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A Mutant Form of Mitochondrial GrpE Suppresses the Sorting Defect Caused by an Alteration in the Presequence of Cytochrome b_2

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Transport of preproteins across the inner mitochondrial membrane requires the action of the matrix heat shock protein Hsp70. Together with its co-chaperone mitochondrial GrpE (Mge1), mtHsp70 transiently binds to the inner membrane translocase subunit Tim44 in a nucleotide-regulated manner, forming an ATP-dependent import driving machinery. We report that a mutant form of Mge1 (Mge1-100) is completely absent in mtHsp70-Tim44 complexes, although its ability to interact with soluble mtHsp70 is only partially reduced. While this *mge1-100* mutation only partially retards preprotein translocation into the matrix, it exerts a selective effect on sorting of cytochrome b_2 to the intermembrane space. A cytochrome b_2 with an altered sorting signal, which is only processed to the intermediate stage and mistargeted to the matrix of wild-type mitochondria, is processed to the mature form and correctly targeted to the intermembrane space of *mge1-100* mitochondria. These results suggest that (1) Mge1-100 discriminates between soluble and membrane-bound mtHsp70 and (2) the membrane-bound mtHsp70-Mge1 driving system competes with the sorting machinery for translocation of preproteins like cytochrome b_2.

Keywords: cytochrome b_2; Hsp70; Mge1; mitochondria; protein sorting

The translocation of preproteins into mitochondria involves translocases in the outer and inner mitochondrial membranes, termed ''Tom'' and ''Tim'', respectively (Lill & Neupert, 1996; Pfanner *et al.*, 1996; Schatz & Dobberstein, 1996; Pfanner & Meijer, 1997). The Tim machinery forms a transport channel (Tim17-Tim23; Blom *et al.*, 1995; Berthold *et al.*, 1995) and mediates binding of the matrix heat shock protein Hsp70 (mtHsp70) to the inner membrane. While the majority of mtHsp70 is a soluble protein of the matrix, a small fraction (~10%) binds to the inner membrane (Rassow *et al.*, 1994). A complex between the peripheral inner membrane protein of 44 kDa (Tim44) and mtHsp70 has been isolated (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). This complex also contains the co-chaperone mitochondrial GrpE (Mge1; Voos *et al.*, 1994; Schneider *et al.*, 1996; Horst *et al.*, 1997). The Tim44-mtHsp70-Mge1 system seems to represent the driving machinery for translocation of preproteins across the inner mitochondrial membrane. mtHsp70 directly binds the precursor polypeptides and is thought to pull the preproteins in. By functioning as a membrane anchor, Tim44 can ensure the directionality of mtHsp70-generated force (Horst *et al.*, 1996; Voos *et al.*, 1996). Mge1 functions as a nucleotide release factor for mtHsp70 and is thought to promote the ATPase cycle of mtHsp70 (Deloche & Georgopoulos, 1996; Dekker & Pfanner, 1997; Miao *et al.*, 1997), although the exact function of Mge1 in the Tim44-mtHsp70 complex is unknown.

The sorting of preproteins to the mitochondrial intermembrane space, in particular the import pathway of cytochrome b_2, in the yeast *Saccharomyces cerevisiae* has received considerable attention

Abbreviations used: b_2-DHFR, fusion protein between the N-terminal 220 residues of cytochrome b_2 and entire dihydrofolate reductase; $\Delta\Psi$, membrane potential; Mge1, mitochondrial GrpE; mtHsp70, mitochondrial heat shock protein of 70 kDa; Tim44, translocase of inner mitochondrial membrane subunit of 44 kDa; BSA, bovine serum albumin; Prot. K, proteinase K.

