The Membrane-spanning Domains of Caveolins-1 and -2 Mediate the Formation of Caveolin Hetero-oligomers

IMPLICATIONS FOR THE ASSEMBLY OF CAVEOLAE MEMBRANES IN VIVO*

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Caveolae, the “little caves” first described in electron micrographs of endothelial cells, have emerged in recent years as the site of the important dynamic regulatory events at the plasma membrane (1–4). Both transcytosis and potocytosis occur within these cell surface organelles, as does the uptake of other organelle density lipoprotein particles via endothelial scavenger receptors, the uptake of cholera toxin and DNA tumor viruses, the processing of Alzheimer disease-related protein APP, and of the Scrapie prion protein PrP (1–4). Caveolae have also been implicated in signal transduction, particularly by receptor tyrosine kinases and G proteins (2, 3).

Caveolins (Cav-1, -2, and -3)† are a family of cytoplasmic membrane-anchored scaffolding proteins that (i) help to sculpt caveolae membranes from the plasma membrane proper, and (ii) participate in the sequestration of inactive signaling molecules (2, 3). In the adult, caveolins-1 and -2 are co-expressed and are most abundant in type I pneumocytes, endothelia, fibroblastic cells and adipocytes, whereas the expression of caveolin-3 is restricted to striated muscle cells.

Caveolae-like vesicles can be generated by expressing caveolin-1 or -3 in insect cells or in mammalian cell lines, providing an in vivo assay for caveolin-dependent vesicle formation (5–8). In addition, caveolin-induced vesicle formation appears to be isoform-specific. Expression of caveolin-2 alone under the same conditions failed to drive the formation of vesicles, either in insect cells or in mammalian cells (7, 8). Thus, caveolin-2 may function as an accessory protein in conjunction with caveolin-1 (3, 9).

In support of this notion, caveolins-1 and -2 form a stable hetero-oligomeric complex of ~200–400 kDa in cell types in which they are co-expressed (9). These caveolin hetero-oligomers are thought to represent the assembly units that drive the formation of caveolae membranes in nonmuscle cells (7, 8). However, caveolin-2 requires caveolin-1 to form high molecular mass oligomers; when caveolin-2 is expressed alone, it behaves as a mixture of monomers and dimers (7, 8). In contrast, caveolin-1 forms high molecular mass homo-oligomers of ~350 kDa (10, 11). Thus, it has been hypothesized that caveolin-2 molecules are embedded within or become tightly associated with high molecular mass homo-oligomers formed by caveolin-1 (9).

The genes encoding murine caveolin-1 and caveolin-2 are co-localized within the A2 region of mouse chromosome 6 (6-A2) (12). Human CAV1 and CAV2 co-map to 7q31 in a region of conserved synteny with murine 6-A2 (13, 14). Similarly, the muscle-specific CAV3 gene is conserved both at the level of sequence and chromosomal context between mouse and human (4). The human CAV3 gene, which underlies an autosomal dominant form of limb-girdle muscular dystrophy (limb-girdle muscular dystrophy-1C), maps to 3p25, corresponding to the mouse region 6-E1 (12, 15).

Given that caveolins-1 and -2 are co-expressed, that they form a hetero-oligomeric complex in vivo, and even their genes are co-localized to the same chromosomal region in mouse and human, it is apparent that this interaction is of vital importance. For example, in cells transformed by activated oncogenes, caveolin-1 levels are selectively down-regulated, but caveolin-2 levels remain relatively constant (9). In these cells, caveolae are not formed. In addition, targeted down-regulation of caveolin-1 expression using an antisense approach prevents caveolae formation, induces cell transformation, and produces a tumorigenic phenotype in NIH 3T3 cells (16). Thus, it is either the absence of caveolin-1 or the presence of unbound caveolin-2 within these cells that is responsible for their transformed phenotype (16). Furthermore, once caveolin-1 expres-
sion is restored in these cells, reconstituting the interaction between caveolins-1 and -2, their transformed phenotype is ablated (16).

