Biogenesis of the preprotein translocase of the outer mitochondrial membrane: protein kinase A phosphorylates the precursor of Tom40 and impairs its import

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ABSTRACT **The preprotein translocase of the outer mitochondrial membrane (TOM) functions as the main entry gate for the import of nuclear-encoded proteins into mitochondria. The major subunits of the TOM complex are the three receptors Tom20, Tom22, and Tom70 and the central channel-forming protein Tom40. Cytosolic kinases have been shown to regulate the biogenesis and activity of the Tom receptors. Casein kinase 2 stimulates the biogenesis of Tom22 and Tom20, whereas protein kinase A (PKA) impairs the receptor function of Tom70. Here we report that PKA exerts an inhibitory effect on the biogenesis of the β-barrel protein Tom40. Tom40 is synthesized as precursor on cytosolic ribosomes and subsequently imported into mitochondria. We show that PKA phosphorylates the precursor of Tom40. The phosphorylated Tom40 precursor is impaired in import into mitochondria, whereas the nonphosphorylated precursor is efficiently imported. We conclude that PKA plays a dual role in the regulation of the TOM complex. Phosphorylation by PKA not only impairs the receptor activity of Tom70, but it also inhibits the biogenesis of the channel protein Tom40.**

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INTRODUCTION

Most mitochondrial proteins are imported from the cytosol. The proteins are synthesized as precursors on cytosolic ribosomes. Targeting signals contained in the precursor proteins direct them to receptors on the mitochondrial surface (Hoogenraad *et al.*, 2002; Dolezal *et al.*, 2006; Neupert and Herrmann, 2007; Chacinska *et al.*, 2009; Endo *et al.*, 2011). The translocase of the outer mitochondrial membrane (TOM complex) functions as the main entry gate for mitochondrial precursor proteins. After passing through the TOM channel, the precursor proteins follow different import routes to the four mitochondrial subcompartments—outer membrane, intermembrane space, inner membrane, and matrix.

The TOM complex consists of seven subunits: three receptors, a central channel-forming protein, and three small subunits (Meisinger *et al.*, 2001; Walther and Rapaport, 2009; Endo *et al.*, 2011). The receptors Tom20 and Tom22 preferentially recognize precursor proteins that carry amino-terminal targeting signals (presequences), whereas the third receptor, Tom70, mainly binds hydrophobic precursor proteins with internal targeting signals (metabolite carriers; Brix *et al.*, 1999; Abe *et al.*, 2000; Young *et al.*, 2003; Li *et al.*, 2009). Each of the receptors is anchored in the outer membrane via a single α-helical transmembrane segment and exposes the preprotein-binding domain to the cytosol. The β-barrel protein Tom40 is the essential core of the TOM complex. Tom40 forms a hydrophilic channel, through which the vast majority of mitochondrial proteins are imported (Hill *et al.*, 1998; Ahting *et al.*, 2001; Suzuki *et al.*, 2004; Becker *et al.*, 2005; Harsman *et al.*, 2010). The small subunits Tom5,

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Abbreviations used: CK2, casein kinase 2; Mim1, mitochondrial import protein 1 of the outer membrane; PKA, protein kinase A; TEV, tobacco etch virus; TOM, translocase of the outer mitochondrial membrane.

Tom6, and Tom7 are involved in the assembly and stability of the TOM complex (Schmitt *et al.*, 2005; Sherman *et al.*, 2005; Meisinger *et al.*, 2006b; Kato and Mihara, 2008; Becker *et al.*, 2010, 2011; Yamano *et al.*, 2010). Each subunit of the TOM complex is encoded by a nuclear gene and thus is synthesized as precursor in the cytosol and imported into mitochondria (Model *et al.*, 2001; Walther and Rapaport, 2009).

