Minireview

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Caveolins, a Family of Scaffolding Proteins for Organizing "Preassembled Signaling Complexes" at the Plasma Membrane*

Takashi Okamoto‡, Amnon Schlegel§¶, Philipp E. Scherer‖, and Michael P. Lisanti§**

From the ‡Department of Neurosciences, Cleveland Clinic Foundation, Cleveland, Ohio 44195 and §Departments of Molecular Pharmacology and ‖Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Caveolae are vesicular invaginations of the plasma membrane. The chief structural proteins of caveolae are the caveolins. Caveolins form a scaffold onto which many classes of signaling molecules can assemble to generate *preassembled signaling complexes*. In addition to concentrating these signal transducers within a distinct region of the plasma membrane, caveolin binding may functionally regulate the activation state of caveolae-associated signaling molecules. Because the responsibilities assigned to caveolae continue to increase, this review will focus on: (i) caveolin structure/function and (ii) caveolae-associated signal transduction. Studies that link caveolae to human diseases will also be considered.

The Caveolin Gene Family: Caveolin-1, -2, and -3

Molecular cloning has identified three distinct caveolin genes (1–6), caveolin-1, caveolin-2, and caveolin-3. Two isoforms of caveolin-1 (Cav-1α and Cav-1β) are derived from alternate initiation during translation. Caveolin-1 and -2 are most abundantly expressed in adipocytes, endothelial cells, and fibroblastic cell types, whereas the expression of caveolin-3 is muscle-specific.

Caveolin proteins interact with themselves to form homo- and hetero-oligomers (7–9), which directly bind cholesterol (10) and require cholesterol for insertion into model lipid membranes (10, 11). Caveolin oligomers may also interact with glycosphingolipids (12). These protein-protein and protein-lipid interactions are thought to be the driving force for caveolae formation (7). In addition, the caveolin gene family is structurally and functionally conserved from worms (*Caenorhabditis elegans*) to man (13), supporting the idea that caveolins play an essential role.

Caveolin-1 assumes an unusual topology. A central hydrophobic domain (residues 102–134) is thought to form a hairpin-like structure within the membrane. As a consequence, both the N-terminal domain (residues 1–101) and the C-terminal domain (residues 135–178) face the cytoplasm. A 41-amino acid region of the N-terminal domain (residues 61–101) directs the formation of caveolin homo-oligomers (7), whereas 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 (14).

Recent co-immunoprecipitation and dual labeling experiments directly show that caveolin-1 and -2 form a stable hetero-oligomeric complex and are strictly co-localized (9). Caveolin-2 localization corresponds to caveolae membranes as visualized by immunoelectron microscopy (9). Thus, caveolin-2 may function as an "accessory protein" in conjunction with caveolin-1.

Caveolin-interacting Proteins

A number of studies support the hypothesis that caveolin proteins provide a direct means for resident caveolae proteins to be sequestered within caveolae microdomains. These caveolin-interacting proteins include G-protein α subunits, Ha-Ras, Src family tyrosine kinases, endothelial NOS,[1] EGF-R and related receptor tyrosine kinases, and protein kinase C isoforms (11, 15–18, 20–32).

Heterotrimeric G-proteins—G-proteins are dramatically enriched within caveolae membranes, where caveolin-1 directly interacts with the α subunits of G-proteins (18). Mutational or pharmacological activation of $G_s\alpha$ prevents its co-fractionation with caveolin-1 and blocks its direct interaction with caveolin-1 *in vitro*, indicating that the inactive GDP-bound form of $G_s\alpha$ preferentially interacts with caveolin-1. G-protein binding activity is located within a 41-amino acid region of the cytoplasmic N-terminal domain of caveolin-1 (residues 61–101). A polypeptide derived from this region of caveolin-1 (residues 82–101) effectively suppresses the basal GTPase activity of purified G-proteins by inhibiting GDP/GTP exchange. In contrast, the analogous region of caveolin-2 possesses GTPase-activating protein activity with regard to heterotrimeric G-proteins (3). However, both of these activities (GDI and GAP) actively hold or place G-proteins in the *inactive* GDP-liganded conformation (3).

