Palmitoylation of Caveolin-1 at a Single Site (Cys-156) Controls Its Coupling to the c-Src Tyrosine Kinase

TARGETING OF DUALLY ACYLATED MOLECULES (GPI-LINKED, TRANSMEMBRANE, OR CYTOPLASMIC) TO CAVEOLAE EFFECTIVELY UNCOUPLES c-Src AND CAVEOLIN-1 (TYR-14)*

Dispersed within the “sea” of phospholipids that constitute the plasma membrane are cholesterol/sphingolipid-enriched microdomains called caveolae (1–4). Caveolin proteins are the principal structural components of caveolae membranes (5–8). There are now three known caveolin genes (Cav-1, Cav-2, and Cav-3) that correspond to four different subtypes of caveolin proteins. Caveolin-1 (α and β) and caveolin-2 proteins have nearly identical tissue distributions, being most abundantly expressed in terminally differentiated cell types (adipocytes, endothelial cells, fibroblasts, type I pneumocytes, and smooth muscle) (9–14). The caveolin-3 protein, however, is mainly expressed in muscle tissues (15–17). Several independent lines of evidence now indicate that the expression of either caveolin-1 or caveolin-3 is sufficient to induce the morphological formation of caveolae membranes; however, caveolin-2 lacks the ability to induce caveolae formation (18–24).

Caveolin-1 was first identified as a tyrosine-phosphorylated protein in v-Src-transformed cells (5). Soon after, caveolin-1 was shown to be the protein component that stipples the caveolar membrane (7). The discovery that many signaling molecules interact with the Src homology 2 and/or phosphotyrosine binding domains of Grb7, the only characterized downstream mediator of its function. Taken together, our data identify a series of novel lipid-lipid-based interactions as important regulatory factors for coupling caveolin-1 to the c-Src tyrosine kinase in vivo.

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Caveolin-1 was initially identified as a phosphoprotein in Rous sarcoma virus-transformed cells. Previous studies have shown that caveolin-1 is phosphorylated on tyrosine 14 by c-Src and that lipid modification of c-Src is required for this phosphorylation event to occur in vivo. Phosphocaveolin-1 (Tyr(P)-14) localizes within caveolae near focal adhesions and, through its interaction with the c-Src tyrosine kinase. Furthermore, upon evaluating a battery of nonreceptor and receptor tyrosine kinases, we demonstrate that the tyrosine phosphorylation of caveolin-1 by c-Src is a highly selective event. We show that Src-induced tyrosine phosphorylation of caveolin-1 can be inhibited or uncoupled by targeting dually acylated molecules (GPI-linked, transmembrane, or cytoplasmic) to the caveolar membrane, respectively. Conversely, when these proteins are not properly targeted or lipid-modified, the ability of c-Src to phosphorylate caveolin-1 remains unaffected. In addition, when purified caveolae preparations are preincubated with a myristoylated peptide derived from the extreme N terminus of c-Src, the tyrosine phosphorylation of caveolin-1 is abrogated; the same peptide lacking myristoylation has no inhibitory activity. However, an analogous myristoylated peptide derived from c-Yes also has no inhibitory activity. Thus, the inhibitory effects of the myristoylated c-Src peptide are both myristoylation-dependent and sequence-specific. Finally, we investigated whether phosphocaveolin-1 (Tyr(P)-14) interacts with the Src homology 2 and/or phosphotyrosine binding domains of Grb7, the only characterized downstream mediator of its function. Taken together, our data identify a series of novel lipid-lipid-based interactions as important regulatory factors for coupling caveolin-1 to the c-Src tyrosine kinase in vivo.

