGHLYTVP IREQGN IYK	6-26
3	PNNK	27-30
4	AMAEEMSEK	31-39
5	QVYDAHTK	40-47
6	EIDLNVNRDPK	48-57
7	HLNDDVVK	58-65
8	IDFEDVIAEPEGTHSFDGIWK	66-86
9	ASFTTFTVTK	87-96
10	YWFYRLLSALFGIPMALIWGIYF AILSFLHIWAVVPCIK	97-135
11	SFLIEIQ CISRVYSI YVHTFCDPFF EAVGK	136-165
12	IFSNIRINMQK	166-176
13	ET	177-178

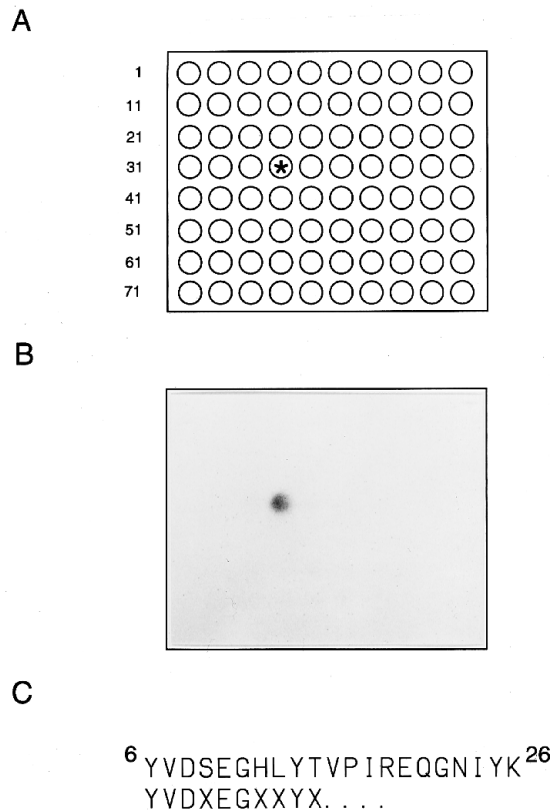


FIG. 2. **Microsequence analysis of Src-phosphorylated caveolin.** Caveolin was phosphorylated by c-Src as in Fig. 1 and subjected to microsequence analysis as we have described previously for other proteins (5, 21). Briefly, after SDS-PAGE and transfer to nitrocellulose, the caveolin band was excised and digested with Lys-C. A, after digestion, peptides were separated by HPLC and 80 column fractions were collected. B, an aliquot of each column fraction was then spotted onto polyvinylidene difluoride membranes and subjected to autoradiography. Only fraction 34 contained a radiolabeled peptide. C, microsequencing of this peptide revealed that it corresponds to caveolin residues 6-26. This indicates that tyrosine residues 6, 14, and/or 25 are the most likely sites for Src phosphorylation.

Lys-C and separated the peptides by HPLC. A total of thirteen peptides are expected from digestion of caveolin with Lys-C; only four of these peptides contain tyrosine residues (Table I). Eighty column fractions were collected, and a single column fraction containing a radiolabeled peptide was identified (Fig. 2, A and B). Microsequencing of this peptide revealed that caveolin is phosphorylated at its extreme N terminus (tyrosine at amino acid position 6, 14, or 25) (Fig. 2C).

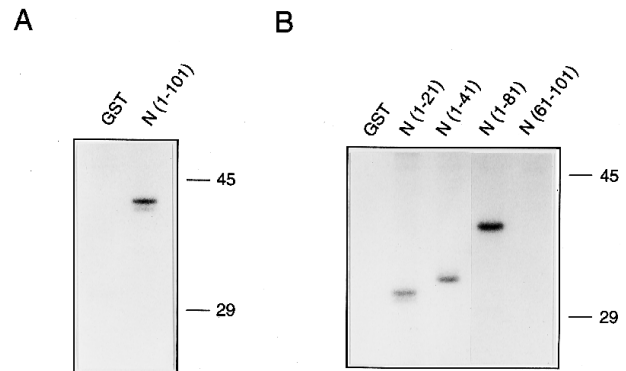


FIG. 3. **Caveolin residues 1-21 are sufficient for phosphorylation of caveolin by Src.** Recombinant fusion proteins encoding various portions of the N-terminal cytoplasmic domain of caveolin (A, residues 1-101; B, residues 1-21, 1-41, 1-81, and 61-101) or GST alone were subjected to *in vitro* phosphorylation by purified c-Src kinase. Each reaction contained equivalent amounts of GST and GST-caveolin fusion proteins. Src-phosphorylated proteins were visualized after SDS-PAGE by autoradiography. Note that caveolin residues 1-21 are sufficient to support Src phosphorylation, whereas residues 61-101 are not phosphorylated.

