Assembly of the TOB Complex of Mitochondria*

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All mitochondrial precursor proteins studied so far are recognized initially at the surface of the organelle by the translocase of the outer membrane (TOM complex). Precursors of β-barrel proteins are transferred further to another complex in the outer membrane that mediates their topogenesis (TOB complex). Tob55 is an essential component of the TOB complex in that it constitutes the core element of the protein-conducting pore. The other two components of the TOB complex are Tob38, which builds a functional TOB core complex with Tob55, and Mas37, a peripheral member of the complex. We have investigated the biogenesis of the TOB complex. Reduced insertion of the Tob55 precursor in the absence of Tom20 and Tom70 argues for initial recognition of the precursor of Tob55 by the import receptors. Next, it is transferred through the import channel formed by Tom40. Variants of the latter protein influenced the insertion of Tob55. Assembly of newly synthesized Tob55 into preexisting TOB complexes, as analyzed by blue native gel electrophoresis, depended on Tob38 but did not require Mas37. Surprisingly, both the association of Mas37 precursor with mitochondria and its assembly into the TOM complex were not affected by mutation in the TOB complex. Mas37 assembled directly with the TOB core complex. Hence, the biogenesis of Mas37 represents a novel import pathway of mitochondrial proteins.

A number of membrane-embedded β-barrel proteins made up from antiparallel β-sheets constitute a distinct group of mitochondrial outer membrane proteins (1, 2). Members of this group include Tom40, porin, Mdm10, and Tob55/Sam50. Like all outer membrane precursors, precursors of β-barrel proteins are synthesized in the cytosol and have to be imported into mitochondria. At the mitochondrial surface, they interact initially with the translocase of the outer membrane (TOM complex) and then are transferred to the TOB complex at the outer membrane (3–7). This latter complex is specifically involved in the insertion of β-barrel precursors into the outer membrane (8–10).

The major component of the TOB complex is Tob55 (also named Sam50). Tob55 is essential for viability of yeast cells and promotes the insertion of β-barrel proteins into the mitochondrial outer membrane (8, 9, 11). The other known components of the TOB complex are the outer membrane proteins Mas37 and Tob38/Sam35. Mas37 interacts with Tob55, and its role in the biogenesis of β-barrel proteins is yet undefined (8–10). Tob38 is essential for viability in yeast and crucial for the biogenesis of mitochondrial β-barrel proteins (12–14). Together with Tob55, Tob38 forms a functional TOB core complex and is essential for the integrity and function of the TOB complex (12). Despite its central role in the biogenesis of mitochondria, the biogenesis of the TOB complex itself has not been resolved so far.

In the present study, we investigated the mechanisms by which Tob55 and Mas37 are targeted to the mitochondria and assembled into the TOB complex. Our results demonstrate that Tob55 interacts initially with the Tom surface receptors. Its assembly into preexisting TOB complexes is facilitated by the small Tim1 proteins in the intermembrane space (IMS) and depends on Tob38 and Tob55, components of the TOB complex. In sharp contrast, the initial import steps of the precursor of Mas37 are independent of the TOM complex and seem to be mediated directly by the TOB core complex. Thus, our results suggest a unique mechanism of targeting and assembly of Mas37.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Methods—Standard genetic techniques were used for growth and manipulation of yeast strains (15). The wild-type strains BY4743, YPH499, and 273-10B were used. For construction of mas37 mutant strain, the MAS37 gene was deleted by replacement with HIS3 gene cassette. PCR-mediated gene manipulation was used to replace the chromosomal copy of TOB38 by a gene expressing HA tag after the coding sequence of Tob38. The resulting strain, Tob38^HA, grew like the wild-type strain (data not shown). Yeast strains containing temperature-sensitive alleles of tomo40 were described previously (16). The yeast strains containing the mutated forms of Tom22 or Tim10 were kind gifts of Drs. N. Pfanner and C. Koehler, respectively (17, 18). Yeast cells were grown under aerobic conditions on lactate-containing liquid medium.