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Dispersed within the “sea” of phospholipids that constitute the plasma membrane are cholesterol/sphingolipid-enriched microdomains called caveolae (1–4). Caveolin proteins are the principal structural components of caveolae membranes (5–8). There are now three known caveolin genes (Cav-1, Cav-2, and Cav-3) that correspond to four different subtypes of caveolin proteins. Caveolin-1 (α and β) and caveolin-2 proteins have nearly identical tissue distributions, being most abundantly expressed in terminally differentiated cell types (adipocytes, endothelial cells, fibroblasts, type I pneumocytes, and smooth muscle) (9–14). The caveolin-3 protein, however, is mainly expressed in muscle tissues (15–17). Several independent lines of evidence now indicate that the expression of either caveolin-1 or caveolin-3 is sufficient to induce the morphological formation of caveolae membranes; however, caveolin-2 lacks the ability to induce caveolae formation (18–24).

Caveolin-1 was first identified as a tyrosine-phosphorylated protein in v-Src-transformed cells (5). Soon after, caveolin-1 was shown to be the protein component that stipples the caveolar membrane (7). The discovery that many signaling molecules reside within caveolae (25) and that caveolin-1 can regulate their function and antagonize cellular transformation (26, 27) made the initial observation that caveolin-1 is a substrate of v-Src all the more salient.

Many of the features of caveolins suggest that they serve as scaffolding proteins able to concentrate signaling molecules within caveolae and integrate signaling processes: 1) caveolins hetero- and homo-oligomerize to form high molecular mass structures at the membrane (17, 28–31); 2) both the N- and C-terminal domains of caveolin-1 face the cytoplasm (29, 32–35); 3) cytoplasmic lipid-modified signaling molecules co-purify with caveolins (25, 36–39); and 4) recombinant caveolin-1 can directly interact with acylated signaling molecules (38, 40–42), and 5) caveolins are also required to regulate certain integrin-mediated processes as well as other signaling events (42–45).

The packing of cholesterol and sphingolipids into caveolae imposes a certain lateral organization to the caveolar mem-
brane that makes it biophysically distinct from the rest of the “fluid” phospholipid membrane (4, 46). This lipid order is thought to energetically favor the intercalation of saturated fatty acyl groups on proteins. Thus, one might suppose that the three palmitoylation sites on caveolin-1 would play an important role in its interaction with the caveolar membrane. However, the palmitoyl groups on caveolin are not necessary for its membrane attachment or for targeting of caveolin-1 to caveolae (34, 47). This suggests that these palmitoyl groups may serve other functions, perhaps in regulating the interaction of caveolin-1 with other caveolar protein components. Here, we directly explore this hypothesis.

Many acylated signaling proteins, such as eNOS, Ga subunits, and Src family kinases also reside within caveolae (1, 3, 36, 48). However, unlike caveolin-1, these molecules localize to noncaveolar cell compartments when their sites of myristoylation and/or palmitoylation are eliminated. Thus, it appears that lipid modification of certain signaling molecules serves to target them to caveolae, where they can interact with caveolin-1 (49).

Here, we show that c-Src-mediated phosphorylation of caveolin-1 is highly selective and is strictly dependent on the palmitoylation of caveolin-1 at a single site (Cys-156). We speculate that this coupling event occurs through an interaction between the N-terminal myristoyl moiety of c-Src and the palmitoyl group attached to caveolin-1 at Cys-156. A prediction of this hypothesis is that targeting of other acylated proteins to caveolae should be able to competitively uncouple c-Src from caveolin-1. In direct support of this hypothesis, we demonstrate that targeting of three distinct classes of dually acylated proteins (GPT-linked, transmembrane, and cytoplasmically oriented) to caveolae is sufficient to inhibit Src-induced phosphorylation of caveolin-1 in vivo. Similarly, in vitro competition experiments show that a peptide encoding the myristoylated N terminus of Src prevents the tyrosine phosphorylation of caveolin-1 in purified preparations of caveolae membranes. We also show that the inhibitory effects of the myristoylated c-Src peptide are both myristoylation-dependent and sequence-specific. Thus, our current studies demonstrate that the lipid-modified nature of certain proteins not only serves to target them to caveolae but may also function to modulate the balance of protein-protein interactions occurring within caveolae membranes.