In further support of these observations, loss of heterozygosity analysis implicates 7q31.1 in the pathogenesis of multiple types of cancer, including breast, ovarian, prostate, and colorectal carcinomas, as well as uterine sarcomas and leiomyomas (17–32). The locus of the presumed 7q31.1 tumor suppressor gene has been narrowed to a ~1-megabase region that includes the highly polymorphic marker D7S252 (24, 28, 30, 33). Given the usefulness of 7q31.1 and D7S252 loss of heterozygosity as markers for carcinogenesis, many laboratories are currently searching this chromosomal region for a novel tumor suppressor gene. Recently, we have shown that the human caveolin-1 and caveolin-2 genes map within ~100 kilobases of D7S252, in the middle of the 1 megabase smallest common deleted region for the presumed tumor suppressor gene (4, 13). Thus, we have proposed that the caveolin genes may represent the missing tumor suppressor genes at this locus (4, 13, 14).

Despite the emerging importance of caveolins-1 and -2, little is known about the mechanism by which they form a heterooligomeric complex in vivo. Here, we have addressed this issue using a variety of complementary approaches. Our results indicate that complex formation between caveolins-1 and -2 is mediated by interactions between their respective membrane-spanning domains. This is the first demonstration that these unusual membrane-spanning regions play a critical role in specific protein-protein interactions.

**EXPERIMENTAL PROCEDURES**

**Materials—**Antibodies and their sources were as follows: anti-caveolin-1 IgG (mAb 2234 and mAb 2297 (34); gifts of Dr. Roberto Campos-Gonzalez, Transduction Laboratories); anti-caveolin-2 IgG (mAb 65 (9); gift of Dr. Roberto Campos-Gonzalez, Transduction Laboratories); anti-Myc epitope IgG (mAb 9E10; Santa Cruz Biotechnology); and anti-caveolin-1 (polyclonal antibody; rabbit anti-peptide antibody directed against caveolin-1 residues 2–21; Santa Cruz Biotechnology). Anti-fatty acyl CoA synthase IgG were the generous gift of Dr. Jean Schaffer (Washington University, St. Louis, MO). The blots containing caveolin-1 and -2 were separated from free 125I using a G-25 column.

**Purification of GST-Caveolin-1 Fusion Proteins and in Vitro Binding Assays—**GST-caveolin-1 fusion proteins were constructed and purified as we described previously (10, 34, 40). Briefly, regions of caveolin-1 were subcloned into the MCS of the vector pGEX-4T-1 (Amersham Pharmacia Biotech) and expressed in a suitable Escherichia coli strain (BL21, lacking lon and ompT proteases; Novagen, Inc.). GST-caveolin-1 fusion proteins were purified by affinity chromatography using glutathione-agarose (41). The purified proteins bound to glutathione-agarose were incubated with 3T3-L1 adipocyte extracts. After binding, the beads were washed and analyzed by SDS-PAGE/Western blotting with anti-caveolin-2 IgG.

**Immunoblotting—**Samples were separated by SDS-PAGE (12% acrylamide) and transferred to nitrocellulose. After transfer, nitrocellulose sheets were stained with Ponceau S to visualize protein bands and subjected to immunoblotting with anti-caveolin-1 IgG (mAb 2297; Molecular Laboratories) or with anti-caveolin-2 IgG (mAb 65 (9); gift of Dr. Roberto Campos-Gonzalez, Transduction Laboratories). Blots were incubated in blocking solution containing 5% bovine serum albumin and 1% nonfat dry milk (Carnation). Primary antibodies were as described by the manufacturer (Promega; Amersham Pharmacia Biotech), except we supplemented our blocking solution with 1% bovine serum albumin and 1% nonfat dry milk (Carnation).

**Far-Western Analysis—**Peptide blots (peptides on paper) containing a panel of peptides derived from the protein sequence of murine caveolin-2 were custom made by Research Genetics. Peptides were designed so that each peptide is 10 amino acids in length, and each pair of consecutive peptides overlaps by 5 amino acids. The caveolin-2 peptide blot was incubated with radiiodinated GST-caveolin-1 for ~18 h at 4 °C, washed thoroughly with 20 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween 20, and subjected to autoradiography. Purified GST-caveolin-1 fusion proteins were radioiodinated using the chloramine-T method (42). To label, GST-caveolin-1 fusion proteins were dialyzed against 20 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween 20. Approximately 50–100 μg of protein was radioiodinated with 1 mCi of Na125I in the presence of 20 μg of chloramine-T for 5 min at room temperature. After terminating the reaction with unlabeled NaI, the labeled proteins were separated from free 125I using a G-25 column.