Ha-Ras—Ha-Ras and Src family tyrosine kinases also directly interact with caveolin-1 (20, 23). Using a detergent-free procedure and a polyhistidine-tagged form of caveolin-1 for affinity purification of caveolin-rich membranes, G-proteins, Src family kinases, and Ha-Ras were all found to co-fractionate and co-elute with caveolin-1. Wild-type Ha-Ras also interacted with recombinant caveolin-1 *in vitro.* Ras binding activity was localized to a 41-amino acid membrane-proximal region (61–101) of the cytosolic N-terminal domain of caveolin-1, *i.e.* the same caveolin-1 region responsible for interacting with G-protein α subunits. Reconstituted caveolin-rich membranes interacted with a soluble recombinant form of wild-type Ha-Ras but failed to interact with mutationally activated soluble Ha-Ras (G12V) (23). Thus, a single amino acid change (G12V) that constitutively activates Ras prevents this interaction. Recombinant overexpression of caveolin in intact cells was sufficient to functionally recruit a non-farnesylated mutant of Ras (C186S) onto membranes (23). This is consistent with the hypothesis that direct interaction with caveolin-1 promotes the sequestration of inactive Ha-Ras within caveolae microdomains.

Src Family Tyrosine Kinases—Caveolin-1 interacts with wild-type Src (c-Src) but does not form a stable complex with mutationally activated Src (v-Src) (20). Thus, caveolin prefers the inactive conformation of Gα subunits, Ha-Ras and c-Src. Deletion mutagenesis indicates that the Src-interacting domain of caveolin is located within residues 61–101. A caveolin peptide derived from this region (residues 82–101) functionally suppressed the autoactivation of purified recombinant c-Src tyrosine kinase and a related Src family kinase, Fyn. Co-expression of caveolin-1 with c-Src shows that caveolin-1 dramatically suppresses the tyrosine kinase activity of c-Src. Thus, it appears that caveolin-1 functionally interacts with wild-type c-Src via caveolin residues 82–101.

Endothelial Nitric Oxide Synthase (eNOS)—Several independent co-immunoprecipitation and domain-mapping studies demonstrate that eNOS interacts directly with caveolin-1 residues 82–101 (30, 34, 36–38). In support of these data, recombinant co-expres-

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** To whom correspondence should be addressed.

[1] The abbreviations used are: NOS, nitric oxide synthase; EGF-R, epidermal growth factor receptor; eNOS, endothelial NOS; PKC, protein kinase C; MAP, mitogen-activated protein; PDGF, platelet-derived growth factor; PtdIns, phosphatidylinositol; PFK-M, phosphofructokinase-M; GPI, glycosylphosphatidylinositol; G_{M1}, $II^3NeuAcGgOse_4Cer$.

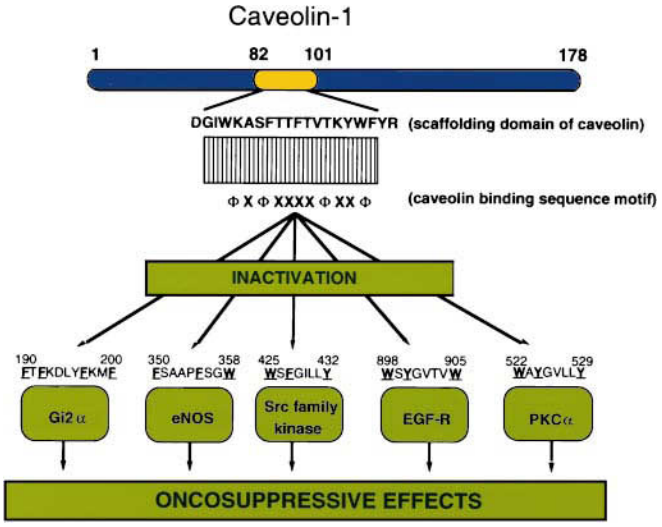

FIG. 1. **The caveolin scaffolding domain and caveolin-binding sequence motifs within G-protein α subunits ($G_{i2}\alpha$), eNOS, Src family tyrosine kinases, receptor tyrosine kinases (EGF-R), and PKC are shown.** See text for details.

sion of caveolin-1 with eNOS can inhibit NOS activity *in vivo* (38). Caveolin has also been shown to interact with other NOS isoforms (37, 38).