For a long time, little has been known about the regulation of the mitochondrial preprotein translocases. Recently, however, mass spectrometric analyses revealed that the subunits of the TOM complex are phosphorylated at multiple sites (Chi *et al.*, 2007; Li *et al.*, 2007; Albuquerque *et al.*, 2008; Gnad *et al.*, 2009; Schmidt *et al.*, 2011). On the basis of prediction programs for kinase target sites and in vitro assays with recombinant Tom proteins and purified kinases, a number of candidate kinases for TOM phosphorylation were identified (Rao *et al.*, 2011; Schmidt *et al.*, 2011). So far, a functional role of TOM phosphorylation has been shown for the Tom receptors in vivo and in organello. 1) Cytosolic casein kinase 2 (CK2) phosphorylates the precursor of Tom22 in the cytosol and thereby stimulates its import into the outer membrane. In addition, CK2 also phosphorylates the mitochondrial import protein Mim1, an outer membrane protein that mediates membrane insertion of the precursors of Tom20 and Tom70 (Becker *et al.*, 2008; Hulett *et al.*, 2008; Popov-Celeketic *et al.*, 2008), and thus promotes the import of these two receptors. The assembly of Tom20 with mature Tom22 in the TOM complex is enhanced when Tom22 is in the phosphorylated state. Taken together, CK2 stimulates the biogenesis of the three Tom receptors (Rao *et al.*, 2011; Schmidt *et al.*, 2011). 2) In contrast, cytosolic protein kinase A (PKA) was found to inhibit the receptor activity of Tom70. Phosphorylation by PKA does not affect the biogenesis of the Tom70 precursor but exerts an inhibitory effect on the mature Tom70 receptor and thus impairs the import of metabolite carriers into mitochondria (Schmidt *et al.*, 2011).

We analyzed a possible effect of kinases on the channel protein Tom40. We show that PKA impairs import of the Tom40 precursor into mitochondria. The inhibitory effect is selectively caused by phosphorylation of a specific serine residue of the Tom40 precursor in the cytosol, whereas mature, imported Tom40 is not accessible to phosphorylation by PKA. We conclude that cytosolic kinases not only regulate the biogenesis and activity of Tom receptors, but they also exert an inhibitory effect on the biogenesis of the channel protein Tom40.

RESULTS

Phosphorylation of Tom40 by yeast PKA

The in vitro screen for TOM phosphorylation by Schmidt *et al.* (2011) led to the prediction of Ser-54 of Tom40 as PKA target site and the demonstration that purified mouse PKA phosphorylates recombinant *Saccharomyces cerevisiae* Tom40 at this site. It has not been determined whether the phosphorylation takes place in yeast and whether it is of functional relevance. PKA consists of two catalytic subunits and two regulatory (inhibitory) subunits. In yeast the catalytic subunits are encoded by the genes *TPK1*, *TPK2*, and *TPK3* and the regulatory subunit by *BCY1* (Cannon and Tatchell, 1987; Toda *et al.*, 1987a, 1987b; Thevelein, 1994; Zaman *et al.*, 2008; Smets *et al.*, 2010). PKA is activated on fermentable growth conditions that lead to increased intracellular cAMP levels (Broach, 1991; Thevelein, 1994). cAMP binds to Bcy1, leading to a release of the active catalytic subunits (Taylor *et al.*, 1990; Thevelein, 1994; Tamaki, 2007; Zaman *et al.*, 2008). *BCY1*-deficient yeast cells lack cAMP-dependent regulation of PKA activity and are unable to grow under nonfermentable conditions (Matsumoto *et al.*, 1983; Toda *et al.*, 1987a; Cameron *et al.*, 1988).

To test for a possible effect of PKA on Tom40 in vivo, we grew yeast cells on fermentable medium containing sucrose as carbon source (Lemaire *et al.*, 2004; Van de Velde and Thevelein, 2008). We compared *bcy1*Δ yeast to rho$^+$ wild-type yeast as well as to rho$^-$ wild-type yeast that lack mitochondrial DNA and are thus also unable to grow on nonfermentable medium. The steady-state protein levels of Tom40 were reduced in *bcy1*Δ yeast cells in comparison to rho$^+$ as well as rho$^-$ wild-type yeast (Figure 1A). The levels of several control proteins, including cytosolic kinases, were not affected. Moreover, the levels of Tom70 were not changed, in agreement with the findings of Schmidt *et al.* (2011) that PKA affects neither the biogenesis nor the level of Tom70 but inhibits the receptor activity of the mature, imported Tom70 (in the study by Schmidt *et al.*, 2011, glucose was used as carbon source). These results suggest that a constitutive activation of PKA exerts an inhibitory influence on the level of Tom40.

