Endoproteolytic Processing and Stabilization of Wild-type and Mutant Presenilin*

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Presenilin 1 (PS1), mutated in pedigrees of early-onset familial Alzheimer's disease, is a polytopic integral membrane protein that is endoproteolytically cleaved into 27-kDa N-terminal and 17-kDa C-terminal fragments. Although these fragments are the principal PS1 species found in normal mammalian brain, the role of endoproteolysis in the maturation of PS1 has been unclear. The present study, which uses stably transfected mouse neuroblastoma N2a cells, demonstrates that full-length polypeptides, derived from either wild-type or A246E FAD-mutant human (hu) PS1, are relatively short-lived (∼1.5 h) proteins that give rise to the N- and C-terminal PS1 fragments, which are more stable (∼24 h). N-terminal fragments, generated artificially by engineering a stop codon at amino acid 306 (PS1–306) of wild-type huPS1, were short-lived, whereas an FAD-linked variant that lacked exon 9 (DE9) and was not endoproteolytically cleaved exhibited a long half-life. These observations suggest that endoproteolytic cleavage and stability are not linked, leading us to propose a model in which wild-type full-length huPS1 molecules are first stabilized then subsequently endoproteolytically cleaved to generate the N- and C-terminal fragments. These fragments appear to represent the mature and functional forms of wild-type huPS1.

A subset of familial Alzheimer's disease (FAD) cases are caused by mutations in two related genes, termed presenilin 1 (PS1) and presenilin 2 (PS2) (1–3), which are functionally interchangeable with sel-12, a Caenorhabditis elegans molecule involved in Notch-mediated cell differentiation (4, 5). PS1 is a highly hydrophobic protein that spans the membrane eight times (6–8), projecting the N- and C-terminal hydrophobic domains, as well as a large internal loop domain, into the cytoplasm. In transiently transfected cultured cells, PS1 protein, a 467-amino acid polypeptide, exhibits a relative mass of 43 kDa (9–12). However, in cultured cells and in transgenic mice expressing either wild-type or FAD mutant PS1, the predominant PS1 species detected are ∼27-kDa N-terminal (NTF) and ∼17-kDa C-terminal fragments (CTF) that appear to be generated by endoproteolysis (9, 11–15). The role of endoproteolysis in PS1 maturation is not well understood as a noncleavable FAD variant (exon 9 deleted (DE9)) rescues the egg-laying defects of sel-12 mutant C. elegans (5, 16). Thus, it has been difficult to resolve whether function is provided by full-length PS1 or the endoproteolytic fragments.

Previous studies of multiple lines of transgenic mice expressing wild-type human (hu) PS1 indicate that the synthesis/accumulation of PS1 NTFs and CTFs is highly regulated; the levels of full-length PS1 increase in parallel with the levels of transgene-derived huPS1 mRNA, but the levels of huPS1 NTFs and CTFs plateau (9). Moreover, in animals expressing high levels of huPS1, the levels of endogenous mouse NTFs and CTFs fall below the threshold of detection (9, 15). These data suggest that the absolute levels of PS1 NTF and CTF in cells is highly regulated, such that the overproduction of human fragments inhibits the synthesis/accumulation of the mouse fragments.

In the present study, we generated mouse neuroblastoma N2a cell lines that harbor wild-type and FAD-mutant huPS1 cDNA genes under the transcriptional control of a butyrate-inducible cytomegalovirus (CMV) promoter. Using this system, we demonstrate that the processed 27 and 17 kDa derivatives of huPS1 are far more stable than full-length polypeptides, indicating that endoproteolysis is a step in the maturation of full-length wild-type PS1 precursors into stable molecules. Because we did not observe short-lived pools of PS1 proteolytic fragments, it is likely that endoproteolysis occurs after the full-length protein has been targeted for stabilization. Consistent with this idea was the finding that recombinant polypeptides, engineered to terminate near the site of endoproteolysis at residue 306, were short-lived. However, and interestingly, the noncleavable DE9 huPS1 variant was capable of being stabilized, suggesting that endoproteolytic cleavage is not an obligatory step in maturation. These new observations, taken together with our previous studies, lead us to conclude that newly synthesized wild-type PS1 is first stabilized (probably via interactions with limiting cellular proteins), and then endoproteolytically cleaved into two long-lived fragments. In our view, the more stable endoproteolytic derivatives are likely to represent the fully mature, functional, forms of PS1.
CCTCGAGCTTATGAGCTCAGGCCTC). The antisense primer hpPS1-R307atop-AS also encoded a BamHI restriction endonuclease site. The PCR product was digested with PstI and BamHI, gel-purified, and ligated with an Asp718-PstI fragment of wild-type huPS1 cDNA and Asp718-BamHI-digested pcB6 expression plasmid, which encodes the gene for conferring resistance to the neomycin analog G418 (19). The inserts and junctions were sequenced with Sequenase (United States Biochemical Corp.).