In summary, a short cytosolic domain derived from the N-terminal region of caveolin-1: (i) is required to form multivalent homooligomers of caveolin; (ii) mediates the interaction of caveolin-1 with Gα subunits, Ha-Ras, Src family tyrosine kinases, and eNOS; (iii) a peptide encoding this region can functionally inactivate the enzymatic activity of G-protein, Src family kinases, and eNOS but does not affect the activity of Ha-Ras; and (iv) it is membrane-proximal, suggesting that this caveolin domain may be involved in other potential protein-protein interactions. As a consequence, this caveolin-derived protein domain has been termed the *caveolin scaffolding domain* (Fig. 1).

The Caveolin Scaffolding Domain

What is the mechanism by which the caveolin scaffolding domain recognizes this diverse group of signal transducers? Perhaps the caveolin scaffolding domain recognizes a common sequence motif within caveolin-binding signaling molecules. To investigate this possibility, we have used the *caveolin scaffolding domain* as a receptor to select *caveolin-binding peptide ligands* from random peptide sequences displayed at the surface of bacteriophage. Two related caveolin-binding motifs (Φ*X*Φ*XXXX*Φ and Φ*XXXX*Φ*XX*Φ, where Φ is aromatic amino acid Trp, Phe, or Tyr) were elucidated, and these motifs exist within most caveolae-associated proteins (31). Thus, caveolin-binding motifs mediate the interaction of caveolin-binding proteins with the scaffolding domain of caveolin. These caveolin-binding motifs are present within most G_α subunits and the kinase domains of many distinct families of tyrosine and serine/threonine protein kinases (Src family kinases; PKCα; MAP kinase; EGF-R; insulin receptor; and PDGF receptor).

As many known caveolae or caveolin-associated proteins contain caveolin-binding motifs (see Table II in Ref. 31), this may be a general mechanism for caveolin-mediated sequestration and inactivation of a diverse group of signaling molecules within caveolae membranes for regulated activation by receptor ligands. Thus, the caveolin scaffolding domain may function like other modular protein domains (Src homology-2, Src homology-3, Pleckstrin homology, WW, and others) to generate *preassembled* membrane-bound oligomeric complexes that contain signaling molecules and cytoskeletal elements. In essence, caveolin may act as molecular "Velcro" to nucleate the formation of signal transduction complexes, holding these molecules in the off state (Fig. 1).

Additional molecular mapping studies have identified functional caveolin-binding sequence motifs within the catalytic region of G-protein α-subunits and the kinase domains of EGF-R and PKC (32). Interaction of the caveolin scaffolding domain with these caveolin-binding sequence motifs inhibits the kinase activity of EGF-R and PKC, suggesting that caveolin may indeed function as a general kinase inhibitor (32, 39).

eNOS contains a well conserved predicted caveolin-binding motif (**F**SAAP**F**SG**W**) within its catalytic domain (31, 38). Does this represent a functional caveolin-binding motif? Two independent lines of evidence suggest that this region binds to caveolin-1 directly. *First,* caveolin competes with calmodulin for binding at this site (30, 40, 41). This may have functional significance, as calmodulin binding activates eNOS activity, whereas caveolin binding represses eNOS activity (30, 40, 41). This suggests that caveolin-1 and calmodulin have a reciprocal relationship with respect to eNOS functioning (40, 41). *Second,* Sessa and colleagues (38) have performed site-directed mutagenesis to modify the predicted caveolin-binding motif (from **F**SAAP**F**SG**W** to **A**SAAP**A**SG**A**) within eNOS. It is known from *in vitro* studies that aromatic residues (Trp, Phe, or Tyr) are required for the proper recognition of the caveolin-binding motif (31). In their work, Sessa and colleagues (38) show that mutation of the caveolin-binding motif within eNOS blocks the ability of caveolin-1 to inhibit eNOS activity *in vivo*. These findings provide the first demonstration that a caveolin-binding motif is relevant and functional *in vivo*.

