Palmitoylation of Caveolin-1 in Endothelial Cells Is Post-translational but Irreversible*

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Caveolin-1 is a palmitoylated protein involved in assembly of signaling molecules in plasma membrane subdomains termed caveolae and in intracellular cholesterol transport. Three cysteine residues in the C terminus of caveolin-1 are subject to palmitoylation, which is not necessary for caveolar targeting of caveolin-1. Protein palmitoylation is a post-translational and reversible modification that may be regulated and that in turn may regulate conformation, membrane association, protein-protein interactions, and intracellular localization of the target protein. We have undertaken a detailed analysis of [3H]palmitate incorporation into caveolin-1 in aortic endothelial cells. The linkage of palmitate to caveolin-1 was hydroxylamine-sensitive and thus presumably a thioester bond. However, contrary to expectations, palmitate incorporation was blocked completely by the protein synthesis inhibitors cycloheximide and puromycin. In parallel experiments to show specificity, palmitoylation of aortic endothelial cell-specific nitric-oxide synthase was not significant even after 24 h. These results show that [3H]palmitate incorporation is limited to newly synthesized caveolin-1, not because incorporation only occurs during synthesis but because the continuous presence of palmitate on caveolin-1 prevents subsequent repalmitoylation.

Originally named to describe membrane invaginations at the cell surface, caveolae are specialized plasmalemman domains rich in glycosphingolipids, cholesterol, and lipid-anchored membrane proteins. In endothelial cells (EC), caveolae have a striated coat, the major protein of which is caveolin-1 (1). Caveolin-1 is involved in cholesterol trafficking (2), oligomerizes to form a scaffold for assembly of signaling molecules including receptors, signal transducers, and effectors (3), and regulates the activation state of these signaling complexes (4).

Lipid modification, including palmitoylation, is an important mechanism targeting signaling proteins to caveolae. Among the palmitoylated proteins in caveolae are heterotrimeric G-protein α-subunits (e.g. αs, αi, αo, and αq), G-protein linked receptors, regulator of G-protein signaling proteins, p21ras, nonreceptor tyrosine kinases, and EC-specific nitric-oxide synthase (eNOS) (5). Palmitoylation of several of these proteins has been shown to be a post-translational and reversible modification that is also subject to regulation (6). The post-translational nature of palmitoylation has been shown by cell culture experiments in which palmitate labeling occurs even in the presence of protein synthesis inhibitors (7). These experiments clearly distinguish protein palmitoylation from protein myristoylation, which is cotranslational and blocked by protein synthesis inhibitors. The reversibility of protein-palmitate bonds has been demonstrated by the rapid turnover of the palmitoyl group and may be caused by the relative lability of the thioester linkage (8–10). Protein palmitoylation and de-palmitoylation reactions are considered to be primarily enzymatic and catalyzed by unidentified membrane-bound palmitoyl acyltransferases (11, 12) and by cytoplasmic and lysosomal forms of palmitoyl thioesterases (13–15), respectively. Regulation of the protein palmitoylation and de-palmitoylation cycle is thought to be important in regulating protein localization, conformation, protein-protein interaction, and activity (16–18).

Caveolin-1, the most abundant caveolin family member in EC, is a 21–24-kDa integral membrane protein with a hairpin-like structure and with both N and C termini facing the cytoplasm (19, 20). Caveolin-1 is palmitoylated on three cysteine residues located near the C terminus of the protein (20). Two isoforms, the slower migrating caveolin-1α and the faster migrating caveolin-1γ, differ by 31 N-terminal amino acids because of alternate translation initiation sites (21). Their C termini are identical and it is likely that both isoforms are palmitoylated similarly. The function of caveolin-1 palmitate chains is not well understood. They may increase the stability of the oligomers and therefore the scaffold structure of caveolae (22). In addition, caveolin-1 palmitate residues may regulate its interaction with other proteins. A palmitate-deficient mutant of caveolin-1 exhibits normal caveolar localization (20); however, its interaction with Go,αi is diminished greatly (23). A recent report suggests that at least two palmitate chains on caveolin-1 are required for binding and transport of cholesterol (24).