To analyze whether Tom40 is a substrate of PKA in a homologous system, we generated a yeast strain expressing protein A–tagged Bcy1. Purification of Bcy1 by affinity chromatography led to the efficient copurification of PKA catalytic subunits, shown here with antibodies directed against Tpk1 (Figure 1B; the protein A tag was removed by cleavage with tobacco etch virus [TEV] protease). On stimulation with 8Br-cAMP, the purified yeast PKA was active and phosphorylated the PKA model substrate Kemptide and the receptor domain of Tom70 in the presence of [γ-^{33}P]ATP (Figure 1C, lane 2; Schmidt *et al.*, 2011). Purified recombinant yeast Tom40 was phosphorylated by yeast PKA in the presence of 8Br-cAMP (Figure 1C, lane 4). The phosphorylation was specific for the predicted PKA target residue Ser-54 since replacement of this serine by glutamic acid blocked the phosphorylation of Tom40 (Figure 1C, lane 6). As an independent assay, we used a yeast strain containing tagged Tpk1 and purified the enzyme. Purified wild-type Tom40, but not the mutant form Tom40^{S54E}, was phosphorylated by Tpk1 (Figure 1D, lanes 3 and 4). We conclude that Tom40 is a substrate of yeast PKA.

PKA phosphorylates the precursor of Tom40

Phosphate-affinity (Phos-tag) SDS–PAGE leads to a lower gel mobility of many phosphorylated proteins compared with the nonphosphorylated forms (Kinoshita *et al.*, 2006). We asked whether the phosphorylation of Tom40 at Ser-54 by PKA could be directly monitored by Phos-tag SDS–PAGE. We incubated purified Tom40 with PKA and indeed observed a slower-migrating form that was generated in a PKA- and time-dependent manner (Figure 2A, lanes 2–4). When Ser-54 was replaced by alanine, no phosphorylated form of Tom40 was detected (Figure 2A, lanes 6–8).

When isolated yeast mitochondria were incubated with PKA, however, a phosphorylated form of Tom40 was not detected (Figure 2B; neither mammalian PKA nor yeast PKA in the presence of 8Br-cAMP led to a detectable phosphorylation of Ser-54). Thus mature Tom40 that is stably integrated into the mitochondrial outer membrane was not accessible to PKA, whereas purified Tom40 in detergent was phosphorylated by PKA (Figures 1, C and D, and 2A). We therefore asked whether Tom40 can be phosphorylated in the cytosol. We synthesized the precursor of Tom40 in reticulocyte lysate in the presence of [^{35}S]methionine. When the synthesis was performed in the presence of PKA, a slower-migrating form of Tom40 was observed by Phos-tag SDS–PAGE (Figure 2C, lanes 2 and 5). This form was sensitive to treatment with alkaline phosphatase (Figure 2C, lane 6). When Tom40^{S54A} was synthesized in reticulocyte lysate, it was not affected by PKA (Figure 2C, lane 4). Taken together, these results indicate that the precursor of Tom40 is phosphorylated by