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in the past years. Cytochrome b_2 possesses a bipartite presequence of 80 residues (Guiard, 1985; Hurt & van Loon, 1986; Hartl *et al.*, 1987). The N-terminal 31 residues form a typical positively charged matrix targeting signal that is cleaved off by the processing peptidase in the matrix. The remainder of the presequence contains an intramitochondrial sorting signal that includes a hydrophobic segment and a preceding cluster of positively charged residues. This second part of the presequence directs the sorting of the protein to the intermembrane space and is cleaved off by an inner membrane-bound peptidase from the intermembrane space side (Pratje & Guiard, 1986; Schneider *et al.*, 1991). Inactivation of the sorting signal leads to a mistargeting of the preprotein into the matrix (Beasley *et al.*, 1993; Schwarz *et al.*, 1993). The mechanism of sorting of cytochrome b_2 is still unclear. The currently discussed models range from a complete import into the matrix and export across the inner membrane (Hartl *et al.*, 1987; Koll *et al.*, 1992) to a translocation arrest of the hydrophobic sorting signal in the inner membrane, which prevents further translocation of the preprotein into the inner membrane and promotes a lateral movement out of the import channel (Hurt & van Loon, 1986; Glick *et al.*, 1992). Also, several variations of these models have been proposed (Gruhler *et al.*, 1995; Gärtner *et al.*, 1995). mtHsp70 interacts with the N-terminal presequence of cytochrome b_2 and promotes unfolding of domains located close to the presequence (Voos *et al.*, 1993; Glick *et al.*, 1993). In contrast to matrix-targeted preproteins, mtHsp70 does not exert an unfolding effect on domains of cytochrome b_2 that are located at the C terminus of the preprotein (Gärtner *et al.*, 1995). These results suggest an early divergence of the precursor of cytochrome b_2 from the general matrix import pathway (Gärtner *et al.*, 1995).

Here, we asked if the co-chaperone Mge1 is involved in the sorting of cytochrome b_2. We analyzed a temperature-sensitive mutant *mge1-100* that was found to retard preprotein translocation into the mitochondrial matrix (Laloraya *et al.*, 1995). After a pretreatment of mitochondria at 37°C, the mutant protein Mge1-100 is partially impaired in interaction with mtHsp70 (Laloraya *et al.*, 1995). This result is shown in Figure 1 for a co-precipitation of Mge1 with antibodies directed against mtHsp70 after lysis of mitochondria with non-ionic detergent. The amount of Mge1-100 co-precipitated with mtHsp70 is reduced by 60 to 70% (Figure 1, lane 4) compared to wild-type Mge1 (Figure 1, lane 3), although the total amount of Mge1 is identical in wild-type and mutant mitochondria (Figure 1, lanes 1 and 2). We asked if the *mge1-100* mutation affected the interaction of Mge1 with the membrane bound Tim44-mtHsp70 complex. We performed a co-precipitation with antibodies directed against Tim44. In wild-type a portion of mtHsp70 and Mge1 was co-precipitated with anti-Tim44 (Figure 1, lanes 5 and 7). With the *mge1-100* mutant mitochondria, however, Mge1-100 was completely absent from the Tim44-mtHsp70 complex (Figure 1, lanes 6 and 8; the lanes 7 and 8 were exposed longer than lanes 1 to 6 to confirm the complete lack of co-precipitated Mge1-100). While the *mge1-100* mutation shows only partial inhibitory effects on protein import into the matrix and the binding of Mge1 to mtHsp70 in total (which is mainly soluble in the matrix; Laloraya *et al.*, 1995), it completely prevents a stable association of Mge1 with the fraction of mtHsp70 that is bound to Tim44. This result suggested that the *mge1-100* mutation should preferentially influence mtHsp70-dependent processes that are occurring at the inner membrane.

Since recent models on the sorting of cytochrome b_2 favor a sorting mechanism closely associated with the inner membrane (Glick *et al.*, 1992; Gruhler *et al.*, 1995; Gärtner *et al.*, 1995), we asked