EXPERIMENTAL PROCEDURES

Materials—The cDNAs encoding wild type caveolin-1 and caveolin-1 palmitoylation mutants were essentially as described previously (25, 32, 34) except that they were not epitope-tagged, and they were subcloned into the β-actin/cytomegalovirus-based vector pCAGGS. The cDNA encoding human c-Src WT in the pUSEKamp cytomegalovirus-based vector was purchased from Upstate Biotechnology, Inc. The cDNAs encoding Fyn, c-Yes (WT), and c-Yes (Y535F) were the generous gifts of Dr. Marius Sudel (Mount Sinai School of Medicine). The cDNA encoding the epidermal growth factor receptor (EGF-R), insulin receptor, and hepatocyte growth factor receptor-c-Met were the gifts of Drs. Gordon N. Gill (University of California, San Diego), Jonathan M. Backer (Department of Molecular Pharmacology, Albert Einstein College of Medicine), and George F. Vande Woude (NCI-Frederick Cancer Research and Development Center). Gaα15-GFP fusion protein constructs were as described previously (49). VS epitope-tagged human Lck, Lyn, Hck, Fgr, Blk, Frk, Csk, fibroblast growth factor receptor, and CD36 subcloned into pcDNA3.1 GS were purchased from Invitrogen, Inc. ("Genetorn Clones"). GST fusion proteins encoding the SH2 domain of Grb7 (residues 418–535) and the phosphotyrosine binding (PTB) domain of Grb7 (residues 345–438) were generated by polymer-
RESULTS

c-Src Phosphorylates Caveolin-1 on Tyrosine 14 in a Highly Specific Manner—We previously identified tyrosine 14 of caveolin-1 as the principle site of phosphorylation by c-Src both in vitro and in vivo (52). In agreement with this finding, pretreatment of purified caveolae-enriched membranes with a selective inhibitor of Src-family tyrosine kinases inhibits the phosphorylation of caveolin-1 on tyrosine 14 (50). In the interest of exploring the specificity of the c-Src/caveolin-1 relationship, here we examined the ability of a panel of Src-related nonreceptor tyrosine kinases (NRTKs) to phosphorylate caveolin-1. COS-7 cells were transiently transfected with caveolin-1 and either c-Src, Fyn, c-Yes, Lck, Lyn, Hck, Fgr, Blk, Frk, or Csk. Each sample was then subjected to Western blot analysis with antibodies specific for caveolin-1 or phosphocaveolin-1 (Tyr(P)-14). In addition, the expression of each kinase was confirmed by Western blotting.

Fig. 1A shows that caveolin-1 is robustly phosphorylated when co-transfected with c-Src. Among the other NRTKs tested, only Blk is able to phosphorylate caveolin-1 to a degree comparable with c-Src. It is of interest that only c-Src (MGS) and Blk (MGL) lack the third position cysteine residue (MGC) that is necessary for palmitoylation, and thus myristoylation is their sole lipid modification.

Viral transduction of normal cellular NRTK genes can result in the transformation of cells through the alteration of the normal enzymatic activity of the kinase domain. This can be informative in identifying the function of a NRTK, if the substrates of the kinases are consistent. Such is the case for c-Src and v-Src (53, 54). Expression of either c-Src or constitutively active c-Src phosphorylates caveolin-1 on tyrosine 14, indicating that the localization of c-Src is sufficient to allow for the normal kinase function (50). However, this is not necessarily the case for other Src family NRTKs. For example, we show here that expression of a constitutively active form of c-Yes, c-Yes (Y535F), is able to phosphorylate caveolin-1 as efficiently as c-Src (WT), whereas c-Yes (WT) is unable to mediate this event (Fig. 1B).