We here report results from a kinetic analysis that shows

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1 The abbreviations used are: EC, endothelial cells; eNOS, EC-specific nitric-oxide synthase; DME, Dulbecco’s modified Eagle’s; PAGE, polyacrylamide gel electrophoresis; BFA, brefeldin A.
that caveolin-1 exhibits unique characteristics of palmitate incorporation. Caveolin-1 palmitoylation is essentially irreversible and completely inhibited by cycloheximide. Nonetheless it is a post-translational event that requires protein transport from the endoplasmic reticulum to the plasma membrane. This distinguishes caveolin-1 from other caveolar palmitoylated proteins and suggests that caveolin-1 may be subject to unique regulatory mechanisms of palmitoylation and/or de-palmitoylation that account for its distinctive palmitoylation characteristics.

**EXPERIMENTAL PROCEDURES**

**Materials**—[9,10-3H]Palmitic acid (35 Ci/mmol) and [9,10-3H]myristic acid (49 Ci/mmol) were obtained from PerkinElmer Life Sciences. Trans-[^35]S-labeled (1175 Ci/mmol, referred to here as [^35]S)methionine, was from ICN (Irvine, CA). Polyclonal rabbit anti-human caveolin-1 antibody and mouse monoclonal antibody against EC-specific eNOS were from Transduction Laboratories (Lexington, KY). Nonimmune rabbit and mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Brefeldin A was purchased from Epitope Technologies (Madison, WI). Protein-G-Sepharose was from Zymed Laboratories Inc. Protein A-Sepharose and other assay reagents were from Sigma.

**Cell Culture Conditions**—Bovine aortic EC were isolated as described (25) and maintained in Dulbecco’s modified Eagle’s (DME) medium and F12 medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO₂.

**Metabolic Labeling of Endothelial Cells—**EC in 10-cm dishes were washed with serum-free DME medium and then incubated for 2 h in the same medium. For measurement of incorporation of palmitate into caveolin-1 and other proteins, EC were incubated for 15–30 min with 250 μCi/ml[^3]H]palmitic acid in serum-free medium containing 3.5 mg/ml fatty acid-free bovine serum albumin. For pulse-chase experiments, the medium was removed and the cells were rinsed three times with serum-free DME medium. The radiolabel was chased by the addition of DME medium containing cycloheximide (29 μg/ml) and either unlabeled palmitate (15 μg/ml) bound to fatty acid-free bovine serum albumin or 5% fetal bovine serum as indicated. To measure protein synthesis, EC were incubated for 15–30 min with 60 μCi/ml[^35]Smethionine in cysteine- and methionine-free medium.

**Immunoprecipitation of Caveolin-1 from Endothelial Cell Lysates—**EC were washed twice in phosphate-buffered saline and lysed at 4°C. 0.6 ml of immunoprecipitation buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2.5 mM MgCl₂, 0.5 mM sodium deoxycholate, 1% Nonidet P-40, 20 μg/ml aprotinin, and 5 μg/ml leupeptin) for 90 min. Cells were scraped, and the lysate was cleared by centrifugation at 16,000 × g for 3 min. An aliquot of the lysate was subjected to 12% SDS-PAGE under nonreducing conditions. The remainder of the lysate was added to SDS (0.1% final concentration). Anti-caveolin-1 antibody was added, followed by protein-A-Sepharose for 60 min. A control cell lysate was immunoprecipitated with rabbit nonimmune IgG. Immunoprecipitated complexes were washed three times with immunoprecipitation buffer and analyzed by 15% SDS-PAGE. In some experiments, the lysates were subjected to a second immunoprecipitation with anti-eNOS antibody or mouse nonimmune IgG as described above except that the antibody was added for 15 h and analyzed by SDS-PAGE (7.5% acrylamide). Gels were analyzed by fluorography.

**Determination of[^3]H/Palmitate Incorporation into Caveolin-1—**To verify that the radiola beled fatty acid incorporated into caveolin-1 was linked by a thioester bond, susceptibility to hydroxylamine treatment was examined (20). EC were incubated with 250 μCi/ml[^3]H]palmitate for 30 min. The cells were lysed, and caveolin-1 was immunoprecipitated with anti-caveolin-1 antibody. Aliquots of the immunoprecipitated material were fractionated by SDS-PAGE. Gel lanes were separated and soaked in either 1 M hydroxylamine, pH 7.0, or 1 M Tris-HCl, pH 7.0 for 4 h and then subjected to fluorography.