Because our microsequencing results suggest that the extreme N terminus of caveolin is the target for Src phosphorylation, we evaluated whether various portions of the N-terminal caveolin domain expressed as GST fusion proteins were capable of supporting Src-mediated phosphorylation. Fig. 3 shows that caveolin residues 1-21 are sufficient to undergo Src-mediated phosphorylation. This suggests that tyrosine at either position 6 or position 14 is the primary site of Src phosphorylation. These observations are also consistent with our results from microsequence analysis.

Tyrosine 14 Is the Principal Site of Caveolin Phosphorylation by Src—To determine if tyrosine 6, 14, or 25 is the preferred substrate for Src phosphorylation, we next generated a series of eight overlapping caveolin-derived peptides that include these tyrosine residues. Their sequences are listed in Table II. Peptides were directly synthesized as discrete spots on a membranous support and are covalently coupled to the support via their C-terminal amino acid. These immobilized peptides were then incubated with purified Src kinase in the presence of nonradioactive ATP. After extensive washing with SDS-containing solutions, phosphotyrosine residues were visualized by immunoblotting with the mAb 4G10 that recognizes phosphorylated tyrosine residues. Fig. 4A shows that only the peptide containing tyrosine 14 underwent phosphorylation under these conditions despite the fact that four other caveolin peptides

TABLE II
In vitro phosphorylation of caveolin peptides by Src kinase

Peptide	Sequence	Caveolin residues	Tyrosine residues	Phosphorylation
1	MSGGKYVDSEGH	1–12	Y6	–
2	DSEGHLYTVPIR	8–19	Y14	+
3	TVPIREQGNIYK	15–26	Y25	–
4	NIYKPNNKAMAE	23–34	Y25	–
5	KAMAEEMSEKQV	30–41	–	–
6	EKQVYDAHTKEI	38–49	Y42	–
7	HTKEIDLVRNDP	45–56	–	–
8	VNRDPKHLNDDV	52–63	–	–

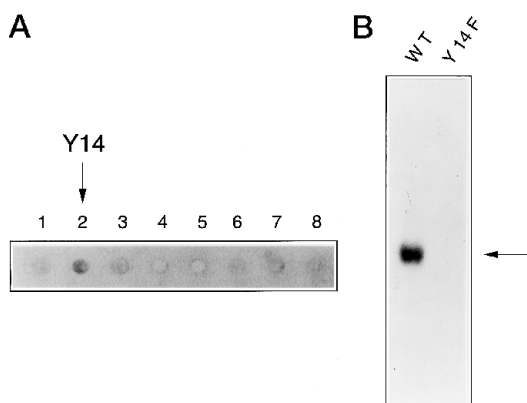


FIG. 4. **Tyrosine 14 is the principal site of phosphorylation by Src kinase *in vitro*.** A, tyrosine phosphorylation of caveolin-derived peptides by Src kinase. Caveolin-derived peptides 1–8 (detailed in Table II) were directly synthesized as discrete spots on a membranous support. These immobilized peptides were then incubated with purified Src kinase in the presence of 1 mM ATP. Phosphotyrosine residues were visualized by immunoblotting with the mAb 4G10. Only peptide 2 (the Tyr¹⁴-containing peptide) underwent tyrosine phosphorylation under these conditions. B, *in vitro* phosphorylation of the Y14F mutant by Src kinase. Recombinant GST-fusion proteins containing residues 1–41 of the N-terminal domain of caveolin were subjected to *in vitro* phosphorylation by purified c-Src kinase. WT, wild type; Y14F, tyrosine 14 to phenylalanine mutant. Each reaction contained equivalent amounts of wild type and the Y14F mutant. Src-phosphorylated proteins were visualized after SDS-PAGE by autoradiography. Note that the Y14F mutant does not undergo phosphorylation, although it contains tyrosine residues 6 and 25.

tested contained tyrosine residues.

To confirm the relative importance of tyrosine 14 as a substrate for Src phosphorylation, we used a second independent approach. We mutated tyrosine 14 of GST-caveolin to phenylalanine by site-directed mutagenesis (Y14F). In accordance with the above peptide experiments, *in vitro* kinase assays revealed that the Y14F mutant failed to undergo phosphorylation by Src (Fig. 4B). This suggests that tyrosine 14 is the primary site of Src phosphorylation *in vitro*. These results are also consistent with our results independently obtained through microsequencing and deletion mutagenesis.