Receptor Tyrosine Kinases Do Not Phosphorylate Caveolin-1 as Efficiently as c-Src—Many receptor tyrosine kinases are
localized to caveolae and interact with caveolin-1 (3). Therefore, we examined the capacity of a panel of well characterized receptors to phosphorylate caveolin-1 on tyrosine 14. COS-7 cells were transiently transfected with caveolin-1 and either insulin receptor, EGF-R, fibroblast growth factor receptor, or hepatocyte growth factor receptor/c-Met. Each sample was then subjected to Western blot analysis using antibodies specific for caveolin-1 or phosphocaveolin-1. Fig. 2 shows that phosphorylation of caveolin-1 is not associated with the co-expression of any of the receptors tested. The results of our EGF-R co-expression experiments are in agreement with our previous data showing that inhibitors that preferentially inhibit EGF-R fail to reduce the in vitro phosphorylation of caveolin-1 using purified caveolae-enriched membranes (50).

### Palmitoylation of Caveolin-1 at Cys-156 Is Required for Coupling Caveolin-1 to the c-Src Tyrosine Kinase

**A**

Palmitoylation-deficient caveolin-1 mutants. A schematic representation of the N-terminal, membrane-spanning, and C-terminal domains of caveolin-1 is shown. Each cysteine of caveolin-1 that is palmitoylated is represented as C. The residue at which a cysteine was mutated to a serine is listed to the left of the construct, using standard nomenclature. B, caveolin-1 tyrosine phosphorylation. Each caveolin-1 mutant was individually co-expressed with c-Src in COS-7 cells. Thirty-six hours post-transfection, cell lysates were prepared and subjected to SDS-PAGE/Western blot analysis with anti-phosphocaveolin-1 (Tyr(P)-14) (upper panel) or anti-caveolin-1 (lower panel) antibodies. Note that mutation of cysteine 156 to serine (C156S) prevents the Src-induced phosphorylation of caveolin-1 on tyrosine 14. C, possible lipid-lipid interactions. A schematic diagram showing the proposed interaction of the N-terminal myristoyl moiety of c-Src with the palmitoyl group attached to caveolin-1 at Cys-156. It has been previously shown that myristoylation of c-Src is required for it to mediate the tyrosine phosphorylation of caveolin-1 (50).
previously demonstrated that palmitoylation-deficient caveolin-1 (C133S, C143S, C156S) cannot be phosphorylated on tyrosine 14 by c-Src (50). However, it remains unknown which of the three palmitoylation sites within caveolin-1 is critical for this phosphorylation event to occur. Thus, each cysteine within the C terminus of caveolin-1 was individually mutated to serine (Fig. 3A) and co-expressed with c-Src in COS-7 cells. These four Cav-1 mutants (C133S, C143S, C156S, and the triple mutant) have been previously shown to be correctly targeted to caveolae by two independent laboratories (34, 47). Interestingly, the C156S mutation was sufficient to ablate the phosphorylation of caveolin-1 on tyrosine 14 by c-Src (Fig. 3A). In contrast, caveolin-1 (C133S) and caveolin-1 (C143S) were tyrosine-phosphorylated as efficiently by c-Src as wild type caveolin-1. Since myristoylation of c-Src is necessary for its targeting to caveolae and phosphorylation of caveolin-1, these data are consistent with the idea that the myristoyl group at the N terminus of c-Src and the palmitoyl group attached to cysteine 156 of caveolin-1 may self-associate to mediate the productive interaction of c-Src with caveolin-1 (Fig. 3C).

Targeting of a GPI-linked Protein (CEA) to the Exoplasmic Leaflet of Caveolae Membranes Disrupts Caveolin-1 Phosphorylation by c-Src—Given that lipid modification of both caveolin-1 and c-Src is required for the phosphorylation of the former by the latter, we entertained the hypothesis that alteration of the lipid-lipid interactions within the caveolar membrane could regulate this phosphorylation event. We speculated that we could uncouple this interaction by altering the balance of acylated proteins within the caveolar membrane.

GPI-anchored proteins contain a C-terminal glycolipid membrane anchor or “greasy foot” that attaches them to the exoplasmic leaflet of the lipid bilayer. Upon cross-linking with antibodies or ligands to their extracellular domains, GPI-anchored proteins are concentrated within caveolae membranes (55, 56). Thus, we used CEA as a model GPI-anchored protein to assess the effect of GPI-protein clustering within caveolar membranes.