Caveolin-1 Is Down-regulated in Transformed Cells

Direct interaction of caveolin with signaling molecules leads to their inactivation (18). Since many signaling molecules can cause cellular transformation when constitutively activated, it is reasonable to speculate that caveolin may possess transformation suppressor activity. Consistent with this hypothesis, both caveolae and caveolin are most abundantly expressed in terminally differentiated cells: adipocytes, endothelial cells, and muscle cells. In addition, caveolin-1 mRNA and protein expression are lost or reduced during cell transformation by activated oncogenes such as v-*abl* and Ha-*ras* (G12V); caveolae are absent from these cell lines (42).

The potential "transformation suppressor" activity of caveolin-1 has recently been evaluated by using inducible expression in oncogenically transformed cells. Induction of caveolin-1 expression in v-Abl- and Ha-Ras (G12V)-transformed NIH 3T3 cells abrogated the anchorage-independent growth of these cells in soft agar and resulted in the *de novo* formation of caveolae (43). Thus, down-regulation of caveolin-1 expression and caveolae organelles may be critical to maintaining the transformed phenotype. These findings may also have relevance to human cancers. Sager and co-workers (44) identified caveolin-1 as one of 26 gene products whose mRNAs were down-regulated in human mammary carcinoma cell lines.

G-protein-coupled Receptors in Caveolae

Several G-protein-coupled receptors, *i.e.* endothelin, bradykinin, muscarinic acetylcholine, and β-adrenergic receptors, have been localized to caveolae using a combination of morphological and biochemical techniques (45–49). Caveolar localization may be ligand-dependent or -independent depending on the receptor (Fig. 2).

Binding of bradykinin to B2 bradykinin receptors on smooth muscle cells promotes the sequestration of the occupied receptors within caveolae (47). Receptor antagonists did not affect the distribution of these molecules, suggesting that only the activated receptor undergoes translocation to caveolae. Similarly, in cardiac myocytes, the muscarinic cholinergic agonist carbachol promotes the translocation of the muscarinic acetylcholine receptor into caveolin-rich membrane domains that contain caveolin-3 (46). Translocation of this receptor subtype was responsible for the radioligand binding detected in the caveolin-rich fractions. Atropine, a muscarinic cholinergic antagonist, did not induce translocation of the m2 muscarinic acetylcholine receptor to caveolae. Thus, agonist-induced translocation of G-protein-coupled receptors to caveolae membranes may be an essential step in the initiation of signaling cascades, as many downstream transducers of G-protein-coupled receptors have been localized to caveolae membranes.

Receptor Tyrosine Kinases in Caveolae

EGF initiates activation of the Ras-MAP kinase cascade. Using Rat-1 cells Mineo *et al.* (27) observed that EGF-R and Ras are greatly enriched within caveolae membranes. Thirty seconds after adding EGF, Raf-1 appears in caveolae but not in other membrane domains. Expression of constitutively active Ha-Ras (Gly-12 → Val,

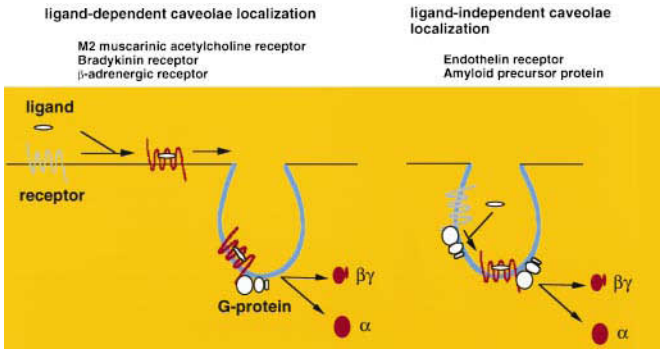

FIG. 2. **Ligand-dependent and ligand-independent localization of G-protein-coupled receptors within caveolae microdomains.** *Red* indicates activated, and *gray* or *white* indicates inactive. See text for details.

Ha-Ras^{G12V}) causes Raf-1 membrane recruitment to occur independently of EGF stimulation.

Liu *et al.* (26) observed that the PDGF receptor localizes to and undergoes PDGF-induced phosphorylation in caveolae. Binding of PDGF to its receptor stimulates phosphorylation of several caveolar targets including the tyrosine phosphatase Syp, the adapter molecule Shc 66, and MAP kinase. Similar results have recently been obtained by Schnitzer and colleagues (50).