**Synthesis of Immobilized Caveolin-2-Derived Peptides—**Caveolin-2-derived polypeptides were synthesized directly onto an activated poly-acylamide membrane by the standard Fmoc with coupling mediated through HOBr/DIC and Fmoc removal with 1:3 piperidine/DMF. For final peptide protecting group removal, the membrane was placed in a bath of 50:47.5:2.5:5:1.5:1.5 DCM/TFH/thioanisole/EDT/anisole for 1 h and finally washed and dried. These sheets were probed by immunoblotting with anti-caveolin-2 IgG or with radioiodinated GST-caveolin-1 as described above.

**Expression of a GST Fusion Protein Carrying the Caveolin-1 Mem-
brane-spanning Domain in Cos-7 Cells—The membrane-spanning domain of caveolin-1 (residues 102–134) fused to GST was amplified by polymerase chain reaction using a previously constructed GST fusion construct as the template. This polymerase chain reaction product then was subcloned into the pCB7 vector downstream of the cytomegalovirus promoter and termed pGST-Cav-1-MS. pGST-Cav-1-MS was co-transfected with (i) N-terminally Myc-tagged caveolin-1 and (ii) untagged caveolin-2 in Cos-7 cells using the Effectene transfection reagent (Qia-gen, Inc), as per the manufacturer’s instructions. Forty-eight hours post-transfection, cells were scraped into lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 60 mM octyl-glucoside). Myc-tagged caveolin-1 was immunoprecipitated with the monoclonal antibody 9E10, which recognizes the Myc epitope (EQKLISEEDLN). Immunoprecipitates were washed with washing buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100) and analyzed by SDS-PAGE/Western blotting using anti-caveolin-2 IgG.

RESULTS

Cloning of the Complete cDNA for Murine Caveolin-2

The full-length cDNA for mouse caveolin-2 was cloned by screening a day 8 3T3-L1 adipocyte library. Twenty-two individual clones were analyzed and characterized by restriction digestion/DNA sequencing. The clone with the longest 5’-end contained a 2.3-kilobase cDNA fragment and was completely sequenced. This 2.3-kilobase insert contained the complete coding sequence of murine caveolin-2 and has been deposited in GenBank™ under accession number AF141322. Only the cDNAs for human and canine caveolin-2 have been previously cloned (9, 39, 43).

The open reading frame has an in frame stop codon preceding the first ATG, confirming the authenticity of the starting methionine. Fig. 1A shows an alignment of murine caveolin-2 with other members of the caveolin gene family. To demonstrate that the clone isolated contains the complete cDNA for murine caveolin-2, we transiently expressed the cDNA in Cos-7 cells (Cos-7 cells express extremely low levels of endogenous caveolin-2). Fig. 1B shows that the expressed protein product was immunoprecipitated with anti-caveolin-2 IgG (mAb 65) and co-migrates with the major α-isofrom of caveolin-2 found in differentiated murine 3T3-L1 adipocytes.
Fig. 2. Biogenesis of caveolin hetero-oligomers. A, velocity gradient analysis of caveolin hetero-oligomers in 3T3-L1 adipocytes. Extracts from 3T3-L1 adipocytes were loaded atop a 5–20% sucrose density gradient and subjected to centrifugation for 4 h at 100,000 rpm in a Beckman TLS-55 rotor. Fractions were collected and analyzed by SDS-PAGE/Western blotting with either anti-caveolin-1 or anti-caveolin-2 IgG. The distribution of recombinantly overexpressed caveolin-2 after transient expression in Cos-7 cells is shown for comparison. Note that in 3T3-L1 adipocytes, caveolins-1 and -2 form high molecular mass oligomers of \( \sim 150 \text{ kDa} \). In contrast, the \( \alpha \)-isoform of caveolin-2 (residues 1–162; the predominant caveolin-2 protein product) only forms monomer or dimers when expressed alone (9, 39). Arrows indicate the position of molecular mass standards.