Fig. 4 shows that neither CEA expression without antibody treatment nor antibody treatment without CEA expression affected the c-Src-induced phosphorylation of caveolin-1. However, when CEA is expressed in cells treated with the cross-linking antibody, caveolin-1 phosphorylation is greatly reduced. This suggests that targeting this dually acylated extracellular protein to the site of the c-Src/caveolin-1 interaction (caveolar membranes) disrupts their lipid-lipid interaction, thus blocking caveolin-1’s ability to serve as a substrate for the c-Src tyrosine kinase.

Targeting the Transmembrane Protein, CD36, to Caveolae Abrogates the Phosphorylation of Caveolin-1 by c-Src—CD36 (also known as glycoprotein IV) is a transmembrane receptor that resides within caveolae membranes (36). It has multiple lipid modification sites at both its N and C termini, consistent with its dual transmembrane structure. To examine the effect of a lipid-modified transmembrane protein on c-Src-phosphorylated caveolin-1, COS-7 cells were co-transfected with caveolin-1, c-Src, and CD36. Fig. 5 shows that co-expression with CD36 is sufficient to eliminate caveolin-1 tyrosine phosphorylation by c-Src.

Targeting the Dually Acylated N-terminal Domain of Gi1 fused to the Cytoplasmic Leaflet of the Caveolar Membrane Disrupts Caveolin-1 Phosphorylation by c-Src—The 32-amino acid N terminus of Gi1 fused to GFP (32aaWT-GFP) is efficiently targeted to caveolae membranes (49). The loss of either its myristoylation site (32aaG2A-GFP) or palmitoylation site (32aaC3S-GFP) eliminates its interaction with caveolin-1 (49). We therefore hypothesized that expression of the fully acetylated 32aaWT-GFP would compete for the lipid-lipid interaction between c-Src and caveolin-1 and thus reduce the phosphorylation of caveolin-1.

COS-7 cells were co-transfected with caveolin-1/c-Src and either the acylated or acylation-deficient Gi1 fusion proteins (Fig. 6A). Western blot analysis of these samples shows that expression of only the 32aaWT-GFP fusion protein significantly reduces the ability of c-Src to phosphorylate caveolin-1 on tyrosine 14, while neither of the acylation-deficient fusion proteins significantly reduces caveolin-1 phosphorylation (Fig. 6B). Taken together, our data indicate that targeting of three distinct classes of dually acylated proteins (GPI-linked, transmembrane, and cytoplasmically oriented) to caveolae mem-
branes is sufficient to inhibit the Src-induced phosphorylation of caveolin-1 in vivo.

An N-terminal Myristoylated c-Src Peptide Inhibits the Tyrosine Phosphorylation of Caveolin-1 (Tyr-14) in Vitro—Myristoylation of c-Src is necessary for its caveolar membrane association and for phosphorylation of caveolin-1 (50, 57). Therefore, we next examined whether a myristoylated N-terminal fragment of c-Src (N-Myr-c-Src-(2–12); see Table I for specific sequences) could competitively uncouple the phosphorylation of caveolin-1 by endogenous c-Src in vitro.

Caveolae-enriched domains were purified from murine lung tissue and incubated in kinase reaction buffer in the presence of exogenous ATP. Myristoylated or nonmyristoylated N-terminal c-Src peptides were preincubated with the purified caveolae

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**TABLE I**

Peptides derived from the N-terminal region of Src family tyrosine kinases

| Name                  | Sequence       | Inhibitory activity |
|-----------------------|----------------|---------------------|
| N-Myr-c-Src-(2–12)    | Myr-GSSKSKPDKPS | +                   |
| c-Src-(2–12)          | GSSKSKPDKPS    | −                   |
| N-Myr-c-Yes-(2–12)    | Myr-GCIRSKGEDKGP | −                 |
| c-Yes-(2–12)          | GCIKSKEDKGP    | −                   |