Caveolar Components Undergo Lipid Modification

Caveolin co-purifies with lipid-modified signaling molecules, including Gα subunits, Ha-Ras, eNOS, and Src family tyrosine kinases (23). As many acylated and prenylated proteins are known to be targeted to caveolae, it has been suggested that lipid modification is required or greatly facilitates this targeting event. In support of this notion, myristoylation (G2A) and palmitoylation (C3S) mutants of $G_{i1}\alpha$ were poorly targeted to caveolae-enriched membrane fractions (51). Similarly, a myristoylation minus mutant of c-Src was quantitatively excluded from caveolae. The caveolar targeting of $G_{i1}\alpha$ was dramatically augmented approximately 4-fold by palmitoylation. Therefore, it is likely that both myristoylation and palmitoylation are important for their correct caveolar localization. The caveolar targeting of Hck and Fyn tyrosine kinases as well as eNOS also supports this hypothesis (21, 25, 30, 35, 36, 38, 52). The C-terminal domain of caveolin-1 also undergoes palmitoylation on three residues, 133, 143, and 156 (53).

Lipid Mediators of Apoptosis within Caveolae

Cholesterol and sphingolipids are two major lipid components of caveolae membranes, and both of these lipid classes have been found to interact directly with caveolin-1 (10–12, 54). Sphingolipids serve as substrates to generate ceramide, a newly characterized intracellular second messenger (55). Caveolin-1 has been shown to physically associate with the cytoplasmic domain of p75, and nerve growth factor-dependent production of ceramide takes place within caveolae (56). Thus, caveolae may play a role in mediating apoptotic cell death.

Phosphoinositol Metabolism within Caveolae

Prompted by the finding that the inositol trisphosphate receptor targets to caveolae (57) and their own observation that PtdIns 4,5-P_2 is enriched in detergent-insoluble lipid domains containing caveolin-1, Pike and Casey (58) determined whether caveolar PtdIns 4,5-P_2 is degraded following treatment with specific growth factors. In A431 cells, at least half of the PtdIns 4,5-P_2 is present within caveolin-rich membrane domains. Treatment with EGF or bradykinin reduces this pool of phospholipids in a time-dependent manner (58). These results underscore how caveolae can function as a focal point for the signaling machinery of the cell, as both a second messenger-progenitor (*i.e.* PtdIns 4, 5-P_2) and the receptor of the second messenger (*i.e.* inositol trisphosphate receptor) are housed within these cell membrane invaginations. Consistent with these findings, the target of diacylglycerol, *i.e.* protein kinase Cα, has also been localized to caveolae membranes (16, 59).

How Are Caveolae Different in Striated Muscle?

Muscle cells predominantly express a muscle-specific isoform of caveolin, termed caveolin-3. In adult skeletal muscle, caveolin-3 is confined to the sarcolemma (muscle cell plasma membrane) and coincides with the distribution of another muscle-specific plasma membrane marker protein, dystrophin (60, 61). Expression of caveolin-3 protein is dramatically induced during the differentiation of C2C12 skeletal myoblasts in culture. In differentiated C2C12 cells, caveolin-3 co-fractionates with cytoplasmic signaling molecules and members of the dystrophin complex. Also, caveolin-3 co-immunoprecipitates with dystrophin, suggesting that caveolin-3 and dystrophin are physically associated. These results are consistent with immunoelectron microscopic studies demonstrating that dystrophin is localized to caveolae in smooth muscle cells (4). In addition, dystrophin contains numerous caveolin-binding motifs (31).

Phosphofructokinase-M (PFK-M) is a key regulatory enzyme in the glycolytic pathway. Recently, a series of co-immunoprecipitation experiments identified PFK-M as a major caveolin-3-associated protein (62). This association is highly regulated by extracellular glucose and can be stabilized by intracellular metabolites, which are allosteric activators of PFK. Glucose-dependent plasma membrane recruitment of activated PFK-M by caveolin-3 could have important implications for understanding the mechanisms that regulate energy metabolism in skeletal muscle. As caveolin-3 is a component of the dystrophin complex (61), these findings with PFK-M may also have implications for understanding the pathogenesis of Duchenne's and related muscular dystrophies.