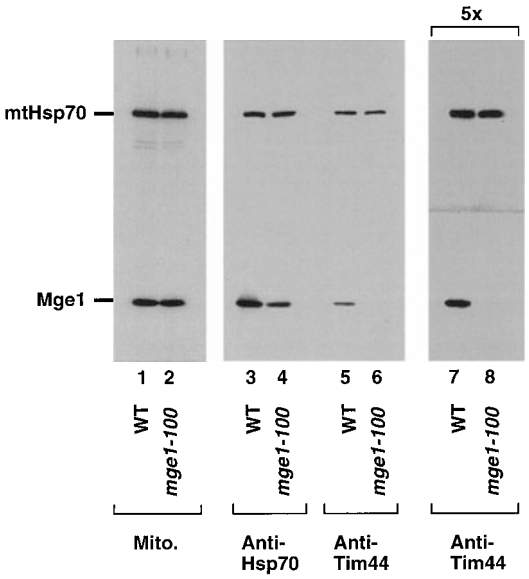

Figure 1. The mutant protein Mge1-100 is absent from isolated Tim44-mtHsp70 complexes. The *S. cerevisiae* strains SL22 (WT, wild-type; *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2*$^+$ *met2-Δ1 lys2-Δ2 mge1-Δ2* pSL20; expressing wild-type *MGE1* from plasmid pSL20) and SL20 (*mge1-100; trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2*$^+$ *met2-Δ1 lys2-Δ2 mge1-Δ2* pSL40; expressing the mutant allele *mge1-100* from plasmid pSL40) were used (Laloraya *et al.*, 1995). Mitochondria were isolated and incubated for 15 minutes at 37°C. The mitochondria were lysed in 250 mM sucrose, 80 mM KCl, 20 mM Mops-KOH (pH 7.2), 0.1% (v/v) Triton-X-100 and 5 mM EDTA and subjected to co-immunoprecipitation with antibodies directed against mtHsp70 or Tim44 (Rassow *et al.*, 1994; Alconada *et al.*, 1995; von Ahsen *et al.*, 1995; Voos *et al.*, 1996). After three washing steps, the precipitated material was eluted at pH 2.5 and analyzed by SDS-PAGE and immunodecoration with antisera directed against mtHsp70 and Mge1 (samples 3 to 8). Of the material added to the co-precipitation, 20% was directly analyzed by SDS-PAGE and immunodecoration (samples 1 and 2). Lanes 7 and 8 were exposed five-times longer than lanes 1 to 6.

if the *mge1-100* mutation exerted a particular influence on the sorting of cytochrome b_2.

We used a fusion protein containing the complete sorting information of cytochrome b_2 and entire dihydrofolate reductase (pb_2-DHFR) (Figure 2A; Koll *et al.*, 1992; Voos *et al.*, 1993). The fusion protein was processed to the intermediate-sized and mature-sized forms by both wild-type and *mge1-100* mitochondria (Figure 2B, upper panel). The import strictly depended on a membrane potential ($\Delta\Psi$) across the inner membrane; dissipation of $\Delta\Psi$ blocked import into wild-type and mutant mitochondria (Figure 2B, upper panel, lanes 3, 6, 9 and 12). The processing and transport to a protease-protected location in *mge1-100* mitochondria (Figure 2B, upper panel, lanes 7, 8, 10 and 11) were slower than in wild-type mitochondria (Figure 2B, upper panel, lanes 1, 2, 4 and 5), as was observed for matrix-targeted preproteins (Laloraya *et al.*, 1995; shown here for F_1-ATPase subunit β ($F_1\beta$); Figure 2B, lower panel). Authentic cytochrome b_2 behaved in a similar manner as b_2-DHFR (not shown). We then used a fusion protein where 19 residues of the intermembrane space sorting signal of cytochrome b_2 were deleted. Thereby the sorting signal is completely inactivated and the