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**Fig. 6.** Targeting of the dually acylated N-terminal domain of Gi1α to the cytoplasmic leaflet of caveolae membranes disrupts caveolin-1 (Tyr-14) phosphorylation by c-Src. A, G-protein/GFP fusions and corresponding mutants. A schematic diagram summarizing the construction and the properties of these fusion proteins is shown. The lipid modification of these constructs (WT, G2A, and C3S) has been previously evaluated (49). Note that prevention of myristoylation (G2A) also inhibits the incorporation of palmitate. B, caveolin-1 tyrosine phosphorylation. Caveolin-1 was co-transfected with either the N-terminus of Gi1α fused to GFP (32aaWT-GFP) or myristoylation-deficient (32aaG2A-GFP) or palmitoylation-deficient (32aaC3S-GFP) forms of this fusion protein. Thirty-six hours post-transfection, cell lysates were subjected to SDS-PAGE/Western blot analysis with anti-phosphocaveolin-1 (Tyr(P)-14) (upper panel) or anti-caveolin-1 (middle panel) antibodies. Expression of GFP fusion proteins was detected with anti-GFP IgG (lower panel). Note that only the dually acylated 32aaWT-GFP is able to inhibit the c-Src-induced phosphorylation of caveolin-1.

**Fig. 7.** An N-terminal myristoylated c-Src peptide reduces the phosphorylation of caveolin-1 (Tyr-14) in vitro. Caveolae-enriched membrane domains were purified from murine lung tissue and incubated in kinase reaction buffer in the presence of exogenous ATP (1 mM). Different concentrations (10 or 50 μM) of myristoylated or nonmyristoylated peptides were added to the kinase reaction (detailed in Table I). Samples were then subjected to SDS-PAGE/Western blot analysis with anti-phosphocaveolin-1 (Tyr(P)-14) (upper panel) or anti-caveolin-1 (lower panel) antibodies. Note that only the N-Myr-c-Src peptide (50 μM) reduced the phosphorylation of caveolin-1 by an endogenous caveolar tyrosine kinase. In contrast, the N-Myr-c-Yes peptide had no effect. Similarly, the nonmyristoylated c-Src peptide also had no effect. Thus, the inhibitory activity of the N-Myr-c-Src peptide is both myristoylation-dependent and sequence-specific.
prior to the initiation of the kinase reaction. Since c-Yes is unable to effectively phosphorylate caveolin-1 in vivo (Fig. 1B), myristoylated and nonmyristoylated N-terminal peptides derived from c-Yes were employed as critical negative controls for these studies (Table I).

Fig. 7 shows that preincubation of purified caveolae membranes with the N-Myr-c-Src peptide greatly reduced the tyrosine phosphorylation of caveolin-1, and this occurred in a concentration-dependent fashion. Importantly, the nonmyristoylated c-Src peptide at the same concentration failed to affect the level of phosphocaveolin-1 (Tyr(P)-14). Neither the myristoylated nor the nonmyristoylated c-Yes peptides had any effect on the phosphorylation of caveolin-1. Thus, uncoupling of this event by the myristoylated c-Src peptide requires myristoylation and is peptide sequence-specific. These data provide the first evidence of a function for the unique

Fig. 8. The SH2 and PTB domains of Grb7 show differential binding capacities for phosphocaveolin-1 (Tyr(P)-14). A, construction of Grb7 GST fusion proteins. Shown is a schematic representation of the Grb7 molecule showing the relative orientation of its SH2 and PTB domains. In addition, the construction of GST-Grb7 fusion proteins harboring the SH2 domain or the PTB domain is illustrated. B, caveolin-1 tyrosine phosphorylation. Caveolin-1 and c-Src were co-expressed in COS-7 cells. Thirty-six hours post-transfection, cell lysates were prepared and incubated for 4 h at 4 °C with purified GST, GST-Grb7-SH2, or GST-Grb7-PTB immobilized on glutathione-agarose beads. The bound material was subjected to SDS-PAGE/Western blot analysis with anti-phosphocaveolin-1 (Tyr(P)-14) antibodies. Phosphocaveolin-1 (Tyr(P)-14) bound to GST-Grb7-SH2 avidly, while a much longer exposure showed that GST-Grb7-PTB was also functional.