**Antibodies and Immunoblot Analyses**—All antibodies used in this study have been described previously and are specific for PS1 epitopes. The rabbit polyclonal antisera Ab14, a gift of Drs. Mary Seeger and Samuel Gandy (Cornell University Medical College, New York, NY), recognizes amino acids 3–15 of human and mouse PS1 (9). The monoclonal antibody N-terminus, a gift of Dr. Allan I. Levey (Department of Neurology, Emory University School of Medicine, Atlanta, GA), is a rat monoclonal antibody that specifically recognizes huPS1 N-terminal 27-kDa fragments and full-length huPS1 (15, 18, 20). The rabbit polyclonal antisera pS1Loop specifically recognizes epitopes in the hydrophilic loop domain of PS1 (9).

For Western blot analysis, cultured cells were lysed as described previously (9), followed by determination of protein concentration by bicinchoninic acid protein assay (Pierce). Generally, 10 μg of protein extract were separated by SDS-PAGE (samples were not heated), then transferred to nitrocellulose membranes and incubated with 1:1000 dilutions of Ab14 and pS1Loop antisera or 1:1000 dilutions of monoclonal antibody N-terminus. Bound primary antibodies were detected by horseradish peroxidase-conjugated antibody A (protein A-HRP) (Sigma) or horseradish peroxidase-conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and chemiluminescence (Pierce). For quantitative immunoblotting studies, blots were incubated with 125I-protein A (NEN Life Science Products), followed by phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

**Generation of Stable Cell Lines with Inducible Expression of huPS1**—Mouse N2a neuroblastoma cells were maintained in 1:1 OptiMEM (Life Technologies, Inc.) and Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with 5% fetal bovine serum.

Stable N2a cells expressing wild-type and mutant huPS1 were generated by transfecting 0.1 μg of pcB6-PS1 expression plasmids, encoding wild-type, A246E, ΔE9, and PS1ΔLM, followed by selection in G418 (400 μg/ml). The level of huPS1 expression in isolated colonies was determined following induction for 24 and 48 h with 10 mM butyrate (21, 22). Four separate cell lines expressing wild-type huPS1 (WT-7, WT-9, WT-15, and WT-18), two cell lines expressing A246E (A246E-25 and A246E-26), one cell line expressing ΔE9, and one cell line expressing PS1ΔLM were used in these studies to demonstrate, in part, that the observations were consistent among different cell lines.

**RNA Isolation and Northern Blotting**—Total RNA was isolated from cells by extraction in Trizol (Life Technologies, Inc.) and quantified by observation. Generally, 10 μg of total RNA were fractionated on agarose gels containing 2.2 M formaldehyde, stained with ethidium bromide, photographed, and transferred to nitrocellulose membranes. Following hybridization at 65 °C for 14–16 h with a random-primed 32P-labeled huPS1 cDNA in 0.5 M phosphate buffer (pH 7.2), 1 mM EDTA, 1% bovine serum albumin, and 7% SDS, membranes were washed at 50 °C in 2 × SSC and 0.1% SDS for 30 min, then exposed to film.