***In Vivo* Analysis of the Tyrosine Phosphorylation of Caveolin Isoforms**—*In vivo* caveolin exists as two isoforms that differ in their extreme N-terminal protein sequence (21). α -Caveolin contains residues 1–178, whereas β -caveolin contains residues 32–178. These isoforms derive from a single gene through alternate initiation during translation. More specifically, methionine 32 serves as an internal start site to generate β -caveolin (21). Functionally, both caveolin isoforms are targeted to caveolae, form homo-oligomers, and interact with G proteins. However, α - and β -caveolin assume a distinct but overlapping subcellular distribution in intact cells (21). These results suggest that co-expression of α - and β -caveolin within a single cell

may be used to generate at least two distinct subpopulations of caveolae (21).

Because our *in vitro* data suggest that Src phosphorylates caveolin at its extreme N terminus on tyrosine 14, our results predict that *in vivo* only α -caveolin should be phosphorylated by Src because β -caveolin lacks tyrosine 14 because it does not contain caveolin residues 1–31. To test this prediction, we immunoprecipitated caveolin from v-Src-transformed NIH 3T3 cells and blotted these immunoprecipitates with an antibody (4G10) that recognizes phosphotyrosine residues. Fig. 5 shows that although both α - and β -caveolin isoforms are present in these immunoprecipitates, α -caveolin is selectively phosphorylated on tyrosine. These results directly demonstrate the *in vivo* relevance and the validity of our *in vitro* studies.

DISCUSSION

The oncogene v-Src arose by viral transduction of the normal cellular gene c-Src (25, 26). Thus, it is thought that viral tyrosine kinases largely induce transformation by intercepting cell regulatory mechanisms that are normally under the control of tyrosine phosphorylation. In support of this notion, v-Src and c-Src appear to differ primarily in enzymatic activity but not in their substrate specificity (27, 28). For example, both c-Src and v-Src phosphorylate Ras GAP at the same major and minor sites both *in vitro* and *in vivo* (29, 30). This difference in enzymatic activity can be attributed to the loss of tyrosine residue 527 within v-Src. Phosphorylation at this C-terminal site within c-Src by CSK kinase normally inactivates c-Src (31, 32).

Few transformation-dependent v-Src substrates are known. Caveolin is one of these transformation-dependent substrates. Tyrosine phosphorylation of caveolin by v-Src requires the membrane attachment of v-Src via myristoylation (19). Similarly, membrane attachment of v-Src is required for its transforming activity. Thus, Glenney (19) has suggested that caveolin may represent a critical target for v-Src. Also, caveolin co-purifies as a hetero-oligomeric complex with c-Src and other Src family tyrosine kinases (4, 5). This is consistent with the general idea that v-Src phosphorylates the normal targets of c-Src or related Src family tyrosine kinases but in an unregulated fashion.

Here, we have reconstituted the tyrosine phosphorylation of caveolin *in vitro* using purified recombinant components: c-Src and GST-caveolin fusion proteins. By using a combination of microsequencing, deletion mutagenesis, synthetic peptide substrates, and site-directed mutagenesis, this *in vitro* approach has allowed us to identify tyrosine 14 within caveolin as the principal site of phosphorylation by Src. The specific phosphorylation of a given tyrosine residue by Src family kinases is dependent on the surrounding amino acid sequences (28). In this regard, it is interesting to note that tyrosine 14 is the only tyrosine-containing site within caveolin that bears any homology to the known consensus sites for v-Src and c-Abl tyrosine kinases (Fig. 6).