Fig. 9. Schematic diagram summarizing the uncoupling of c-Src phosphorylation of caveolin-1 by dually acylated proteins (GPI-linked or cytoplasmically oriented). We speculate that the palmitoyl group on cysteine 156 of caveolin-1 and the myristoyl group on the N terminus of c-Src interact to facilitate the phosphorylation of caveolin-1 on tyrosine 14 by the c-Src kinase. In accordance with this hypothesis, we show here that targeting of dually acylated proteins to caveolae serves to perturb the ability of c-Src to act on caveolin-1, most likely by disrupting lipid-lipid interactions between caveolin-1 and the c-Src kinase.
N-terminal region of Src family tyrosine kinases (i.e. in coupling c-Src to caveolin-1).

In addition, the above data show that the amino acid sequence directly adjacent to the myristoylation site of c-Src may import some degree of specificity to the lipid-dependent interaction between c-Src and caveolin-1, since other “myristoylated-only” peptides/proteins (N-Myr-c-Yes (in vitro; Fig. 6) and 32aaC3S-GFP (in vivo; Fig. 5) failed to inhibit the c-Src-induced phosphorylation of caveolin-1.

The SH2 and PTB Domains of Grb7 Show Differential Binding Capacities for Phosphocaveolin-1 (Tyr(P)-14)—Grb7 is the only protein known to bind and mediate the functions of phosphocaveolin-1 (Tyr(P)-14) (50). Grb7 has both an SH2 and a PTB domain (58), each capable of binding to motifs containing a phosphorylated tyrosine (Fig. 8A).

In order to determine which of these domains binds phosphocaveolin-1 (Tyr(P)-14), we created GST fusion proteins harboring either the Grb7-SH2 domain or the Grb7-PTB domain. Cell lysates were then exposed to the bound GST fusion proteins or GST alone. Western blot analysis of these samples shows that phosphocaveolin-1 associates with both GST-Grb7-SH2 (Fig. 8B, upper panel) and GST-Grb7-PTB (Fig. 8B, lower panel). However, phosphocaveolin-1 (Tyr(P)-14) preferentially interacts with the SH2 domain of Grb7. Importantly, GST alone fails to interact with phosphocaveolin-1 (Tyr(P)-14).

**DISCUSSION**

Lipid modification of certain proteins serves to target them to caveolae. It is ironic that the defining protein of caveolae (i.e. caveolin-1), while palmitoylated on three residues, does not require lipid modification for its proper targeting or caveolar membrane association (34). Thus, we explored the possibility that the acyl groups of caveolar proteins, including caveolin-1 itself, can regulate the tyrosine phosphorylation of caveolin-1 by c-Src.

Here, we show through a detailed mutational analysis that the terminal palmitoylation site of caveolin-1, cysteine 156, is the single palmitoylation site required for coupling the c-Src tyrosine kinase to caveolin-1. This finding is of interest, since the only other experimentally derived function for the acyl groups of caveolin-1 shows that removal of the cysteine 156 palmitoylation site eliminates caveolin-1 association with cholesterol and the chaperone protein HSP56 (47). These effects are specific, since the loss of cysteines at positions 133 and 143 either did not affect caveolin-1 cholesterol binding or resulted in its inability to bind other proteins, respectively. Taken together with our current findings, it is clear that the palmitoylation of specific residues on caveolin-1 affects its interaction with other proteins in vivo. These data suggest that interactions with cholesterol via caveolin-1’s terminal palmitoyl group may also be important for caveolin-1 to serve as a substrate for c-Src. In support of this notion, we have shown that insertion of oxidized cholesterol within caveolae membranes in vivo blocks the tyrosine phosphorylation of caveolin-1 in response to cellular stress (51).