Levels of PS1 mRNA in stably transfected N2a cells following butyrate induction were assayed by PCR of reverse-transcribed RNA (RT-PCR). Degenerate primers, capable of hybridizing to human and mouse PS1 mRNA, were used to amplify a 351-bp fragment PS1 that encompasses amino acids 290–407 (sense primer, CCTCAACAATGGTGTGGCCG; antisense primer, GGTTGTGTTCCAGTCTCCAC). The resulting PCR product was digested with AsaI, which recognizes a site present only in human PS1 mRNA, generating two fragments of 199 and 152 base pairs. To confirm that hyperexpression of huPS1 does not affect the level of mouse PS1 mRNA, we also PCR-amplified reverse-transcribed RNA with primers (sense, CGGAAATTCTATGTTGGACG 5'-3'; antisense, CGGAGACGAGAGTGTTGCAGT). The resulting PCR product was ligated with AsaI, which recognizes a site present only in human PS1 mRNA, generating two fragments of 199 and 152 base pairs. To confirm that hyperexpression of huPS1 does not affect the level of mouse PS1 mRNA, we also PCR-amplified reverse-transcribed RNA with primers (sense, CGGAAATTCTATGTTGGACG 5'-3'; antisense, CGGAAATTCTATGTTGGACG 5'-3'). The resulting PCR product was ligated with AsaI, which recognizes a site present only in human PS1 mRNA, generating two fragments of 199 and 152 base pairs. To confirm that hyperexpression of huPS1 does not affect the level of mouse PS1 mRNA, we also PCR-amplified reverse-transcribed RNA with primers (sense, CGGAAATTCTATGTTGGACG 5'-3'; antisense, CGGAAATTCTATGTTGGACG 5'-3'). The resulting PCR product was ligated with AsaI, which recognizes a site present only in human PS1 mRNA, generating two fragments of 199 and 152 base pairs. To confirm that hyperexpression of huPS1 does not affect the level of mouse PS1 mRNA, we also PCR-amplified reverse-transcribed RNA with primers (sense, CGGAAATTCTATGTTGGACG 5'-3'; antisense, CGGAAATTCTATGTTGGACG 5'-3').

**RESULTS**

**Inducible Expression of Wild-type and A246E Mutant huPS1**—Due to the low synthetic rate of PS1 in cultured cells, we and others (10, 11) have been unable to detect endogenous PS1 polypeptides, using conventional metabolic radiolabeling and immunoprecipitation approaches. To overcome this technical limitation, we chose to express huPS1 polypeptides, using an inducible expression system, in mouse neuroblastoma N2a cells, and then examine the catabolism of PS1 polypeptides by immunoblotting approaches.

huPS1 cDNAs were inserted downstream of the huCMV promoter and enhancer elements in the expression plasmid pcB6 (19), which also contains sequences encoding resistance to the neomycin analog G418. The transcription of CMV promoter-driven cDNAs in the pCB6 vector is markedly induced by butyric acid (21, 22). We initially identified cell lines that showed marked induction of human wild-type and A246E mutant PS1 after 24- and 48-h incubations in 10 mM butyrate (Fig. 1). Analyses of the mRNA levels in selected cell lines demonstrated remarkable increases in the levels of huPS1 after butyrate induction (Fig. 1A, lanes 3 and 4). Notably, the levels of endogenous PS1 mRNA were not affected by butyrate treatment (Fig. 1A, compare lanes 1 and 2). In parallel with increased mRNA expression after butyrate, immunoblots with PS1Loop antiserum, which is specific for epitopes in the loop domain of PS1 (9), revealed robust increases in the steady-state levels of wild-type and mutant full-length huPS1 and in the PS1 17-kDa CTF (Fig. 1, B, lanes 2, 3, 5, 6, 8, and 9; C, lanes 2, 3, 5, 6, 8, and 9). Butyrate treatment of untransfected cells did not change the levels of full-length endogenous PS1 polypeptides or the levels of endogenous NTFs and CTFs (data not shown).