The specific fatty acid esterified to caveolin-1 was identified by reverse-phase thin layer chromatography essentially as described (26). EC were incubated with 250 μCi/ml[^3]H]palmitate for 90 min. The cells were lysed, and caveolin-1 was immunoprecipitated with polyclonal anti-caveolin-1 IgG and separated by SDS-PAGE. The caveolin-1 band was excised and subjected to in-gel alkaline hydrolysis by treatment with 0.1 M KOH for 30 min. After acidification of the extract to pH 4.5, the free fatty acids were extracted with hexane, dried under N₂, and dissolved in chloroform/methanol (2:1, v/v). The extracted lipids were fractionated by reverse-phase thin layer chromatography (RP-18, Whatman) in a resolving solvent system of acetonitrile/acetate acid (1:1, v/v). Standards containing[^1]H)palmitate and[^1]H)myristate were fractionated in parallel lanes. The lanes were scraped at 1-cm intervals, and radioactivity was determined by scintillation counting.

**RESULTS**

**Incorporation of Palmitate into Caveolin-1 Requires de Novo Protein Synthesis—**Protein palmitoylation is considered generally to be a post-translational modification independent of de novo protein synthesis (7, 8). This characteristic differentiates palmitoylation from myristoylation, a cotranslational modification that is blocked by protein synthesis inhibitors (27). To investigate the cellular mechanism of caveolin-1 palmitoylation, bovine aortic EC were pretreated with cycloheximide for 60 min before incubation with[^3]H]palmitate for 20 min. Caveolin-1 was immunoprecipitated from EC lysates using polyclonal anti-caveolin-1 IgG, and[^3]H]palmitate-labeled protein was detected by fluorography. Surprisingly, cycloheximide treatment blocked essentially all incorporation of[^3]H]palmitate into caveolin-1 (Fig. 1A, upper panel). This inhibition was also seen after longer labeling periods in the presence of cycloheximide up to 6 h (data not shown). Previous results by Baker et al. (28) showed substantial labeling of caveolin-1 by[^3]H]palmitate in cycloheximide-treated NIH 3T3 cells (28). We found that the complete inhibition of palmitoylation of caveolin-1 in either EC (Fig. 1A, upper panel) or mouse NIH 3T3 cells (data not shown) was observed only in cells pretreated with cycloheximide. When cycloheximide was added only during the labeling period, substantial incorporation of[^3]H]palmitate into caveolin-1 was observed, which agreed with the previous report (28). In parallel dishes to determine the effectiveness of the inhibitor, cycloheximide completely blocked de novo synthesis of caveolin-1 in EC metabolically labeled with[^35]S)methionine (Fig. 1A, middle panel). Interestingly, cycloheximide added without pretreatment inhibited caveolin-1 synthesis completely but inhibited its palmitoylation only partially. These findings suggest the existence of a newly synthesized pool of caveolin-1 lacking palmitoyl chains and that incorporation of palmitate into caveolin-1 in this pool is post-translational but limited to a period 60 min or less after synthesis. Coomassie Blue staining of the gel served as a loading control and showed that the total amount of cellular caveolin-1 was not changed by cycloheximide treatment (Fig. 1A, lower panel).