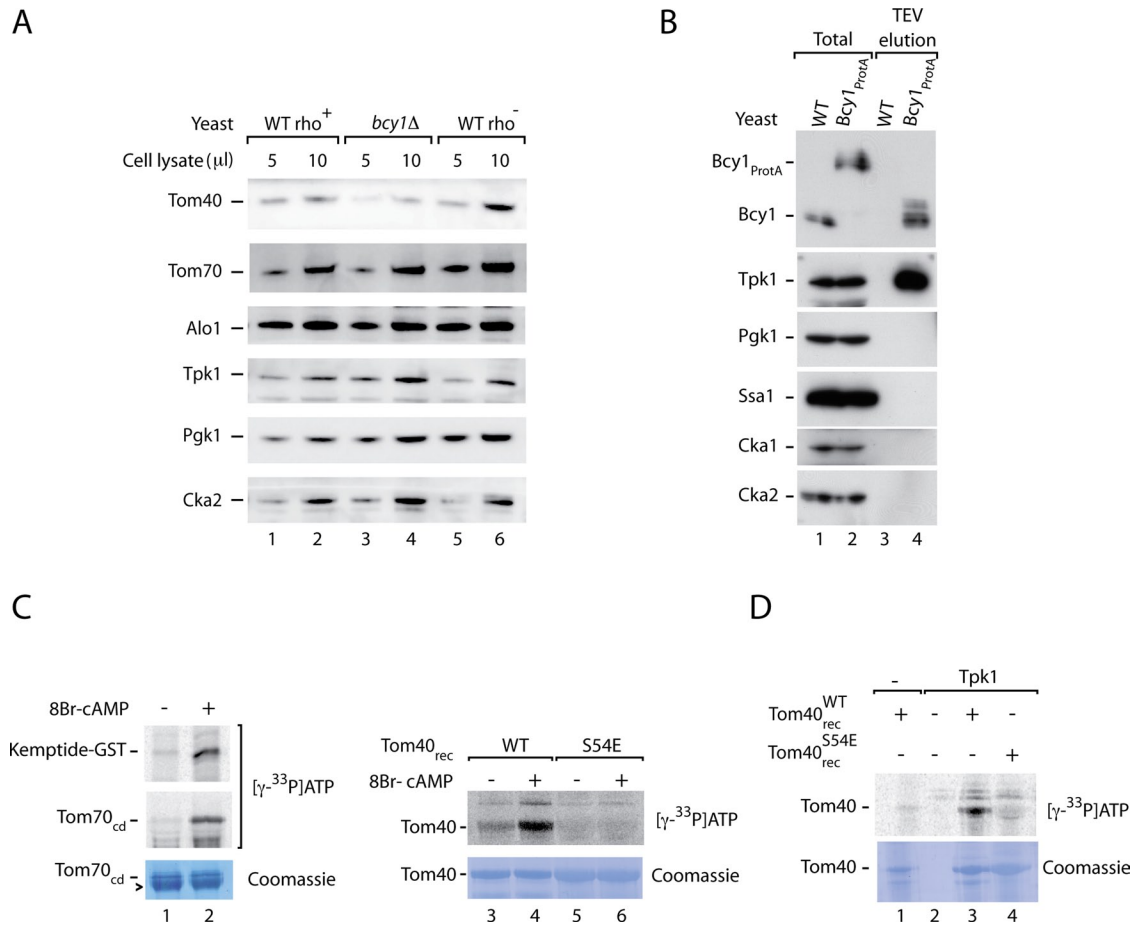

FIGURE 1: Phosphorylation of Tom40 by yeast PKA. (A) Yeast cells from rho$^+$ wild-type (WT), rho$^-$ wild type, and *bcy1*Δ were grown on sucrose-containing medium at 30°C. Protein extracts were prepared by postalkaline lysis and analyzed by SDS–PAGE and Western blotting. Alo1, D-arabinono-1,4-lactone oxidase; Pgk1, 3-phosphoglycerate kinase. (B) Yeast PKA (Bcy1-Tpk) was purified by affinity chromatography using a $Bcy1_{ProtA}$ yeast strain as described in *Materials and Methods*. Total (yeast lysate) and TEV eluate were analyzed by SDS–PAGE and Western blotting. Total, 10%; eluate, 100%. Cka1, Cka2, catalytic subunits of CK2; Ssa1, cytosolic member of heat shock protein 70 family. (C) Kemptide, the cytosolic domain of Tom70, and recombinant Tom40 (WT and S54E mutant form) were incubated with purified yeast PKA (Bcy1-Tpk) and [γ-^{33}P]ATP in the presence of 8Br-cAMP as indicated. The samples were analyzed by SDS–PAGE, digital autoradiography, and staining with Coomassie brilliant blue R-250. Arrowhead, bovine serum albumin. (D) Recombinant $Tom40^{WT}$ and $Tom40^{S54E}$ were incubated with affinity-purified yeast Tpk1 as indicated and [γ-^{33}P]ATP. The samples were analyzed as described for C.

PKA in the cytosol, whereas mature imported Tom40 is not a substrate of PKA.

Phosphorylation impairs the biogenesis of Tom40

To analyze the biogenesis of Tom40, we incubated the ^{35}S-labeled precursor with isolated mitochondria. We used the wild-type precursor of Tom40 and two mutant forms of Ser-54. When serine was replaced by alanine, the binding of Tom40 to mitochondria was only mildly affected compared with the wild-type precursor (Figure 3A, lanes 4–9). When serine was replaced by the phosphomimetic residue glutamate, however, binding of Tom40 to mitochondria was reduced (Figure 3A, lanes 1–3). Treatment of mitochondria with sodium carbonate at alkaline pH leads to the extraction of soluble and peripheral membrane proteins, whereas integral membrane proteins remain in the membrane sheets (Fujiki *et al.*, 1982; Stojanovski *et al.*, 2007). Mitochondria were incubated with [^{35}S]Tom40 and then treated at alkaline pH, demonstrating that the replacement of Ser-54 by glutamate considerably reduced the membrane integration of Tom40 (Figure 3B, lanes 1–3).