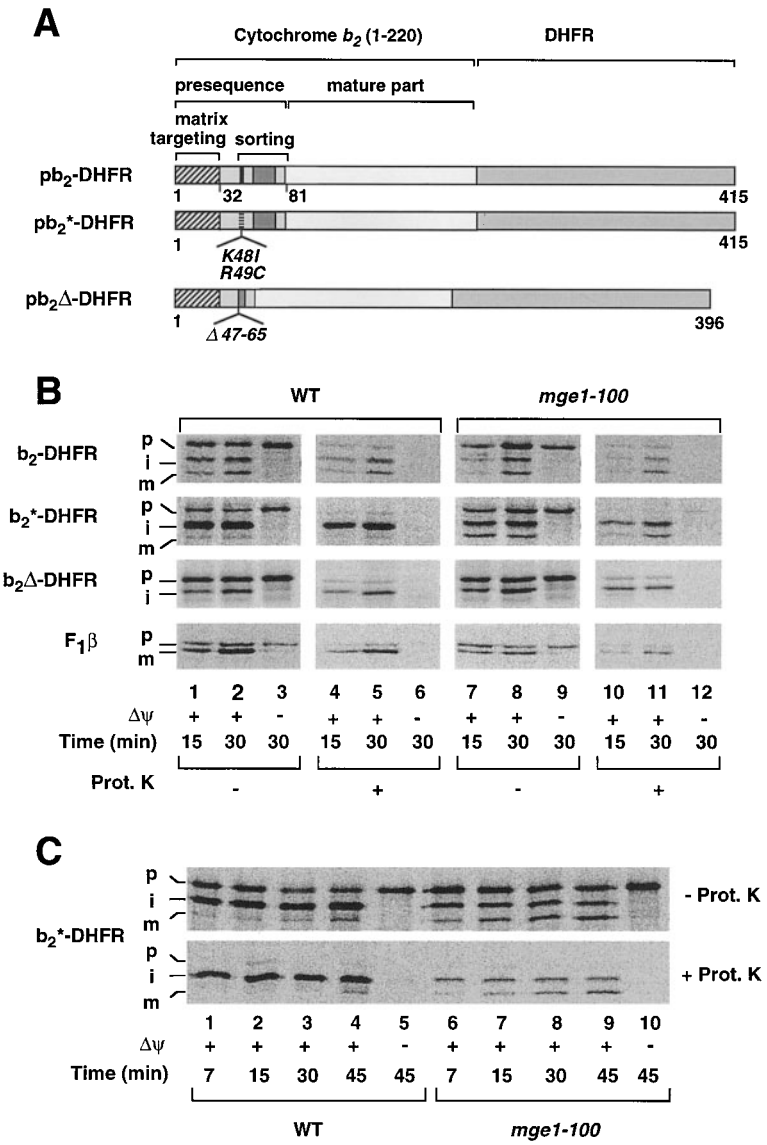

Figure 2. *mge1-100* mitochondria suppress the maturation defect of a cytochrome b_2-fusion protein with altered sorting signal. A, Fusion proteins employed. pb_2-DHFR contains the N-terminal 220 amino acid residues of the cytochrome b_2 precursor, an eight-residue linker and entire DHFR (Koll *et al.*, 1992; Voos *et al.*, 1993). In pb_2^*-DHFR the residues lysine 48 and arginine 49 of pb_2-DHFR were replaced by isoleucine and cysteine, respectively; for this purpose the nucleotides AAACGC for codons 48 and 49 of pb_2-DHFR were changed to ATATGC by PCR mutagenesis (Barik, 1997); the sequences were confirmed by DNA sequencing. $pb_2\Delta$-DHFR lacks the amino acid residues 47 to 65 (Voos *et al.*, 1996). The matrix-targeting sequence (residues 1 to 31) is shown hatched. The sequence region required for intramitochondrial sorting of cytochrome b_2 (Beasley *et al.*, 1993; Schwarz *et al.*, 1993) is indicated including the cluster of positively charged residues (residues 47 to 49; black line) and the hydrophobic segment (~residues 58 to 71; dark box). The mature part of cytochrome b_2 starts with residue number 81. B, Import of pb_2-DHFR, pb_2^*-DHFR and $pb_2\Delta$-DHFR into wild-type (WT) and *mge1-100* mitochondria. Isolated mitochondria were preincubated for 15 minutes at 37°C (Laloraya *et al.*, 1995). Preproteins were expressed from pGEM4Z by *in vitro* transcription and translation in rabbit reticulocyte lysates in the presence of [^{35}S]methionine/[^{35}S]cysteine and incubated with the mitochondria (20 µg protein per sample) in bovine serum albumin (BSA)-containing buffer (3% (w/v) BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 6 mM ATP, 2 mM KP_i, 10 mM Mops-KOH (pH 7.2)) at 25°C for the times indicated (Alconada *et al.*, 1995). For samples 3, 6, 9 and 12 the membrane potential ($\Delta\Psi$) was dissipated by additon of 1 µM valinomycin before the 25°C incubation. After the incubation at 25°C, samples were divided into halves, and one half was treated with 50 µg/ml proteinase K (Prot. K) for 20 minutes at 0°C to remove preproteins which were not imported (Alconada *et al.*, 1995). The samples were analyzed by SDS-PAGE and digital autoradiography. C, Import kinetics of pb_2^*-DHFR. The import reaction was performed as described above. p, i, m, Precursor, intermediate and mature-sized forms of a protein, respectively.