![Fig. 1. Inducible expression of huPS1.](Image)

A. Northern blots of total RNA (7.5 μg) from untransfected cells (lanes 1 and 2) and cells transfected with wild-type (WT) huPS1 expression plasmids (lanes 3 and 4). Lanes 1 and 3, without butyrate induction; lanes 2 and 4, after 24 h of induction with 10 mM butyrate. The positions of human (Hs) and mouse (Mo) PS1 mRNA are marked by arrowheads. B and C, detergent lysates, containing 10 μg of total protein, from multiple independent cell lines expressing wild-type (WT) PS1 (cell lines 7, 9, 15, and 18) and A246E huPS1 (cell lines 25 and 26) were prepared after 0, 24, and 48 h of induction with butyrate. Lysates were electrophoresed on 14% polyacrylamide gels and immunoblotted with pS1Loop antiserum. The prominent 43-kDa protein is full-length huPS1, whereas the 17-kDa polypeptide is huPS1 CTF. The endogenous 16-kDa mouse CTF can be seen just below the hCTF. The relative positions of mouse and huPS1 CTF are illustrated by the bars to the left of the panel.
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To examine the kinetics of butyrate-induced expression of full-length huPS1 and proteolytic processing, cells were induced for varied intervals followed by detergent lysis, SDS-PAGE, and immunoblotting (Fig. 2). huCTFs migrate slightly slower than murine CTFs, whereas huNTFs migrate slightly faster than the murine equivalent (9). In cells expressing either wild-type or A246E huPS1, full-length huPS1 polypeptides as well as NTFs and CTFs were first detectable at 6–10 h post-induction (Figs. 2, A and B, lanes 3, 4, 10, and 11). By 24 h post-induction, the levels of both wild-type and mutant full-length huPS1, and proteolytic derivatives, began to plateau, with no further increase observed after 48–80 h of induction (Fig. 2, A and B, lanes 5, 6, 12, and 13). Immunoblots with Ab14 (Fig. 2B), which is specific for a shared mouse/human epitope between amino acids 3 and 15 of PS1 (9), revealed that the maximal levels of huNTFs were ~2–3-fold higher than mouse NTFs in uninduced cells. Although the observed increase in huCTFs after induction appears much more robust (Fig. 2A), the PS1Loop antiserum binds huCTFs 2.3 times more avidly than the murine CTF (9). Thus, relative to uninduced huPS1-N2a cells, the total steady-state levels of proteolytically processed fragments increased by only 2–3-fold after butyrate induction, whereas the levels of full-length protein increased dramatically. These data are entirely consistent with our earlier analysis of huPS1 expression in the brains of transgenic mice, where we demonstrated a highly regulated, saturable, accumulation of huNTF and huCTF (9, 15).

The NTFs and CTFs of PS1 Exhibit Extended Half-lives Relative to Full-length Polypeptide—To examine the relative stabilities of the full-length polypeptide versus proteolytic derivatives, we employed two complementary strategies. First, we examined the decay of full-length PS1, over a 6-h interval, following 24 h of butyrate induction and blockade of protein synthesis with cycloheximide (Fig. 3, A and B). Immunoblots of lysates from cells expressing both wild-type and mutant PS1 revealed that the levels of full-length polypeptide decline relatively rapidly (within 2 h) after cycloheximide addition (Fig. 3, A and B, lane 4). From multiple immunoblots developed with [125I]-labeled protein A and quantified by PhosphorImager analysis, we estimate that the half-life of both wild-type and A246E mutant, full-length, polypeptides in these cells is ~1.5 h (data not shown). Similar data for full-length PS1 have been obtained by metabolic radiolabeling studies of transfected cells and immunoprecipitation of PS1 (12). Likewise, using strategies very similar to those used here, Kim et al. (23) demonstrated that the endoproteolytic fragments of PS2 are much more long-lived than full-length precursor protein. Notably, a short-lived pool of CTFs was not observed as the levels of CTF were unchanged over the 6-h incubation period in cycloheximide.

To examine huPS1 metabolism over much longer intervals, we adopted a second strategy, which was to transiently induce huPS1 expression with butyrate then chase in standard medium. Ten-hour butyrate inductions markedly increased the levels of full-length huPS1 and CTFs (Fig. 4, lanes 7 and 8). Upon withdrawal of butyrate and subsequent incubation for 15 h, the levels of full-length huPS1 declined dramatically, whereas the levels of both wild-type and mutant 17-kDa huPS1 CTF were unchanged (Fig. 4, lanes 4 and 9). These data suggested that the endoproteolytically processed CTFs are much longer lived than full-length polypeptide.