Biochemical Procedures—Mitochondria were isolated from yeast cells by differential centrifugation as described (19). Blotting to polyvinylidene difluoride or nitrocellulose membranes and immunodecoration was according to standard procedures and visualization was by the ECL method (Amersham Biosciences). Radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [35S]methionine (Amersham Biosciences) after in vitro transcription by SP6 polynucleotide from pGEM4 vectors containing the gene of interest. Import experiments were performed in a buffer containing 250 mM sucrose, 0.25 mg/ml bovine serum albumin, 80 mM KCl, 5 mM MgCl₂, 10 mM MOPS-KOH, 2 mM NADH, and 2 mM ATP, pH 7.2.

Blue Native Gel Electrophoresis (BNGE)—Mitochondria (50–150 μg) were lysed in 40 μl of buffer containing 1% digitonin, 20 mM Tris-HCl, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl

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1 The abbreviations used are: TIM, translocase of the inner mitochondrial membrane; IMS, intermembrane space; HA, hemagglutinin; MOPS, 4-morpholinopropanesulfonic acid; BNGE, blue native gel electrophoresis; bis-Tris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxymethyl)propane-1,3-dio; PK, protease K; DHFR, dihydrofolate reductase.

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embedded in the outer membrane, and they could not be extracted by alkaline solution (Fig. 1B). Thus, this protection assay can be employed to study the mechanism of insertion of Tob55 into the mitochondrial outer membrane. The intensity of the 30-kDa fragment served in further experiments to quantify membrane insertion of Tob55. To study the association of Mas37 with the mitochondrial outer membrane, we used the observation that treatment of mitochondria with PK results in the formation of two proteolytic fragments (14 and 20 kDa) of Mas37 (10).

We first asked whether the TOM complex plays a role in the import of these two precursor proteins. Using the in vitro assays described above, we examined the requirement for the import receptors, Tom20 and Tom70, in the insertion/association pathways of Tob55 and Mas37. Mitochondria isolated from strains lacking either Tom20 or Tom70 were incubated with radiolabeled Tob55, and the amounts of inserted precursor were analyzed. Tob55 was inserted into these mutated mitochondria with a strongly reduced efficiency as compared with wild-type organelles (Fig. 2). In contrast, deletion of the import receptors did not affect the association of Mas37 with mitochondria (Fig. 3). Similar results were obtained when the import experiments were performed at 15°C (not shown). Hence, although the import receptors Tom20 and Tom70 appear to play only a minor role, if any, in the association of Mas37 with the outer membrane, they have a crucial function in the recognition of Tob55 precursor.

Can the import of the two proteins be competed out by a matrix-destined precursor? To investigate this possibility, a large molar excess of pSu9(1–69)-DHFR, a recombinant preprotein that is known to use the import pore, was added to the import reaction containing the radiolabeled precursors. Similar experiments had shown previously that import of radiolabeled matrix-destined precursors as well as precursors of outer membrane β-barrel proteins was competed out by recombinant pSu9(1–69)-DHFR (3, 4, 9). Consistent with these observations, the insertion of Tob55 was strongly reduced upon the addition of excess pre-protein (Fig. 4A). In contrast, excess recombinant pSu9(1–69)-DHFR did not reduce the association of radiolabeled Mas37 with mitochondria (Fig. 4A). To further investigate the involvement of Tom40 in the import of both precursor proteins, we investigated their import into mitochondria isolated from strains harboring temperature-sensitive alleles of tom40. These strains (tom40-3 and tom40-4) were used previously to study the insertion of porin into the outer membrane. The mutation in the tom40-3 strain did not affect the insertion of porin, whereas those in tom40-4 resulted in lower efficiency of porin insertion into the membrane (Fig. 4B) (4). The insertion of Tob55 into the Tom40-4 mitochondria was significantly less efficient in comparison to the wild-type mitochondria, whereas no difference was observed with Tom40-3 mitochondria (Fig. 4B). The association of Mas37 with mitochondria from the mutated strains and from wild-type strain was similar (Fig. 4B). Thus, Tom40 does play a role in the insertion of Tob55, whereas the same mutational alterations of Tom40 do not affect the association of Mas37 precursor with mitochondria.