B, pulse-chase analysis of caveolin hetero-oligomer formation. 3T3-L1 adipocytes (on day 6 of differentiation) were pulse-labeled for 10 min with \([^35S]\text{methionine/cysteine}\) and chased for either 0, 10, 30, or 60 min. Cell extracts were then immunoprecipitated with anti-caveolin-1 IgG (left panel). In addition, immunoprecipitates were denatured with 1% SDS, renatured by 10-fold dilution with TNET, and then re-immunoprecipitated with anti-caveolin-2 IgG (right panel). Samples were analyzed by SDS-PAGE, followed by autoradiography. Note that caveolin-1 and -2 associate with pre-existing caveolin-1.

### Biogenesis of Caveolin Hetero-oligomers

#### That Contain Caveolins-1 and -2

We have previously shown that caveolins-1 and -2 form a hetero-oligomeric complex in fibroblasts (9), but it remains unknown whether they form similar high molecular mass oligomers in 3T3-L1 adipocytes. To address this issue, we employed a well characterized velocity gradient system used previously to characterize homo-oligomers of caveolins-1, -2, or -3 (9, 10, 37, 39). Fig. 2A shows that in 3T3-L1 adipocytes, caveolins-1 and -2 form high molecular mass oligomers of \( \sim 150 \text{ kDa} \). Note that two isoforms of caveolins-1 and -2 are endogenously expressed in 3T3-L1 adipocytes (Cav-1a, Cav-1b, Cav-2a, and Cav-2b). For both caveolins-1 and -2, these \( \alpha \) - and \( \beta \)-isoforms differ in their translational start sites and are generated by alternate initiation during translation (7, 9, 34). Note also that both isoforms of caveolins-1 and -2 are present within these high molecular mass oligomers.

In contrast, the \( \alpha \)-isoform of caveolin-2 (residues 1–162; the predominant caveolin-2 protein product) only forms monomers or dimers when overexpressed in Cos-7 cells. This is consistent with previous reports showing that the \( \beta \)-isoform of caveolin-2 (residues 14–162; a much less abundant isoform of caveolin-2) only forms monomer or dimers when expressed alone (9).

We next examined the kinetics of caveolin hetero-oligomer formation in murine 3T3-L1 adipocytes. Although both caveolins-1 and -2 are quite abundant in 3T3-L1 adipocytes, they have long half-lives and are difficult to visualize with short radioactive pulses. As a consequence, day 6 adipocytes were labeled with \([35S]\text{methionine/cysteine}\) for 10 min and chased for 0, 10, 30, and 60 min in the presence of cycloheximide. After lysis, caveolin-1 was retrieved by immunoprecipitation with monoclonal antibodies that recognize caveolin-1 but not caveolin-2. We have previously shown that caveolin-2 can be retrieved almost quantitatively with caveolin-1 antibodies when they form a complex (9).

Caveolin-1 immunoprecipitates were denatured with SDS to dissociate any noncovalent protein-protein interactions and either analyzed directly or subjected to reprecipitation with antibodies directed against caveolin-2. Fig. 2B shows that caveolins-1 and -2 associate either co-translationally or posttranslationally with rapid kinetics as judged by the recovery of caveolin-2 with caveolin-1 antibodies even at the earliest time point, i.e. 10-min pulse without any chase. Alternatively, newly synthesized caveolin-2 might associate with preexisting caveolin-1. However, this is unlikely as \( >90\% \) of caveolin-1 is localized at the plasma membrane at steady-state, and newly synthesized caveolin-2 would be initially located within the endoplasmic reticulum.

### Reconstitution of the Interaction between Caveolins-1 and -2 in Vivo

To reconstitute complex formation between caveolins-1 and -2 in vivo, we transiently transfected Cos-7 cells with Myc-tagged forms of caveolin-1 and untagged caveolin-2. Extracts from doubly transfected cells were prepared and immunoprecipitated with anti-Myc IgG to retrieve caveolin-1. These immunoprecipitates were then probed by Western blot analysis with antibodies directed against caveolin-2, to detect the amount of caveolin-2 associated with caveolin-1.