Caveolae Purification and Caveolae-related Domains

Caveolae Purification—Several independent methods have been developed for purifying caveolae (15, 16, 22–24, 29). Initially, these methods took advantage of the *natural* detergent insolubility of caveolae membranes. More recently, detergent-free methods have become available that are dependent on the intrinsic light buoyant density of caveolae membranes (22, 23). Purification of caveolae by all of these methods reveals the dramatic enrichment of caveolins and signaling molecules relative to plasma membrane. Caveolar localization of these signaling molecules has been confirmed in many cases by Schnitzer and colleagues (24, 50, 63). Also, these molecules appear to form a tight complex with caveolin-1, as shown using a polyhistidine-tagged form of caveolin-1 to affinity purify caveolae membranes from cultured cells (23).

Caveolae have also been shown to be detergent-resistant structures *in vivo*. For example, when intact cells were fixed with paraformaldehyde, extracted with cold Triton X-100, and then examined by electron microscopy, the insoluble membranes that remained were found to be caveolae (64). In support of these morphological findings, caveolin proteins are Triton-insoluble in caveolae (15, 16) but Triton-soluble in the Golgi (65). This compartment-specific Triton insolubility is most likely due to the association of caveolin with cholesterol and sphingolipids, which form a Triton-insoluble microenvironment, termed a liquid-ordered phase.

It has been debated whether GPI-anchored proteins are constitutively associated with caveolae or are only associated with caveolae upon cross-linking with antibody probes. In the case of the tissue factor receptor system, this GPI-anchored protein is only associated with caveolae upon binding its natural ligand (66). Thus, antibody probes may mimic natural endogenous ligands and target clustered GPI-anchored proteins to caveolae. Similarly, clustering of a glycosphingolipid (G_{M1}) with a ligand (the G_{M1} binding B-subunit of cholera toxin) increases its association with caveolin-1 by ~5-fold (12). Several G-protein-coupled receptors are targeted to caveolae in response to receptor agonist but not receptor antagonists (46, 47, 67). Taken together, these results suggest that clustering or receptor activation by ligand binding is sufficient to promote regulated translocation into caveolae membranes.

Caveolae-related Domains or Precaveolae or Both?—A number of investigators have purified "caveolae" from cells and tissues that lack apparent expression of caveolin (69–72). These domains have been termed Triton-insoluble complexes, detergent-resistant membranes, and low density membranes. We now suggest the term *caveolae-related domains* be used to describe these structures. Like caveolae, these microdomains are dramatically enriched in choles-

terol, sphingolipids, and lipid-modified signaling molecules (69, 70). The existence of "caveolae-related domains" that fail to contain caveolin has caused considerable confusion (69). However, this was at a time when only one caveolin gene was known to exist, *i.e.* caveolin (now termed caveolin-1). In addition, it has been shown that caveolin-1 and caveolae are down-regulated in response to cell transformation, whereas caveolin-2 levels remain constant (9). As a consequence, many commonly used cell lines lack caveolin-1 protein expression and visible caveolae, as they are immortalized or transformed. Also, other detergent-insoluble membrane proteins have recently been cloned, and one or more of them may represent functional homologues of the caveolins (63, 68, 69).

Caveolae-related domains can also be produced *in vitro* simply by mixing cholesterol, sphingolipids, and phospholipids in the appropriate ratio (70). Their Triton insolubility is a physical property of their molecular organization that produces a liquid-ordered membrane domain (rather than fluid or liquid crystalline). As caveolin-1 is found associated with glycosphingolipids *in vivo* (12), binds cholesterol directly (10), and requires a high local concentration of cholesterol (>30%) to insert into model lipid membranes (10, 11), these findings suggest that a true functional relationship exists between caveolae-related domains and mature caveolae. For example, during the biogenesis of mature caveolae, caveolae-related domains would need to exist as precursors for proper insertion of caveolins into membranes. Thus, in cells that express caveolins, these caveolae-related domains may represent "precaveolae" that simply lack caveolins. In support of this reductionist model, recombinant expression of caveolin-1 in cells that lack morphologically detectable caveolae is sufficient to drive the formation of mature invaginated caveolae (19, 33, 43). This indicates that cells normally make the ingredients that are necessary for the formation of mature caveolae, and insertion of caveolin proteins may be only a late phase in this process. Insertion of caveolins into these caveolae-related domains may provide a necessary "brake" in signal transduction (43).

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