**The Small Tim Components in the IMS Are Involved in the Assembly of Tob55**—The precursor of Tom40 was transferred from the TOM to the TOB complexes via the IMS (21, 22). Because the C-terminal domain of Tom22 protrudes into the IMS, we asked whether this domain plays any role in the insertion of Tob55 precursor molecules. To investigate that question, we analyzed the insertion into mitochondria isolated from a strain expressing Tom22 molecules without this domain (Fig. 5A) (17). Because such a deletion had no effect on the insertion efficiency, we conclude that this domain does not play an important role in the insertion process.

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**Fig. 1. An assay to study the in vitro insertion of Tob55.** A, two specific proteolytic fragments are formed upon the correct insertion of Tob55 into the outer membrane. Radiolabeled Tob55 was incubated for 20 min at 25°C in import buffer in the absence or presence of isolated mitochondria. After the incubation, the samples were divided into aliquots. Although two aliquots were left intact (lanes 1 and 3), the other aliquots were treated with 50 µg/ml PK (lanes 2, 4, and 5) in the absence or presence of Triton X-100 (+TX). Proteins in the sample that contained Triton X-100 were precipitated with trichloroacetic acid. Mitochondria in the other samples were pelleted. Samples were subjected to SDS-PAGE and autoradiography. The membrane containing lanes 3–5 was immunodecorated with antibodies against the N-terminal peptide of Tob55 (lanes 6–8). F' and F'', specific proteolytic fragments of Tob55, *, a nonprecursor protein expressed from mRNA in the reticulocyte lysate. B, import reaction was performed as above. Mitochondria were suspended in 0.1M Na2CO3 for alkaline extraction (Alk. Ex.). After 30 min on ice, the sample was centrifuged, and the pellet (P) and supernatant (S) were analyzed. As a control, 100% of the input lysate for the import reactions was treated with PK.

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**RESULTS**

The TOM Machinery Is Dispensable for the Import of Mas37—To analyze the import and assembly pathways of the precursor proteins Tob55 and Mas37, radiolabeled precursors were synthesized in vitro and incubated with mitochondria isolated from yeast cells. As a criterion for correct insertion into the outer membrane, we used a protease accessibility assay. In the case of Tob55, treatment of mitochondria with proteinase K (PK) resulted in the formation of a specific proteolytic fragment of 30 kDa (9). This fragment was also observed upon insertion of radiolabeled Tob55 precursor into the outer membrane of isolated mitochondria (Fig. 1A). We observed a second fragment with an apparent molecular mass of ~20 kDa. These fragments were completely degraded upon solubilization of the mitochondria with the detergent Triton X-100 and were not observed when reticulocyte lysate containing the Tob55 precursor was treated with PK (Fig. 1A). The two fragments are
We checked whether the small Tim proteins in the IMS (Tim9/10 and Tim8/13 complexes) are involved in the insertion pathway of a precursor of Tob55. We tested the insertion of Tob55 precursor into mitochondria isolated from a strain lacking Tom8/13 (A) or Tom70 (B). After import, the samples were treated with proteinase K, and mitochondria were re-isolated. Inserted proteins were analyzed by SDS-PAGE and autoradiography. The proteolytic fragments of Tob55 (F and F') are indicated. The insertion of Tob55 was quantified by analyzing the formation of the 30-kDa fragment (F'). The amount of precursor protein imported into wild-type mitochondria for the longest time period was set to 100%.