Fig. 3 shows that retrieval of caveolin-1 with anti-Myc IgG co-precipitates caveolin-2 as expected. Using this approach, we observed that the naturally occurring \( \beta \)-isoform of caveolin-1, which lacks residues 1–31, co-precipitated caveolin-2 as efficiently as the full-length \( \alpha \)-isoform of caveolin-1. These results indicate that residues 1–31 of caveolin-1 are not required for its interaction with caveolin-2 and that both isoforms of caveolin-1 are competent to form complexes with caveolin-2. Importantly, anti-Myc IgG did not immunoprecipitate caveolin-2 from cells transfected with untagged caveolin-2 alone.

Interestingly, when Myc-tagged caveolin-3 was co-expressed with untagged caveolin-2, no complex formation could be detected. This indicates that the interaction between caveolins-1 and -2 is highly selective, as complex formation does not occur between caveolins-2 and -3, despite the fact that caveolins-1 and -3 are 85% identical and 65% similar at the amino acid level (37).

As our pulse-chase experiments demonstrated that caveolins-1 and -2 form a complex shortly after or during their synthesis, this suggests that they may have to be co-synthesized within the same cell in order to undergo complex formation in vivo. To test this hypothesis directly, Myc-tagged caveolin-1 and untagged caveolin-2 were expressed separately in Cos-7 cells. These extracts were prepared separately and then combined just prior to immunoprecipitation. As expected, no detectable complex formation occurred. These results demonstrate that the interaction between caveolins-1 and -2 is clearly not a post-lysis artifact. In addition, these observations
In vivo strongly suggest that caveolins-1 and -2 may need to associate with caveolin-2. Synthase was observed. These results demonstrate that the synthase was expressed in adipocytes, fatty acyl-CoA synthase (44). Examined the binding of an irrelevant protein that is abundant in adipocytes, fatty acyl-CoA synthase (44). As expected, anti-Myc IgG did not immunoprecipitate the interaction between caveolins-1 and -2 is clearly not a post-lysis artifact.

The Membrane-spanning Domain of Caveolin-1 Recognizes Caveolin-2

In order to identify the region within caveolin-1 that interacts with caveolin-2, we employed a panel of bacterially expressed GST fusion proteins that contain various regions of the caveolin-1 molecule. These GST-caveolin-1 fusion proteins, bound to glutathione-agarose beads, were incubated with cell extracts from 3T3-L1 adipocytes that contain caveolin-1 and -2. We hypothesized that due to the large excess of recombinant caveolin-1 for binding to caveolin-2. After incubation, the beads were washed and subjected to immunoblot analysis with a specific antibody directed against caveolin-2.

Fig. 4 shows that only the GST-caveolin-1 fusion proteins corresponding to full-length caveolin-1 (residues 1–178) and the caveolin-1 membrane-spanning domain (residues 102–132) efficiently retrieved caveolin-2 from these adipocyte-derived protein extracts. In sharp contrast, other regions of caveolin-1 fused to GST, GST alone, or a GST fusion carrying an irrelevant protein (Acrp-30) did not show any binding to caveolin-2. Furthermore, no binding of any of the GST fusions to fatty acyl-CoA synthase was observed.

The Caveolin-2 Membrane-spanning and Scaffolding Domains Interact with Caveolin-1

GST-Caveolin-1 Overlay—Using GST fusion proteins carrying the various caveolin-1 domains, we have shown that both full-length caveolin-1 and the membrane-spanning region of caveolin-1 can bind caveolin-2 from an adipocyte cell extract. However, these results do not exclude the possibility that this interaction is indirect, i.e. mediated by other secondary protein factors within the cell extract.

To evaluate the ability of caveolin-1 to interact directly with caveolin-2, we developed an overlay assay. Radiiodinated GST-caveolin-1 was incubated with a blot containing 32 overlapping 10-amino acid peptides derived from caveolin-2 (see Table 1). Fig. 5 shows that two caveolin-2 peptides specifically bound caveolin-1; these correspond to residues 74–83 and 113–122 within caveolin-2. Interestingly, residues 74–83 lie within the membrane-proximal caveolin-2 scaffolding domain, whereas residues 113–122 are within the distal portion of the caveolin-2 membrane-spanning domain.