The assembly pathway of Tom40 involves several steps. On initial import of the precursor by the TOM complex to the intermembrane space side, intermembrane space chaperone complexes transfer Tom40 to the sorting and assembly machinery (SAM complex) of the outer membrane (Model *et al.*, 2001; Kozjak *et al.*, 2003; Paschen *et al.*, 2003; Wiedemann *et al.*, 2003, 2004; Gentle *et al.*, 2004; Hoppins and Nargang, 2004). The interaction of the Tom40 precursor with the SAM complex can be directly monitored by blue native electrophoresis after lysis of the mitochondria with the nonionic detergent digitonin (Ryan *et al.*, 2001; Wittig *et al.*, 2006; Stojanovski *et al.*, 2007). The SAM intermediate of ~250 kDa is followed by a second intermediate (Int-II) of lower molecular mass and finally the assembly of imported Tom40 into the mature TOM complex of ~400 kDa (Figure 4A, lanes 1–3; Wiedemann *et al.*, 2003; Kutik *et al.*, 2008). When the precursor of Tom40 was phosphorylated by PKA, formation of the assembly steps was considerably impaired. The inhibition occurred already at an early stage, since the generation of the SAM intermediate was impaired (Figure 4A, lanes 4–6). To exclude that PKA inhibited the assembly of the TOM complex in

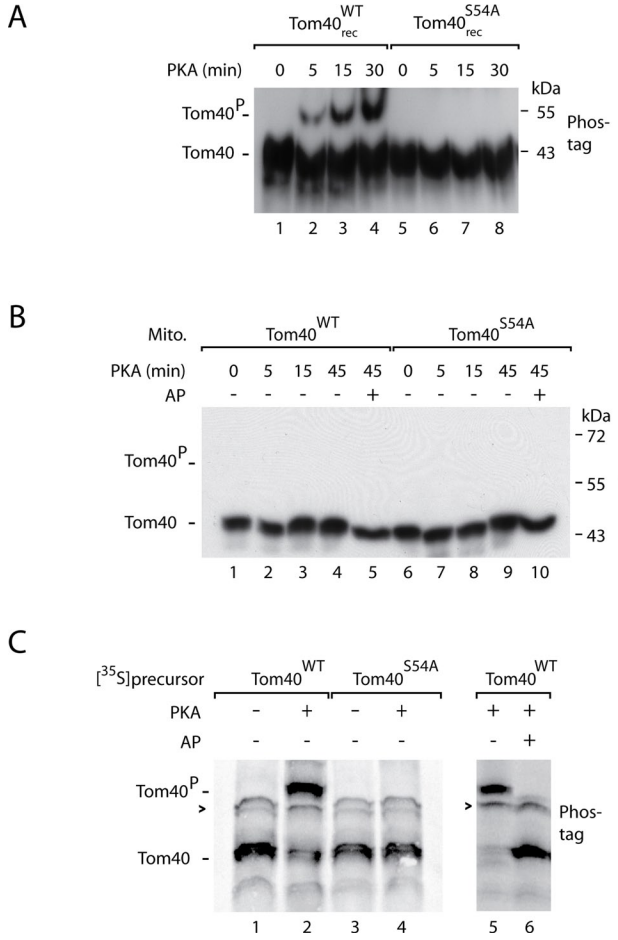

FIGURE 2: PKA phosphorylates the precursor of Tom40 at serine 54. (A) Recombinant Tom40^{WT} or Tom40^{S54A} was incubated with purified PKA (New England BioLabs). The samples were lysed in Laemmli buffer and analyzed by Phos-tag SDS–PAGE and Western blotting using antiserum directed against Tom40. (B) Mitochondria were isolated from WT and Tom40^{S54A} yeast strains and incubated with purified yeast PKA (Bcy1-Tpk) and 8Br-cAMP for the indicated periods. Where indicated, the samples were subsequently incubated with alkaline phosphatase (AP). The samples were analyzed as described for A. Similarly, mammalian PKA did not phosphorylate Ser-54 of Tom40 in intact mitochondria. (C) ^{35}S-Labeled Tom40^{WT} and Tom40^{S54A} were synthesized in reticulocyte lysate in the presence or absence of PKA as indicated. Sample 6 was subsequently treated with AP. The samples were analyzed by Phos-tag SDS–PAGE and digital autoradiography. Arrowhead, nonspecific band; Tom40^{P}, phosphorylated form of Tom40.