To further examine the half-life of wild-type CTFs, cells were incubated in medium containing butyrate for 48 h, then “chased” in standard culture medium for 20-, 32-, 44-, 56-, and 68-h intervals. Initially, we examined the steady-state levels of huPS1 mRNA by RT-PCR analysis to determine when tran-
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BUTYRATE, THEN CHASED IN MEDIUM LACKING BUTYRATE FOR THE INDICATED INTERVALS. DETERGENT LYSATES (10 \( \mu \)G OF TOTAL PROTEIN) WERE ANALYZED BY IMMUNOBLOT WITH A PS1 loop antisera and protein A-HRP, FOLLOWED BY CHEMILUMINESCENCE. THE RELATIVE POSITIONS OF MOUSE AND HU PS1 CTF ARE ILLUSTRATED BY THE BARS TO THE LEFT PANEL.

Fig. 4. The C-terminal derivative of huPS1 persists after removal of butyrate. N2a cells expressing wild-type (cell line 9, lanes 1–5) and A246E (cell line 25, lanes 6–10) huPS1 were induced with butyrate, then chased in medium lacking butyrate for the indicated intervals. Detergent lysates (10 \( \mu \)G OF TOTAL PROTEIN) WERE ANALYZED BY IMMUNOBLOT WITH A PS1loop antis serum and protein A-HRP, FOLLOWED BY CHEMILUMINESCENCE. THE RELATIVE POSITIONS OF MOUSE AND HU PS1 CTF ARE ILLUSTRATED BY THE BARS TO THE LEFT PANEL.

Fig. 5. The C-terminal derivatives of huPS1 have half-lives of \( \approx 24 \) h. A, N2a cells expressing wild-type (line 18) huPS1 were induced with butyric acid (10mM) for 48h, followed by incubation in standard medium (chased) for the indicated intervals. Detergent lysates (10 \( \mu \)G OF TOTAL PROTEIN) WERE ANALYZED BY IMMUNOBLOT WITH A PS1-loop antisera and protein A-HRP, FOLLOWED BY CHEMILUMINESCENCE. THE RELATIVE POSITIONS OF MOUSE AND HU PS1 CTF ARE ILLUSTRATED BY THE BARS TO THE LEFT OF THE PANEL. B AND C, LEVELS OF PS1 mRNA IN N2A CELLS EXPRESSING WILD-TYPE HU PS1 WERE ANALYZED AT VARIED INTERVALS OF INDUCTION AND CHASE AS INDICATED. TOTAL RNA WAS ISOLATED AND PS1 mRNA SEQUENCES WERE AMPLIFIED BY RT-PCR AS DESCRIBED UNDER “MATERIALS AND METHODS.” B, ETHIDIUM BROMIDE-STAINED AGAROSE GEL OF AOD1-DIGESTED RT-PCR PRODUCTS AMPLIFIED WITH DEGENERATE PRIMERS FOR HUMAN AND MOUSE PS1 mRNA. THE RELATIVE POSITION OF THE MOUSE AND HU PS1 AMPLIFIED FRAGMENTS ARE ILLUSTRATED BY THE BARS TO THE RIGHT OF THE PANEL. C, ETHIDIUM BROMIDE-STAINED AGAROSE GEL OF RT-PCR PRODUCTS AMPLIFIED WITH MOUSE PS1 SPECIFIC PRIMERS AND MOUSE APP SPECIFIC PRIMERS.

Fig. 6. \( \Delta E9 \) PS1 Polypeptides Lacking Exon 9 and the Endoproteolytic Cleavage Site Are Stable—Previous studies have demonstrated that the FAD variant that lacks exon 9 (\( \Delta E9 \) PS1) possesses normal function (5, 16) but does not undergo endoproteolytic processing (9, 18). In previous studies of stably transfected cell lines (18) and transgenic mice (15), we observed that the expression of \( \Delta E9 \) polypeptides led to diminished accumulation of endogenous PS1 NTFs and CTFs, indicating that the \( \Delta E9 \) PS1 protein competes with authentic full-length PS1 for entry into the stabilization/cleavage pathway. To determine whether \( \Delta E9 \) PS1 is actually stabilized, the half-life of \( \Delta E9 \) huPS1 was examined in stably transfected N2a cell lines. Forty-eight-hour inductions with butyrate resulted in the appearance of \( \Delta E9 \) huPS1 polypeptides (Fig. 6, compare lanes 1 and 2). In parallel to the increase in \( \Delta E9 \) huPS1, the levels of endogenous mouse CTFs were diminished (Fig. 6, lane 2). Removal of butyrate and further incubation in standard medium demonstrated that a substantial portion of the \( \Delta E9 \) PS1 polypeptide persisted for more than 40h (Fig. 6, lanes 3 and 4) (RNA expression returns to basal levels in less than 20h of chase; see Fig. 5). Thus, we conclude that the \( \Delta E9 \) PS1 polypeptide is capable of being stabilized despite its inability to be endoproteolytically processed.