Caveolin-2 mAb Epitope Mapping—We have previously shown that the interaction of mAb 65 with caveolin-2 is masked when caveolin-2 forms a hetero-oligomeric complex with caveolin-1 in cellular extracts (9). Thus, we hypothesized that the epitope masked by caveolin-1 binding may represent a physical contact site between caveolins-1 and -2.
To test this hypothesis directly, we mapped the epitope recognized by mAb 65 using a blot containing a panel of peptides derived from caveolin-2. Fig. 6 demonstrates that this epitope resides with residues 79–88 of caveolin-2. This region corresponds to the membrane-proximal caveolin-2 scaffolding domain and overlaps directly with one of the two sites (residues 74–83) that we identified by overlay analysis using radioiodinated GST-caveolin-1. As this epitope is masked by the binding of caveolin-1 to caveolin-2 in vivo, these studies provide additional evidence that the binding sites that we identified are relevant in vivo.

### Expression of the Caveolin-1 Membrane-spanning Domain in Mammalian Cells Stabilizes the Formation of Caveolin Hetero-oligomers

In order to begin to assess the in vivo role of the caveolin-1 membrane-spanning domain in facilitating the formation of caveolin hetero-oligomers, we expressed a GST fusion protein carrying the membrane-spanning domain of caveolin-1 in mammalian cells. For this purpose, we used the same assay devised earlier to measure the in vivo formation of caveolin hetero-oligomers. Briefly, Myc-tagged caveolin-1 and untagged caveolin-2 were co-expressed in Cos-7 cells. Caveolin hetero-oligomers were then retrieved using antibodies directed against the Myc epitope and visualized by immunoblotting. No effect was observed with vector alone or with a GST fusion carrying 23 amino acids from an irrelevant protein (pantophysin; data not shown). These results provide evidence that the membrane-spanning domain of caveolin-1 plays a functional role in caveolin hetero-oligomer formation in vivo.

### DISCUSSION

Caveolins-1 and -2 are co-expressed and form a hetero-oligomeric complex in many cell types (9), with particularly high levels in adipocytes, endothelial cells, and fibroblasts (38, 39). These caveolin hetero-oligomers are thought to represent the assembly units that drive the formation of caveolae organelles in nonmuscle cells (6–8). In contrast, caveolin-3 homo-oligomers drive caveolae formation in striated muscle cells (cardiac and skeletal) (7, 37, 45).

The genes encoding murine caveolin-1 and caveolin-2 are co-localized within the A2 region of mouse chromosome 6 (6-A2) (12). Similarly, the human caveolin-1 and caveolin-2 genes co-map to 7q31, in a region of conserved synteny with murine 6-A2 (13, 14). It is intriguing that these two homologous genes
are co-segregated at the genomic level, their gene products are co-expressed in the same cell types, and they form a functional hetero-oligomeric unit in vivo (4). However, the mechanism by which these two distinct caveolin proteins interact remains unknown.

Here, we have shown that the membrane-spanning domains of both caveolins-1 and -2 play an important role in hetero-oligomer formation. These hetero-oligomeric complexes assembled almost immediately after or during the synthesis of caveolins-1 and -2. Next, we reconstituted this reciprocal interaction both in vivo and in vitro. Using GST-caveolin-1 fusion proteins, we localized the caveolin-2 binding site to the membrane-spanning domain of caveolin-1. In addition, we identified two regions within caveolin-2 that interact with caveolin-1: a membrane proximal region of the caveolin-2 scaffolding domain and a region of the caveolin-2 membrane-spanning domain. In further support of these observations, recombinant expression of a GST fusion protein carrying the membrane-spanning domain of caveolin-1 in mammalian cells was able to stabilize or facilitate the formation of these caveolin hetero-oligomers in vivo. These results are summarized in Table II.