The identity of the[^3]H]palmitate-labeled protein was confirmed by mass spectrometric sequence analysis of proteolytic fragments of the eluates. The sequence of fragments in the upper panel from Fig. 1A (upper panel) was identical to that of bovine caveolin-1a (data not shown). The lower band contained sequences found in bovine caveolin-1β and also a protein with a sequence consistent with human caveolin-2 (the bovine sequence has not been reported). It is likely that the observed[^3]H]palmitate in the lower band is due to caveolin-1β because caveolin-2 does not contain the C-terminal Cys residues that are palmitoylated in other caveolin types (and palmitate linked to caveolin-2 has not been reported). Because inhibition of protein acylation by cycloheximide is
considered generally to be evidence for cotranslational myristoylation rather than post-translational palmitoylation, we investigated the identity of the radiolabeled fatty acid and the nature of the linkage to caveolin-1. Protein palmitoylation occurs on Cys residues through a thioester bond sensitive to treatment with hydroxylamine (20). EC were incubated with [3H]palmitate, and caveolin-1 was immunoprecipitated from cell lysates using polyclonal anti-caveolin-1 IgG and subjected to SDS-PAGE. Incubation of the gel in neutral hydroxylamine removed all radioactivity, consistent with a palmitate thioester bond (Fig. 1B). A chromatographic method was used to identify more precisely the fatty acid linked to caveolin-1 (26). Caveolin-1 was immunoprecipitated from [3H]palmitate-labeled EC and fractionated by SDS-PAGE. The fatty acids released by alkaline hydrolysis were extracted and subjected to reverse-phase thin layer chromatography. The mobility of the fatty acid released from caveolin-1 was the same as that of [3H]palmitate (Fig. 1C).
To examine the target specificity of cycloheximide we determined its effect on incorporation of [3H]palmitate into a second palmitoylated EC protein, eNOS. Palmitoylation of eNOS was not decreased by cycloheximide (Fig. 1D). To evaluate the effect of cycloheximide on an array of EC proteins, lysates not subjected to immunoprecipitation were examined. Palmitate incorporation into multiple proteins was diminished by cycloheximide but not inhibited completely. Interestingly, modification of proteins with molecular mass greater than 65 kDa was not decreased (Fig. 1E, left). The effectiveness of the cycloheximide treatment was shown by metabolic labeling with [35S]methionine (Fig. 1E, right). Puromycin, a protein synthesis inhibitor with a mechanism different from cycloheximide, also blocked palmitoylation of caveolin-1 (Fig. 1F) but did not inhibit palmitoylation of eNOS and only partially inhibited palmitoylation of most visible cellular proteins (data not shown). These results suggest that the process of palmitoylation of caveolin-1 differs from that of most proteins studied so far in that it requires de novo protein synthesis. Several mechanisms may be responsible for this requirement, namely (i) caveolin-1 palmitoylation is cotranslational, (ii) caveolin-1 palmitoylation occurs during trafficking to the plasma membrane, (iii) caveolin-1 palmitoylation requires the synthesis of accessory proteins, e.g., chaperones, or (iv) caveolin-1 palmitoylation occurs after synthesis and delivery to the plasma membrane but is irreversible, and therefore incorporation of [3H]palmitate is found only on newly synthesized protein.

Caveolin-1 Palmitoylation Is Not Cotranslational—To examine whether incorporation of palmitate into caveolin-1 is cotranslational, we examined the effects of inhibitors of protein transport between endoplasmic reticulum and plasma membrane. A lack of an effect would be consistent with palmitate incorporation proximal to the inhibited trafficking step and with cotranslational modification. Newly synthesized caveolin-1 travels from the endoplasmic reticulum through the Golgi apparatus, where it is incorporated into detergent-resistant membranes (29, 30), and then to the cell surface (31). Brefeldin A (BFA) reversibly blocks the exit of secretory and membrane proteins from the endoplasmic reticulum by disassembling the Golgi apparatus and mixing it into the endoplasmic reticulum (32). Incubation of EC with BFA dramatically reduced [3H]palmitate labeling of caveolin-1 (Fig. 2A, upper panel). The much smaller decrease in [35S]methionine labeling indicates that the effect of BFA was not caused by decreased neosynthesis of caveolin-1 (Fig. 2A, lower panel). The BFA-induced retrograde transport of vesicles from the Golgi to endoplasmic reticulum is prevented by the addition of nocodazole, which depolymerizes microtubules; thus in combination with BFA, nocodazole can distinguish the effects of inhibition of anterograde and retrograde transport (33). Nocodazole did not prevent the inhibition of caveolin-1 palmitoylation by BFA, indicating that anterograde transport from the endoplasmic reticulum to Golgi is necessary for palmitate incorporation (Fig. 2A). The inhibition of palmitoylation of caveolin-1 by BFA was highly specific as shown by [3H]palmitate labeling in cell lysates; palmitoylation of most visible proteins was not inhibited, whereas palmitoylation of two proteins of 60 and 70–75 kDa was increased markedly (Fig. 2B).