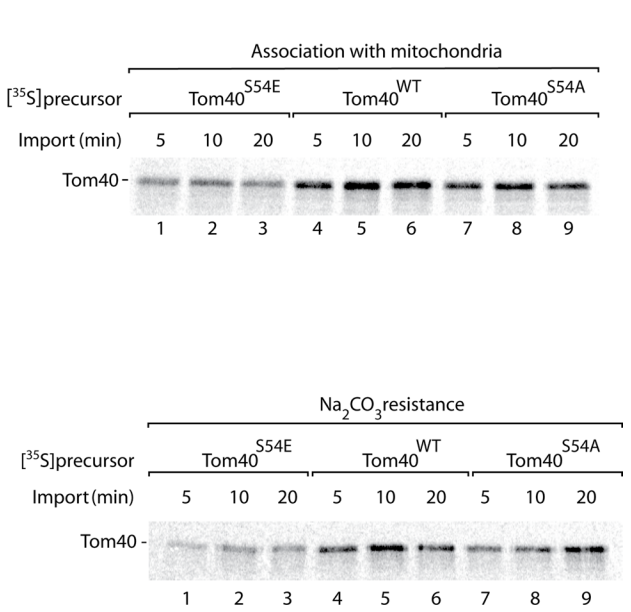

FIGURE 3: Replacement of serine 54 by glutamate impairs import of Tom40 into mitochondria. ^{35}S-Labeled Tom40^{S54E}, Tom40^{WT}, and Tom40^{S54A} precursors were incubated with isolated yeast wild-type mitochondria at 25°C for the indicated periods. The samples were split in half, and the mitochondria were reisolated. (A) One half was lysed in Laemmli buffer and analyzed by SDS–PAGE and digital autoradiography. (B) The other half was resuspended in Na_2CO_3, pH 11.5, and incubated for 30 min on ice. Membrane sheets were pelleted by centrifugation at 100,000 × *g* and analyzed by SDS–PAGE and digital autoradiography.

general, we imported two small Tom precursors—Tom6 and Tom7—whose assembly can be efficiently monitored by blue native electrophoresis (Dembowski *et al.*, 2001; Model *et al.*, 2001). Both proteins were assembled into the TOM complex independent of the presence or absence of PKA (Figure 4B).

To analyze whether the inhibitory effect of PKA was specific for the phosphorylation of Ser-54 of Tom40, we compared formation of the SAM intermediate of the wild-type Tom40 precursor to the mutant precursor Tom40^{S54A}. In the absence of PKA, wild-type precursor and mutant precursor accumulated at the SAM after a short-term import reaction (Figure 4C, lanes 1 and 4). PKA only inhibited the formation of the SAM intermediate of the wild-type precursor and not of the mutant precursor (Figure 4C, lanes 2 and 3), demonstrating that PKA inhibits the biogenesis of Tom40 selectively via phosphorylation of Ser-54. Taking the data together, we conclude that phosphorylation by PKA impairs the biogenesis pathway of Tom40 at an early that is, at or before formation of the SAM intermediate.

Mitochondria import the nonphosphorylated form of Tom40

The initial stage of Tom40 import, that is, translocation via the TOM complex to the intermembrane space side, does not involve a blue native–stable intermediate and thus cannot be directly monitored by native gel analysis (Wiedemann *et al.*, 2004). Therefore we used the accessibility to externally added protease to determine a possible role of PKA phosphorylation in this initial import step. To distinguish between phosphorylated and nonphosphorylated Tom40 precursors, we analyzed the mitochondria by Phos-tag SDS–PAGE. The nonphosphorylated Tom40 was protected against added proteinase K, indicating that it was imported into mitochondria (Figure 5A; Model *et al.*, 2001; Wiedemann *et al.*, 2003). The phosphorylated Tom40 precursor, however, was almost completely degraded by proteinase K (Figure 5A), demonstrating that the precursor was still located on the mitochondrial surface and not imported.