resulting protein $pb_2\Delta$-DHFR is transported into the matrix (Koll *et al.*, 1992; Voos *et al.*, 1996). This is evidenced by a complete lack of processing to the mature-sized form in wild-type and *mge1-100* mitochondria (Figure 2B, second panel from below). With *mge1-100* mitochondria, the transport to a protease-protected location was delayed (Figure 2B, second panel from below) similarly to the effect of the mutation on import of b_2-DHFR and $F_1\beta$. We thus did not observe a specific influence (exceeding that on matrix proteins) of the *mge1-100* mutation on import of b_2-fusion proteins when the intermembrane space sorting signal was either intact or completely inactivated.

We then constructed a b_2-DHFR fusion protein, where only a limited alteration of the sorting signal was introduced. The cluster of three positively charged residues in the presequence of cytochrome b_2 (amino acid residues 47 to 49) is required for proper sorting of cytochrome b_2 (Beasley *et al.*, 1993; Schwarz *et al.*, 1993). Substitution of some of the charged residues by uncharged ones, e.g. lysine 48 and arginine 49 by isoleucine and cysteine (Figure 2A, pb_2^*-DHFR), inactivates the sorting signal (Schwarz *et al.*, 1993). pb_2^*-DHFR was only processed to the intermediate-sized form and mistargeted into the matrix of wild-type mitochondria (Figure 2B, second panel from the top, lanes 1, 2, 4 and 5; see below, Figure 3, lanes 1 to 3). With *mge1-100* mitochondria, however, pb_2^*-DHFR was processed to the mature-sized form (Figure 2B, lanes 7, 8, 10 and 11). To confirm this finding, we performed more detailed time kinetics of import of pb_2^*-DHFR. Figure 2C shows that in wild-type

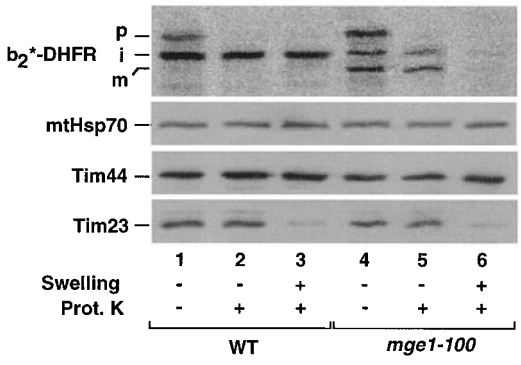

Figure 3. A cytochrome b_2-fusion protein with an altered sorting signal (b_2^*-DHFR) is transported to the intermembrane space in *mge1-100* mitochondria. b_2^*-DHFR was imported into isolated energized mitochondria (10 µg protein) for 30 minutes as described in the legend of Figure 2. Samples 1, 2, 4, and 5 were diluted with nine volumes of SEM-buffer (250 mM sucrose, 10 mM Mops-KOH (pH 7.2), 1 mM EDTA), samples 3 and 6 with nine volumes of hypotonic swelling buffer (10 mM Mops-KOH (pH 7.2), 1 mM EDTA) to generate mitoplasts. Samples 2, 3, 5, and 6 were treated with 50 µg proteinase K (Prot. K) per ml before SDS-PAGE and transfer to nitrocellulose. b_2^*-DHFR was analyzed by digital autoradiography, and marker proteins (mtHsp70, Tim44 and Tim23) were analyzed by immunodecoration.

mitochondria the fusion protein was efficiently processed to the intermediate-sized form (lanes 1 to 4), but only very poorly to the mature-sized form after long import times (lane 4). With *mge1-100* mitochondria the import of pb_2^*-DHFR was slower than in wild-type mitochondria, as observed above for other b_2-fusion proteins and matrix proteins, but the ratio between intermediate and mature forms was strongly shifted such that a considerable amount of mature-sized protein was formed (Figure 2C, lanes 6 to 9). Dissipation of $\Delta\Psi$ blocked the generation of mb_2^*-DHFR (Figure 2B, lanes 9 and 12; Figure 2C, lane 10), demonstrating that the processing depended on the initial insertion of the preprotein into the inner membrane.