To further study the biogenesis of Tob55, its assembly into the TOB complex was analyzed by blue native gel electrophoresis (BNGE). Radiolabeled Tob55 was incubated with mitochondria isolated from either wild-type strain or a strain containing a HA-tagged version of Tob38 (Tob38HA). At the end of the import reactions, the mitochondria were re-isolated, solubilized in buffer containing 1% digitonin, and subjected to BNGE. At short incubation periods, we observed a high molecular mass species of 350 kDa (Fig. 6A, I). At longer incubation periods, we observed additional species with lower molecular mass (Fig. 6A, II–IV). The two upper species (Fig. 6A, I and II) contain the Tob55-interacting partner, Tob38, as they migrated higher in Tob38HA mitochondria than in wild-type organelles (Fig. 6A). Further support for the presence of Tob38 in the upper species (Fig. 6A, I) was obtained by antibody shift experiment. Radiolabeled Tob55 was imported into Tob38HA mitochondria. Antibodies against the HA tag were added after solubilization of the mitochondria. This addition resulted in a shift of the upper band to higher molecular masses (Fig. 6B). To obtain further information about the various species observed upon import of radiolabeled Tob55, we decorated the membrane with antibodies against Tob55. Because the radiolabeled Tob55 are in amounts that are too low to be detected by the antibodies, this immunodecoration can be used to analyze the migration of the preexisting Tob55 protein. The endogenously assembled Tob55 migrates in BNGE in 3–4 distinct bands that represent various oligomeric conformations (10, 14, 23). The exact composition and stoichiometry of these oligomeric states are not resolved yet. Nevertheless, the upper and lower bands of the radiolabeled Tob55 (Fig. 6C, I and IV) co-migrate with the upper and lower species of the assembled Tob55 (Fig. 6C). Hence, at least a fraction of the newly imported radiolabeled Tob55 molecules behave like the endogenously assembled Tob55.
To obtain further support for the notion that components in the IMS are involved in the assembly of Tob55, we imported Tob55 into swollen mitochondria in which the IMS was open. The formation of the assembly intermediate and the TOB complex was strongly reduced when the outer membrane was ruptured (Fig. 6D). As a control, the assembly of Tom20 into the TOM complex was not affected by rupturing the outer membrane. Hence, opening of the IMS interferes with the assembly of Tob55.

Assembly of Tob55 and Mas37 Precursors into Preexisting TOB Complexes—The final stage in the biogenesis of both Mas37 and Tob55 is the assembly of the newly synthesized precursors into TOB complexes. To verify that radiolabeled precursors of Tob55 and Mas37 can indeed assemble into the TOB complex, we imported them into mitochondria containing a His-tagged version of Tob38 (12). Upon lysis of the mitochondria and incubation with nickel-nitrilotriacetic acid beads, both precursors were co-isolated with Tob38His, whereas no such interaction was observed with the control precursor of Tom20 (Fig. 7).

Tob38 and Mas37 are two components of the TOB complex. In the absence of Mas37, Tob55 together with Tob38 forms the TOB core complex (12). To investigate whether these latter two proteins and Mas37 play a role in the insertion into the outer membrane of Tob55 precursor, we performed import experiments with mitochondria isolated from either wild-type strain or from the indicated strains. A, a strain containing a Tom22 variant where the C-terminal domain is deleted (Tom22ΔC). B, a strain lacking tim8 and tim13 (ΔTim8/13). C, a strain containing a mutated allele of tim10 (Tim10–1). The radiolabeled precursor of ADP-ATP carrier protein (35S-AAC) was also imported into these latter mitochondria for comparison. After import, the samples were treated with proteinase K, and mitochondria were re-isolated. The proteolytic fragments of Tob55 and of Mas37 are indicated. The bands corresponding to F' in panels B and C and the protected ADP-ATP carrier protein precursor were quantified, and the amount of precursor protein imported into wild-type mitochondria for 20 min was set to 100%.

In the absence of Mas37, Tob55 together with Tob38 forms the TOB core complex (12). To investigate whether these latter two proteins and Mas37 play a role in the insertion into the outer membrane of Tob55 precursor, we performed import experiments with mitochondria lacking Mas37 or depleted of Tob55 or Tob38 (9, 12). In the absence of its partner protein, Tob38, or upon depletion of preexisting Tob55, the insertion of Tob55 was strongly impaired (Fig. 7, B and C). This reduction could be the outcome of the essential role of Tob55 in the assembly of all β-barrel proteins in the outer membrane and/or the result of the requirement of newly synthesized Tob55 molecules to assemble with preexisting Tob55 molecules. Only moderate re-
Assembly of the TOB Complex

The TOB complex is essential for the biogenesis of mitochondrial \(\beta\)-barrel proteins. It is composed of three known proteins: Tob55, a putative \(\beta\)-barrel protein that is embedded in the outer membrane, and Tob38 and Mas37, two proteins that are peripheral ly associated with the membrane. In this way, the TOB complex belongs to those types of protein machineries that are composed of two different types of proteins. We investigated how components of this complex are recognized at the surface of mitochondria and assembled into functional TOB complex.