Tom20, Tom22, and Tom70 function as receptors for import of nuclear-encoded precursor proteins into mitochondria (Kiebler *et al.*, 1993; Brix *et al.*, 1997, 1999; van Wilpe *et al.*, 1999; Yamano *et al.*, 2008; Rimmer *et al.*, 2011; Shiota *et al.*, 2011). Pretreatment of mitochondria with trypsin removes the receptor domains and inhibits preprotein import into mitochondria (Ryan *et al.*, 2001). Trypsin pretreatment considerably impaired the interaction of the nonphosphorylated precursor of Tom40 with mitochondria but not the phosphorylated precursor (Figure 5B). We thus studied mutant mitochondria deficient in Tom receptors. The import of radiolabeled

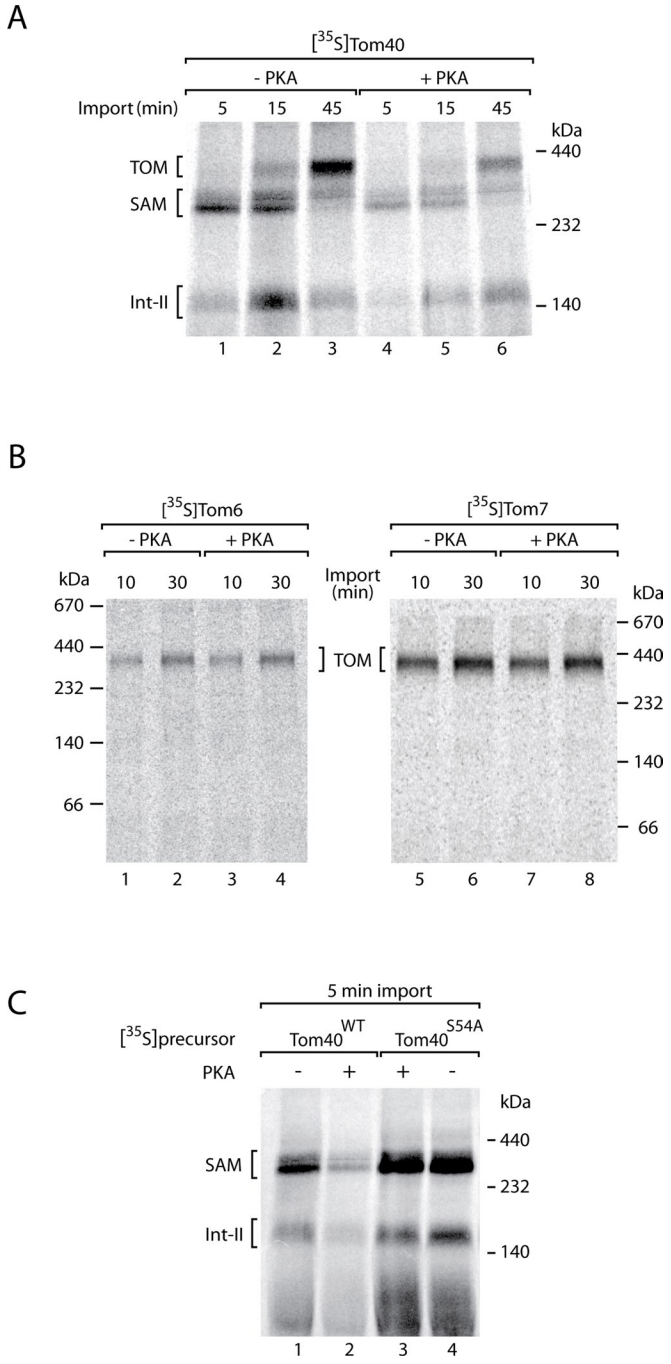

FIGURE 4: Phosphorylation of Tom40 by PKA impairs formation of the SAM import intermediate. (A) ^{35}S-Labeled precursor of Tom40 was synthesized in the presence or absence of PKA (New England BioLabs) as indicated and incubated with isolated yeast wild-type mitochondria for the indicated periods at 25°C. The mitochondria were solubilized in digitonin-containing buffer and analyzed by blue native electrophoresis and digital autoradiography. (B) ^{35}S-Labeled precursors of Tom6 and Tom7 were imported into isolated mitochondria in the presence or absence of PKA. The mitochondria were analyzed as described for A. (C) [^{35}S]Tom40^{WT} and [^{35}S]Tom40^{S54A} precursors were synthesized in the presence or absence of PKA and incubated with isolated mitochondria for 5 min at 25°C. The mitochondria were analyzed as described for A.