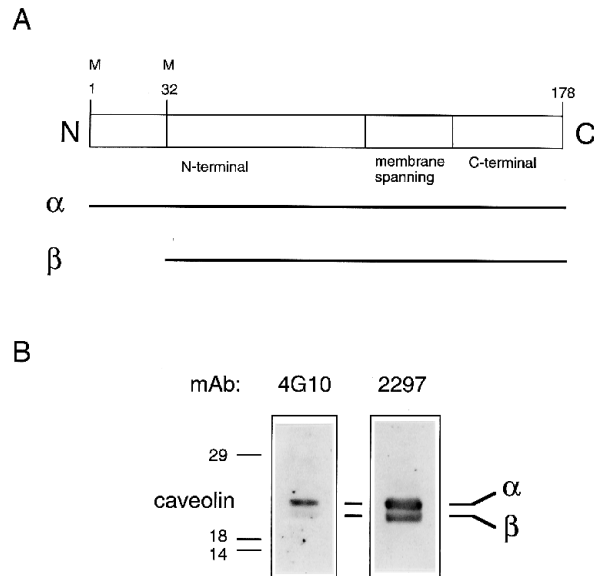


FIG. 5. Only the α -isoform of caveolin is phosphorylated on tyrosine in v-Src transformed NIH 3T3 cells *in vivo*. A, schematic diagram showing the positions of the two start sites within the caveolin coding sequence and the resulting caveolin isoforms that differ in their extreme N-terminal protein sequence. Note that only α -caveolin contains tyrosine 14. β -Caveolin specifically lacks tyrosine 14 because it does not contain caveolin residues 1–31. B, v-Src-transformed NIH 3T3 cells were solubilized in a buffer containing octyl-glucoside and tyrosine phosphatase inhibitors and subjected to immunoprecipitation with a rabbit polyclonal antibody that recognizes both α - and β -caveolin isoforms. After SDS-PAGE and transfer to nitrocellulose, caveolin immunoprecipitates were probed with a monoclonal antibody that recognizes phosphotyrosine residues (4G10) or another monoclonal antibody (2297) that recognizes both α - and β -caveolin isoforms.

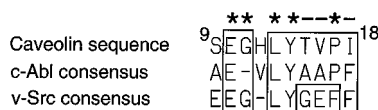


FIG. 6. Comparison of caveolin tyrosine 14 and its surrounding sequence with the preferred recognition sequences of tyrosine kinases. The amino acid sequence of canine caveolin (residues 9–18) is compared with the preferred recognition sequences of c-Abl and v-Src (28). Identical residues and conservative substitutions are boxed. An asterisk indicates that a given caveolin residue is identical in all caveolin molecules across species (from chick to man). A dash indicates that a given caveolin residue is not identical across species but undergoes a conservative substitution. Note that the substrate preferences for v-Src and c-Src are identical and are detailed in Songyang *et al.* (28).

Tyrosine 14 is present at the extreme N terminus of full-length caveolin. However, two isoforms of caveolin exist that differ in their extreme N terminus (21). These two isoforms derive from a single gene through alternate initiation during translation; methionine 32 serves as an internal start site to generate β -caveolin (21). Therefore, α -caveolin contains the complete caveolin sequence, whereas β -caveolin lacks residues 1–31 and therefore tyrosine 14. Because β -caveolin specifically lacks tyrosine 14, it should not undergo phosphorylation by Src. In direct support of this prediction, we observed that α -caveolin is selectively phosphorylated on tyrosine in v-Src-transformed NIH 3T3 cells, although these cells express both α - and β -caveolin. Thus, it appears that α -caveolin is actually the target of v-Src rather than β -caveolin. This is the first time this distinction has been delineated. This may be important because α - and β -caveolin assume a distinct but overlapping subcellular distribution within a single cell, implying the existence of subpopulations of caveolae (21). In addition, α -cave-

lin is preferentially phosphorylated on tyrosine in response to insulin stimulation *in vivo*, although this was not noted by the authors because the structural differences between α - and β -caveolin were not known at the time (12). This insulin-stimulated tyrosine phosphorylation of caveolin is thought to occur via an endogenous member of the Src family of tyrosine kinases (12) rather than directly through the insulin receptor, which also resides within caveolae (33).

Alternate initiation from the same mRNA transcript is used to generate two isoforms of another tyrosine kinase substrate: the 52- and 46-kDa isoforms of Shc (34). In this case, initiation from an internal methionine residue results in an N-terminal 59-amino acid truncation of the 46-kDa isoform relative to the 52-kDa isoform. Like caveolin, these two isoforms are differentially recognized by tyrosine kinases. Specifically, only the 52-kDa isoform is a substrate for the insulin receptor kinase, whereas both the 52- and the 46-kDa isoforms are recognized equally well by the epidermal growth factor receptor kinase (34). Thus, alternate initiation may provide a means for generating distinct forms of the same gene product that can be differentially regulated by a given signaling molecule.

What could be the functional consequences of the tyrosine phosphorylation of caveolin? Caveolin exists as a high molecular mass oligomer containing ~14–16 individual caveolin molecules (16, 17), and these caveolin homo-oligomers have the capacity to self-associate into larger complexes (16). Thus, tyrosine-phosphorylated caveolin could potentially serve as an oligomeric docking site for SH2 domain signaling molecules—much like activated growth factor receptors that oligomerize, undergo tyrosine phosphorylation, and recruit SH2 domain-containing proteins to the cytoplasmic surface of the plasma membrane. We are currently searching for proteins that preferentially bind to Src-phosphorylated caveolin.

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