Similar to other \(\beta\)-barrel proteins, the Tob55 precursor is recognized initially by the surface receptors Tom20 and Tom70. It is translocated across the outer membrane via the general import pore of the TOM complex, and then it is transferred to the TOB complex. The small Tim proteins appear to be involved in the assembly pathway of Tob55. These recognition and insertion steps of Tob55 are shared with other \(\beta\)-barrel precursors in two major aspects. First, all \(\beta\)-barrel precursors are substrates of Tob38, but only Tob55 is an interacting partner of Tob38 in the functional TOB complex. This difference is reflected by the requirement of Tob38 for the stability of assembled Tob55. Although Tob38 is a peripheral membrane protein, its extraction destabilizes the structure of the membrane-embedded Tob55.

A second difference in the assembly pathway of Tob55 in comparison with other \(\beta\)-barrel precursors is the role played by Mas37. Although Mas37 was reported to have an important role in the assembly pathway of Tob55, we performed experiments. Tob55 is embedded in the outer membrane, whereas its partner protein, Tob38, is only associated with the membrane and can be extracted by alkaline solution (9, 12). After import of radiolabeled Tob55 into mitochondria, alkaline extraction was performed, and the membrane pellets were analyzed by BNGE. In mitochondria subjected to alkaline, no assembly intermediates of Tob55 were observed (Fig. 7D). In addition, the endogenous TOB complex was hardly detected upon immunostaining with antibodies against Tob55, whereas the TOM complex and porin assemblies were not affected (Fig. 7E). Taken together, these results lend further support for the importance of Tob38 in the assembly and stability of the TOB complex. It might be that the extraction of Tob38 results in aggregation of its partner protein Tob55, and therefore Tob55 cannot be detected by BNGE.

Fig. 6. The assembly of Tob55 is inhibited upon opening of the outer membrane. A, the radiolabeled precursor of Tob55 was incubated with wild-type and Tob38\(_{\text{HA}}\) mitochondria at 25 °C for the indicated time periods. Mitochondria were re-isolated and analyzed by BNGE followed by autoradiography. Species containing the radiolabeled Tob55 are indicated (I, II, III, and IV). B, Tob38 interacts with precursor of Tob55. Wild-type and Tob38\(_{\text{HA}}\) mitochondria were incubated with radiolabeled Tob55 precursor for 3 min at 25 °C. Mitochondria were re-isolated and resuspended in a buffer containing 1% digitonin. One-half of the samples were analyzed directly by BNGE, whereas the other half were incubated before analysis with antibodies against the HA tag. The radiolabeled bands are indicated as in A. The unspecific band, which is often observed in BNGE, is indicated with an asterisk (9, 23). C, the radiolabeled precursor of Tob55 was incubated with mitochondria isolated from the wild-type strain 273-10B at 25 °C for 3 or 15 min. Mitochondria were re-isolated and analyzed by BNGE followed by autoradiography and immunodecoration with antibodies against Tob55. Species containing the radiolabeled Tob55 are indicated as in A. D, opening of the outer membrane blocks the assembly of Tob55. Isolated mitochondria were preincubated in isotonic buffer (−SW) or in hypotonic buffer (+SW) for 30 min on ice. The mitochondria and mitochondria were re-isolated and analyzed by BNGE and autoradiography. The TOM complex and the assembly intermediates of Tob55 are indicated.

The role of the TOB components in the association and assembly of Mas37 precursor was investigated. Surprisingly, association of Mas37 precursor molecules with mitochondria and their assembly into the TOB complex were increased by at least 2-fold when mitochondria lacking Mas37 were used (Fig. 8). Depletion of either Tob38 or Tob55 had an opposite effect. Mas37 precursor was not assembled into high molecular mass complexes when either Tob38 or Tob55 were depleted (Fig. 8B). Hence, newly synthesized Mas37 molecules do not need preexisting Mas37 molecules on mitochondria but rather require Tob55 and Tob38 for their proper targeting and assembly. These results are consistent with our previous observation that the endogenous levels of Mas37 in mitochondria depleted for either Tob38 or Tob55 are clearly reduced (12). It appears that precursors of Mas37 assemble directly with the TOB core complex, which is composed of Tob38 and Tob55.