To evaluate the role of more distal trafficking in palmitate incorporation into caveolin-1, we incubated EC with monensin. Monensin equilibrates the monovalent cations Na+, K+, and H+ across biological membranes, which perturbs protein transport from medial- to trans-Golgi compartments (34) and also increases intralysosomal pH. Pretreatment of EC with monensin substantially reduced [3H]palmitate labeling of caveolin-1 (Fig. 2C, upper panel) without affecting caveolin-1 neosynthesis (Fig. 2C, lower panel). The inhibition was not due to the lysosomotropic property of monensin because chloroquine, which also increases intralysosomal pH, did not inhibit caveolin-1 palmitoylation (data not shown). Together these results show that an intact trafficking pathway is necessary for caveolin-1 palmitoylation.

Disruption of Caveolin-Chaperone Complexes Does Not Inhibit Caveolin-1 Palmitoylation—Protein synthesis inhibitors may block palmitoylation of caveolin-1 by an indirect mechanism, e.g., by inhibition of chaperones or other accessory proteins required for caveolin-1 trafficking rather than by a direct effect on caveolin-1 itself. Caveolin-1 is present in the cyto-
plasm as a complex with the immunophilins FK506-binding protein 52, cyclophilin A, and cyclophilin 40 (35). These chaperones could be the target of cycloheximide either because their neosynthesis is necessary for caveolin-1 trafficking or because cycloheximide competitively binds immunophilins that may disrupt complex formation with caveolin-1 (35, 36). To test this mechanism, we examined the effect of agents that specifically bind immunophilins on [3H]palmitate labeling of caveolin-1. Cyclosporin A and rapamycin were used to bind cyclophilins and FK506-binding protein 52, respectively, at concentrations known to disrupt the caveolin-chaperone complex (35). Immunophilin binding did not diminish caveolin-1 palmitoylation, suggesting that the synthesis and activity of these chaperones are not critical for palmitoylation of newly synthesized caveolin-1 (Fig. 3).

**Palmitoylation**: An alternative explanation for our observations is that the modification of caveolin-1 by palmitate is essentially irreversible. If this is the case, the apparent dependence of palmitoylation on new protein synthesis would be due to the fact that [3H]palmitate cannot be incorporated into caveolin-1 that has been palmitoylated already but instead is incorporated preferentially into newly synthesized, and thus nonpalmitoylated, caveolin-1. To test this mechanism, the rate of caveolin-1 de-palmitoylation was measured in a pulse-chase experiment. EC were incubated with [3H]palmitate or [35S]methionine for 30 min, and the radiolabel was chased for up to 24 h with medium in the presence of cycloheximide and excess unlabeled palmitate. The release of [3H]palmitate from caveolin-1 was negligible during the 24-h chase period (Fig. 4A, upper panel), as was turnover of the caveolin-1 polyepitope chain (Fig. 4A, lower panel). This result was in marked contrast to the rapid release of [3H]palmitate from eNOS, which was essentially complete after 3 h (Fig. 4B, upper panel); the high rate of palmitate turnover was not caused by eNOS protein turnover, which had a half-life of about 9 h (Fig. 4B, lower panel). Analysis of total [3H]palmitate-labeled proteins in cell lysates shows that the rate of release of [3H]palmitate from EC proteins is rapid with apparent half-lives of less than 6 h for most detectable proteins (Fig. 4C). These results indicate that the process of palmitoylation of caveolin-1 is essentially irreversible in EC and that this characteristic distinguishes it from most other palmitoylated EC proteins.

**DISCUSSION**

Our results show that incorporation of [3H]palmitate into caveolin-1 requires de novo synthesis of the protein. This requirement is not due to cotranslational modification but rather results from the absence of a detectable palmitoylation/de-palmitoylation cycle. Our data also show that palmitate incorporation occurs within 60 min of caveolin-1 synthesis and that an intact trafficking pathway is required, indicating that palmitoylation occurs distal to the Golgi apparatus, most likely in the caveolae or plasma membrane compartments. These findings are not limited to cells of a single type or from a single species because similar results were obtained using murine NIH 3T3 fibroblastic cells (data not shown).