Recombinant PS1 NTFs Are Short-lived—The high stability of \( \Delta E9 \) huPS1 indicates that full-length PS1 possesses the signals for entry into the stabilization/cleavage pathway. To test whether individual NTFs can be stabilized, we inserted recombinant PS1 cDNAs that terminate at amino acid 306
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Several lines of evidence support the view that endoproteolytic cleavage is a late event in the maturation of PS1, occurring after full-length polypeptides are stabilized. First, we failed to demonstrate the presence of short-lived pools of PS1 CTFs, as would be expected had cleavage preceded stabilization. Second, ΔE9 huPS1 was capable of being stabilized. Finally, recombinant N-terminal PS1 polypeptides terminating at residue 306 showed short half-lives. Collectively, these findings suggest that the signals for stabilization reside in the C-terminal domain of full-length polypeptide. Whether an individual CTF is capable of being stabilized is difficult to assess because the membrane topology of the C-terminal domain is dictated by the adjoining N-terminal domain (6), making it difficult to express CTFs alone. However, we believe it likely that only full-length PS1 polypeptides are capable of entering the stabilization/cleavage pathway because such a mechanism is the best way to explain the observed 1:1 stoichiometry of huPS1 NTFs and CTFs in the brains of transgenic mice (9, 15).

To explain our observations in the present and previous studies, we propose a model (Diagram 1) in which newly synthesized full-length PS1 polypeptides first become associated with cellular proteins, which are responsible for its initial stabilization, and only later are endoproteolytically cleaved. Several observations suggest that the factors responsible for targeting and delivery of PS1 into the “stabilization/cleavage” pathway are limiting. First, despite tremendous increases in steady-state levels of PS1 mRNA and full-length protein, following butyrate induction, parallel increases in the total levels of NTF and CTF are not observed (see Figs. 1 and 2). Second, previous analyses of several lines of transgenic mice expressing different levels of huPS1 demonstrated that the levels of full-length PS1 polypeptide increase proportionately with mRNA levels, but the levels of both NTFs and CTFs are maximal at different levels of huPS1 demonstrated that the levels of full-length PS1 polypeptide increase proportionately with mRNA levels but the levels of both NTFs and CTFs are maximal at varying butyrate induction, parallel increases in the total levels of NTF and CTF are not observed (see Figs. 1 and 2). Finally, in transgenic mice (9, 15) and cultured cells (see Figs. 5A and 6), the high level expression of huPS1 leads to decreased accumulation of mouse NTFs and CTFs. Collectively, these data suggest that, under conditions of hyperexpression, excess huPS1 polypeptides compete with mouse PS1 polypeptide for a limited supply of factors that specify targeting to the “stabilization/cleavage pathway.”

Whether endoproteolytic cleavage modifies the function of PS1 is unclear at present. Previous studies have demonstrated that the egg-laying defects in C. elegans, lacking the PS1 homologue sel-12, can be rescued by ΔE9 huPS1 (5). Moreover, we demonstrate here that ΔE9 huPS1 can be stabilized without cleavage. Thus, whether endoproteolytic cleavage modifies function is uncertain. Despite this uncertainty, the high stability of the NTFs and CTFs suggests that these processed derivatives represent the functional entities of PS1. The factors necessary for the targeting of PS1 to the “stabilizing/cleavage” pathway appear to be present in subsaturating levels and thus provide a mechanism to regulate the cellular levels of PS1 post-translationally. Whether these targeting factors are permanently associated with PS1 in some type of stable, functional complex is presently unclear (24). It is anticipated that the molecular characterization of proteins that assist in the stabil-
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