Topologically, all known caveolin family members can be subdivided into three conserved subdomains: (i) a hydrophilic N-terminal domain that varies in length between the three mammalian caveolins and that shows the highest level of diversity among different species, in particular within the residues located at the extreme N terminus; (ii) a membrane-spanning domain consisting of 33 mostly hydrophobic amino acids; and (iii) a hydrophilic C-terminal domain that is also fairly conserved in length (43–44 amino acids in all mammalian caveolins) (3). Little is known about the topological arrangement of the caveolin molecules within the membrane. However, it is clear that both the N-terminal as well as the C-terminal domains face the cytoplasm and that caveolins behave biochemically as integral membrane proteins as judged by their carbonate inextractability (34, 46, 47). In support of this cytoplasmic topology, both the N-terminal domain and C-terminal domain of caveolin-1 undergo cytoplasmic posttranslational modifications. The N-terminal domain of caveolin-1 is phosphorylated by v-Src on tyrosine at position 14 (48), and the C-terminal domain of caveolin is palmitoylated on three cysteine residues at positions 133, 143, and 156 (47).

Most known interactions between caveolins and other proteins have been mapped to the N-terminal domain, including interactions with heterotrimeric G-proteins, H-Ras, c-Src, eNOS and various receptor tyrosine kinases (epidermal growth factor receptor and c-Neu) (3). In all instances, these interactions occur within the juxtamembrane 20 amino acids of caveolin-1 and -3, termed the caveolin scaffolding domain (49, 50). This modular protein domain recognizes a conserved sequence of aromatic residues that is present in many distinct classes of signaling molecules. Binding of signaling molecules to the caveolin scaffolding domain results in their inactivation, suggesting that caveolin-1 functions as a negative regulator of signal transduction. Interestingly, the scaffolding domains of caveolins-1 and -3 recognize the same sequence motif, but the scaffolding domain of caveolin-2 fails to recognize this motif and does not inhibit the activity of signaling molecules (51). Thus, no binding partners have been previously identified for the scaffolding domain of caveolin-2.

Mutational analysis of caveolin-1 has revealed that a 41-amino acid region of the N-terminal domain (residues 61–101) directs the formation of caveolin-1 homo-oligomers (10). In addition, the 44-amino acid C-terminal domain acts as a bridge to allow these homo-oligomers to interact with each other, thereby forming a caveolin-rich scaffold or lattice-work within the plane of the membrane (36). This caveolin-rich scaffold is thought to nucleate the formation of the characteristic caveolar coat observed in electron micrographs.

This is the first report that involves the membrane-spanning domain of any caveolin in a protein-protein interaction. We show that this interaction is specific and not merely a function of overall hydrophobicity, as judged by a lack of interaction with other hydrophobic proteins as well as the inability to observe an interaction between caveolin-2 and caveolin-3 under the same conditions. Interestingly, there is very little flexibility with respect to the length of the membrane-spanning domain. The 32 or 33 hydrophobic amino acids are highly conserved from mammals to invertebrates (52). We and others have suggested a hairpin-like structure that does not traverse the lipid bilayer entirely. Such an arrangement could bring the N- and C-terminal regions near the membrane into close juxtaposition, possibly explaining how these two separate regions could form a binding

**Table II**

| Approach                        | Identified domain         | Residues involved |
|--------------------------------|---------------------------|-------------------|
| GST fusion protein binding *in vitro* and *in vivo* | Membrane spanning domain | Cav-1 (102–134)   |
| GST overlay assay              | Membrane spanning domain | Cav-2 (113–122)   |
| Cav-2 mAb epitope mapping      | Scaffolding domain        | Cav-2 (74–83)     |
|                                | Scaffolding domain        | Cav-2 (79–88)     |
FIG. 8. Schematic diagram summarizing the regions within caveolin-2 that interact with caveolin-1. We and others have suggested that the membrane-spanning domain of the caveolin forms a hairpin-like structure or greasy elbow that does not traverse the lipid bilayer entirely. Such an arrangement would bring the N- and C-terminal regions near the membrane into close juxtaposition, possibly explaining how these two separate regions, each capable of binding caveolin-1 independently, could form a caveolin-1 binding domain (see Fig. 8). In agreement with a “greasy elbow” configuration for the caveolins, there is a highly conserved glycine residue located in the middle of the membrane-spanning region that could be located at the tip of the looped structure within the membrane and accommodate a tight turn. Future studies will have to address the three-dimensional arrangement of this domain and its relationship to the formation of hetero-oligomeric complexes containing caveolins-1 and -2.

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