The observed low rate of depalmitoylation does not imply that the palmitate linkage to caveolin-1, or its pathway of formation, is unique but rather that the enzymatic or nonenzymatic depalmitoylation reaction is extremely slow. The in vitro sensitivity to neutral hydroxylamine (our results and Ref. 20) indicates that palmitate is linked to caveolin-1 by a trans-esterification-labile thioester bond. The resistance to cleavage of the palmitate linkage in caveolin-1 in EC may be caused by the inaccessibility of caveolin-1 to the depalmitoylating enzyme(s) or to the possibility that caveolin-1 is a weak substrate for these enzymes. Little is known about the substrate specificities of the palmitoyl protein thioesterases. Palmitoyl protein thioesterase-1 and its homologue, palmitoyl protein thioesterase-2, are localized in the lysosomes and are thus unlikely to be...
responsible for caveolin-1 deacetylation. However, acyl protein thioesterase-1, which is located primarily in the cytoplasm and increases the turnover rate of palmitate on Goα and eNOS, is a candidate for regulation of caveolin-1 deacetylation (14, 15). In agreement with our results, Yeh et al. (14) recently showed that caveolin-1 is not a substrate for acyl protein thioesterase-1. It is not known whether caveolin-1 lacks a specific sequence required for thioesterase recognition or whether the orientation of the palmitate groups within the membrane renders them inaccessible to the active site of the enzyme. An alternate possibility is that our findings on caveolin-1 palmitoylation are restricted to cells under basal conditions, and the palmitoylation/depalmitoylation cycle is activated by agonists. However, in preliminary studies using thrombin, bradykinin, and several cholinergic and adrenergic ligands, we have not observed agonist-dependent caveolin-1 increases in the rate of palmitoylation (data not shown).

Our results show that [3H]palmitate labeling of newly synthesized caveolin-1 requires an intact trafficking pathway as shown by inhibition by BFA and monensin. Other investigators have reported that palmitoylation of SNAP-25 and GAP-43 is also inhibited by brefeldin A (33). Interestingly, both proteins are modified by palmitate but not by other acyl groups (SNAP-25 and GAP-43 are modified by four and two palmitate groups, respectively). In contrast, palmitoylation of several proteins that are both myristoylated and palmitoylated, e.g. Goα, Goβ, and p55γκ, is insensitive to brefeldin A (33, 37). Thus it is possible that the specific acyl modifications define not just susceptibility to acylation-deacylation cycling but also specific intracellular trafficking pathways.

Because there are not other reports of proteins with similar palmitoylation kinetics, we can only speculate on the cellular consequences of the essentially irreversible nature of the palmitate linkage on caveolin-1. The fact that caveolin-1 remains in a continuous palmitoylated state suggests a tight and possibly irreversible interaction with membranes. The “two-signal model” for membrane binding (6, 38, 39) proposes that the first signal (e.g. lipid modification, poly-basic stretch of amino acids, interaction with a membrane-bound protein) permits transient interaction of a protein with membranes, whereas the second signal (often a palmitate chain) provides a more persistent association with a specific membrane. According to this model, the presence in caveolin-1 of a 33- amino acid hydrophobic domain as well as three palmitate chains may constitute the equivalent of four membrane-binding signals and promote essential irreversibility of the membrane interaction. Thus the persistent palmitate chains may be important for a structural role of caveolin-1 rather than a kinetic role. Indeed, this idea is consistent with the finding (using palmitoylation-deficient mutants of caveolin-1) that palmitate modification is required for optimal stability of the caveolin-1 oligomers (22), which may contribute to the rigid structure of caveolae (40). Likewise, caveolin-1 palmitate residues are required for efficient binding and sequestration of signaling molecules in caveolae (23). Thus unlike most caveolar proteins that require cycles of palmitoylation and depalmitoylation for normal function, e.g. translocation between cellular compartments (17) and regulation of protein interaction (41), caveolin-1 function may require persistent palmitate linkages.

In contrast to its potentially static role in caveolar structure and in protein-protein interactions, caveolin-1 and caveolae have been implicated in dynamic cellular processes including transcytotic and potocytotic endothelial transport (42), polarized vesicular trafficking in epithelial cells (43), and two-way transport of cholesterol (and other lipids) between the endoplasmic reticulum/Golgi system and the plasma membrane.
Caveolin-1 Palmitoylation Is Irreversible

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