Using proteins that localize to caveolae under various conditions, we were able to examine multiple aspects of the role played by fatty acyl-containing proteins on the c-Src-mediated phosphorylation of caveolin-1. Since GPI-linked proteins target to caveolae only when cross-linked or clustered, the caveolar localization of GPI-linked proteins can be specifically induced by the addition of bivalent antibodies to the medium. In accordance with these previous studies, we show here that expression of CEA (a well studied GPI-anchored protein) at the plasma membrane is not sufficient to uncouple the Src-induced tyrosine phosphorylation of caveolin-1. However, after induction of caveolar targeting of CEA by antibody cross-linking, the tyrosine phosphorylation of caveolin-1 is greatly reduced. Thus, the mere co-expression of a dually acylated membrane protein is insufficient to alter caveolin-1 phosphorylation by c-Src, showing that caveolar localization is necessary to block this phosphorylation event.

Since GPI-acyl groups are restricted to the exoplasmic leaflet of the lipid bilayer, while caveolin-1 palmitoyl groups and the c-Src myristoyl group reside within the cytoplasmic leaflet of the lipid bilayer, our results also provide evidence that there is communication between lipid moieties within the exoplasmic and cytoplasmic leaflets of the lipid bilayer. The mechanism by which the clustering of GPI-anchored proteins connects to the function of lipid-anchored proteins on the cytosolic surface has not been thoroughly elucidated (59). It is possible that lipid-lipid interactions between the exoplasmic and cytoplasmic leaflets of the caveolar membrane serve to alter the relationship between the proteins to which they are bound, ultimately affecting their function. This is supported by the fact that there is no known “protein-protein” interaction between GPI-anchored proteins and caveolin-1 or c-Src. The acyl group-dependent association of caveolin-1 with cholesterol may also link the cytoplasmic and exoplasmic leaflets of the lipid bilayer, since cholesterol is present in both leaflets and can potentially form dimers that span the membrane, acting as a bridge (60).

The use of the acylated Ga1-GFP fusion proteins was also quite informative. Given that both the “myristoylated/palmitoylated” and “myristoylated-only” Ga1-GFP fusion proteins localize to caveolae (49), we predicted that expression of either would abrogate c-Src phosphorylation of caveolin-1. However, only the dually acylated form of the protein affected caveolin-1 phosphorylation. This observation can be generalized, since only dually acylated proteins, regardless of the region of caveolae to which they were targeted (exoplasmic or cytoplasmic membrane domains), affected the phosphorylation of caveolin-1 by c-Src (summarized in Fig. 9). Both in vitro and in vivo, proteins that were myristoylated-only were unable to disrupt caveolin-1 phosphorylation, with the exception of the myristoylated c-Src N-terminal peptide lacking the kinase domain, in vitro. This presents the intriguing possibility that the amino acid sequence adjacent to the myristoyl group on c-Src is important for its interaction with caveolin-1. Although differences in substrate specificity and kinase activity must also be taken into account, it is an interesting first observation that the myristoylated-only Src family kinases (c-Src and Blk) were the only two that could efficiently phosphorylate caveolin-1 (Fig. 1A).

Finally, we determined which binding domains of Grb7 interact with phosphocaveolin-1 (Tyr(P)-14). The SH2 and PTB domain of Grb7 each bound Src-phosphorylated caveolin-1, although the SH2 domain showed a much stronger interaction. Similarly, other Grb7-interacting proteins, such as insulin receptor and focal adhesion kinase, either prefer the SH2 domain or interact with both SH2/PTB domains equally well (58, 61). Since Grb7 contains two functional phosphotyrosine binding domains (SH2 and PTB) and Grb7 forms large hetero-oligomeric complexes, Grb7 binding may serve to link phosphocaveolin-1 (Tyr(P)-14) to the focal adhesion machinery. In support of this notion, phosphocaveolin-1 (Tyr(P)-14) is localized within a subpopulation of caveolae membranes that are proximal to focal adhesions in intact cells (50, 51).

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