**DISCUSSION**

The assembly of Tob55 is inhibited upon opening of the outer membrane because radiolabeled Tob55 is also a substrate of Tob38 and Mas37.

To demonstrate the importance of Tob38 for the stability of the assembled Tob55, we performed experiments. Tob55 is embedded in the outer membrane, whereas its partner protein, Tob38, is only associated with the membrane and can be extracted by alkaline solution (9, 12). After import of radiolabeled Tob55 into mitochondria, alkaline extraction was performed, and the membrane pellets were analyzed by BNGE. In mitochondria subjected to alkaline, no assembly intermediates of Tob55 were observed (Fig. 7D). In addition, the endogenous TOB complex was hardly detected upon immunostaining with antibodies against Tob55, whereas the TOM complex and porin assemblies were not affected (Fig. 7E). Taken together, these results lend further support for the importance of Tob38 in the assembly and stability of the TOB complex. It might be that the extraction of Tob38 results in aggregation of its partner protein Tob55, and therefore Tob55 cannot be detected by BNGE.
role in the assembly of Tom40 and porin (10), we observed that the deletion of Mas37 had only a moderate effect on the insertion of Tob55 into the outer membrane. These results are consistent with our previous observations that Tob55 and Tob38 form the TOB core complex in the absence of Mas37 and that the levels of expressed Tob55 are not affected by the deletion of Mas37 (12). The different roles of Mas37 in the assembly of the various \( \beta \)-barrel precursors point to a function downstream of the Tob55-Tob38 core complex. All \( \beta \)-barrel precursors may interact first with Tob55-Tob38 before being transferred further to other elements of the outer membrane in a process that requires Mas37. Because the precursor of Tob55 does not have to leave the TOB complex, it may not require Mas37 for its assembly. Recent findings on the role of Mdm10 in the assembly of the Tom40 precursor support our proposal (23). It was suggested that Mdm10 would interact with Mas37, which is a component of the TOB core complex. The reported interaction of Mdm10 with Mas37 is compatible with a role of Mas37 in mediating the release of \( \beta \)-barrel precursors from the TOB complex.

At present, we can only speculate how Tob55 is actually assembled into the TOB complex. Tob55 is predicted to form a \( \beta \)-barrel structure with an aqueous pore in the middle of its oligomeric structure (9, 10). Hence, one possibility could be the insertion of newly synthesized Tob55 into the pore and lateral release into the complex; but such a mechanism would require major structural rearrangements of the \( \beta \)-barrel and disruption of many hydrogen bonds (1). Hence, we favor a mechanism in which the preexisting Tob55 molecules serve as a scaffold for the newly synthesized Tob55 precursor. In such a mechanism, assembly can follow a concerted partitioning of the bulky \( \beta \)-barrel into the membrane.

The TOM complex was found to be involved in the import of all mitochondrial precursor proteins analyzed thus far. On the other hand, the import pathway of a protein with a topology similar to that of Mas37, namely one that is associated on the surface of the organelle, had not been studied. Despite previous reports about interaction of Mas37 with the import receptor Tom70 (24), we could not find any evidence for an involvement of the TOM complex in the biogenesis of Mas37. Rather, our findings suggest that Mas37 follows a unique import pathway where it is recognized directly by and assembled with the TOB core complex. The levels of Mas37 in mitochondria depleted of either Tob38 or Tob55 were indeed clearly reduced as compared with those in mitochondria from wild-type cells (12, 14). The observed higher efficiencies of association of Mas37 precursor...
with mitochondria lacking endogenous Mas37 molecules are consistent with such a pathway. In wild-type mitochondria, the vast majority of the TOB core complexes are in association with Mas37. In this situation, a site that could serve as a docking point for newly synthesized Mas37 molecules is probably occupied by these Mas37 molecules. In the absence of endogenous Mas37 molecules, this site on the TOB core complex is free, and more newly synthesized Mas37 molecules can dock on the surface of mitochondria. Further studies will be required to clarify what is the targeting information within the sequence of Mas37 and how this information is decoded by the TOB core complex.

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