To determine if mb_2^*-DHFR formed in *mge1-100* mitochondria was correctly transported to the intermembrane space, mitochondria were subjected to swelling to selectively open the intermembrane space. Thereby the marker protein Tim23 of the inner membrane, that exposes its N terminus to the intermembrane space side, became accessible to added proteinase K (Figure 3, lanes 3 and 6; probed with an antibody directed against the N terminus of Tim23) (Kübrich *et al.*, 1994). The inner membrane remained intact as demonstrated by the protection of mtHsp70 and Tim44. In wild-type mitochondria, the intermediate-sized b_2^*-DHFR was protected against proteinase K in swollen mitochondria (Figure 3, upper panel, lane 3), confirming its transport into the matrix. In *mge1-100* mitochondria, the mature-sized b_2^*-DHFR was accessible to proteinase K, demonstrating a location in the intermembrane space (Figure 3, upper panel, lane 6). The intermediate-sized b_2^*-DHFR generated in *mge1-100* mitochondria was partially accessible to proteinase K in intact mitochondria (Figure 3, lane 5; see also Figure 2C, lanes 6 to 9, compare upper panel to lower panel); the remainder of ib_2^*-DHFR was degraded after opening of the intermembrane space (Figure 3, lane 6). ib_2^*-DHFR formed in *mge1-100* mitochondria thus shows a localization distinct from that of ib_2^*-DHFR formed in wild-type mitochondria. We conclude that the *mge1-100* mutation strongly influences the processing and sorting of b_2^*-DHFR. The mutation in *MGE1* suppresses the sorting defect caused by the alteration of the intermembrane sorting signal and permits correct translocation of b_2^*-DHFR into the intermembrane space.

This study has two major implications. (1) The co-chaperone Mge1 distinguishes between soluble mtHsp70 in the matrix and mtHsp70 bound to Tim44 of the inner membrane. A single amino acid substitution, the conserved arginine 216 by lysine (Laloraya *et al.*, 1995), renders Mge1-100 unable to stably interact with mtHsp70-Tim44. While binding of Mge1-100 to soluble mtHsp70 is reduced only by a factor of 2 to 3 compared to wild-type Mge1, the binding to mtHsp70-Tim44 is reduced by a factor of at least 100 (the level dectable with our co-precipitation assay; Figure 1). This suggests that mtHsp70 in the Tim44-bound form has a

different conformation than soluble mtHsp70, supporting models of an active role of mtHsp70 in protein import that proposed conformational changes of the Tim44-bound form (Pfanner & Meijer, 1995; Glick, 1995; von Ahsen *et al*., 1995; Horst *et al*., 1996; Voos *et al*., 1996). (2) A lack of Mge1 in the Tim44-mtHsp70 complex has a striking effect on sorting of a cytochrome b_2 fusion protein with a partial alteration of the intermembrane sorting signal. It suppresses the mistargeting into the matrix and allows correct processing and sorting to the intermembrane space. In contrast, the translocation of matrix-targeted proteins, $F_1\beta$ or a cytochrome b_2 fusion protein with a complete inactivation of the sorting signal, is only retarded as is the translocation of cytochrome b_2 with a fully intact sorting signal. We propose that the inner membrane sorting machinery competes with the general matrix import machinery (that includes Tim44-mtHsp70) for preproteins in transit. The partial inactivation of the sorting signal in b_2^*-DHFR decreases the affinity for the sorting machinery and therefore causes translocation into the matrix when the Tim44-mtHsp70-Mge1 system is fully functional. The *mge1-100* mutation slows down mtHsp70-driven import and thus allows a prolonged interaction of the altered sorting signal with the sorting machinery. The complete lack of Mge1-100 in the mtHsp70-Tim44 complex suggests a dominant role of the membrane-bound form of mtHsp70 for preprotein sorting at the inner membrane. The results presented here are in agreement with the model that the sorting pathway of cytochrome b_2 diverges from the general matrix import pathway at the level of the inner membrane.

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