Tom40 precursor was strongly inhibited in mitochondria lacking the central receptor Tom22 (Figure 5C, lanes 7 and 8; Model *et al.*, 2001). Analysis by Phos-tag SDS–PAGE revealed that interaction of the nonphosphorylated precursor with mitochondria was strongly inhibited when Tom22 was lacking, whereas binding of the phosphorylated form to mitochondria was not affected (Figure 5C, lanes 11 and 12). As observed with wild-type mitochondria, the phosphorylated Tom40 interacting with *tom22*Δ mitochondria was digested by externally added protease (Figure 5C, lanes 13–16), demonstrating that phosphorylated Tom40 was not imported into mitochondria. Similarly, mitochondria lacking Tom20 or Tom70 were impaired in the interaction with nonphosphorylated Tom40, whereas the binding of phosphorylated Tom40 to the isolated mitochondria was not affected by lack of the receptors (Figure 5, D and E).

Swelling of mitochondria leads to a release of intermembrane space chaperones and thus inhibits the biogenesis of Tom40 (Wiedemann *et al.*, 2004). Swollen mitochondria (mitoplasts) were inhibited in the interaction with nonphosphorylated Tom40 but not phosphorylated Tom40 (Figure 5F). *tim10-2* mutant mitochondria are impaired in the activity of the Tim9–Tim10 intermembrane space chaperone and thus in the import of Tom40 (Truscott *et al.*, 2002; Wiedemann *et al.*, 2004). Only nonphosphorylated Tom40, and not the phosphorylated form of Tom40, was affected by the *tim10-2* mutant (Figure 5G).

Taking the data together indicates that the nonphosphorylated precursor of Tom40 shows the characteristics of specific import into mitochondria, including dependence on Tom receptors and intermembrane space chaperones. In contrast, phosphorylated Tom40 remains on the mitochondrial surface in a receptor-independent manner and is not imported into mitochondria, indicating that the binding observed with mitochondria is nonproductive. We conclude that mitochondria specifically import the nonphosphorylated form of Tom40.

PKA inhibits Tom40 import independently of Tom70 phosphorylation

Phosphorylation of the receptor Tom70 by PKA impairs the interaction of the cytosolic chaperone Hsp70 with Tom70 (Schmidt *et al.*, 2011). Hsp70 delivers hydrophobic precursor proteins such as the inner membrane metabolite carriers to Tom70 (Young *et al.*, 2003; Li *et al.*, 2009; Zara *et al.*, 2009). PKA selectively phosphorylates Ser-174 of Tom70, which is located close to the chaperone-binding site of Tom70, and thus disturbs the Hsp70–Tom70 interaction (Schmidt *et al.*, 2011). We asked whether the phosphorylation of Tom70 influenced the import of Tom40. In yeast mitochondria in which Ser-174 of Tom70 was replaced by alanine, the import of carrier precursors was enhanced, whereas a replacement of Ser-174 by glutamate inhibited carrier import (Schmidt *et al.*, 2011). In the case of Tom40, however, neither replacement of Ser-174 by alanine nor replacement by glutamate affected the import of the precursor in comparison to wild-type mitochondria (Figure 6, lanes 1, 2, 5, 6, 9, and 10). Phosphorylation of the Tom40 precursor by PKA inhibited its import into Tom70^{S174A} and Tom70^{S174E} mitochondria like that into wild-type mitochondria (Figure 6, lanes 3, 4, 7, 8, 11. and 12). Given that Ser-174 is the only PKA target site of Tom70 (Schmidt *et al.*, 2011), these results demonstrate that the inhibitory effect of PKA on the import of Tom40 occurs independently of the phosphorylation of Tom70.

DISCUSSION

We report a new mechanism of how cytosolic kinases regulate the preprotein translocase of the outer mitochondrial membrane. PKA phosphorylates the precursor of Tom40, the channel-forming core component of the TOM complex, and thus inhibits the import of Tom40 into mitochondria. So far, cytosolic kinases had been shown

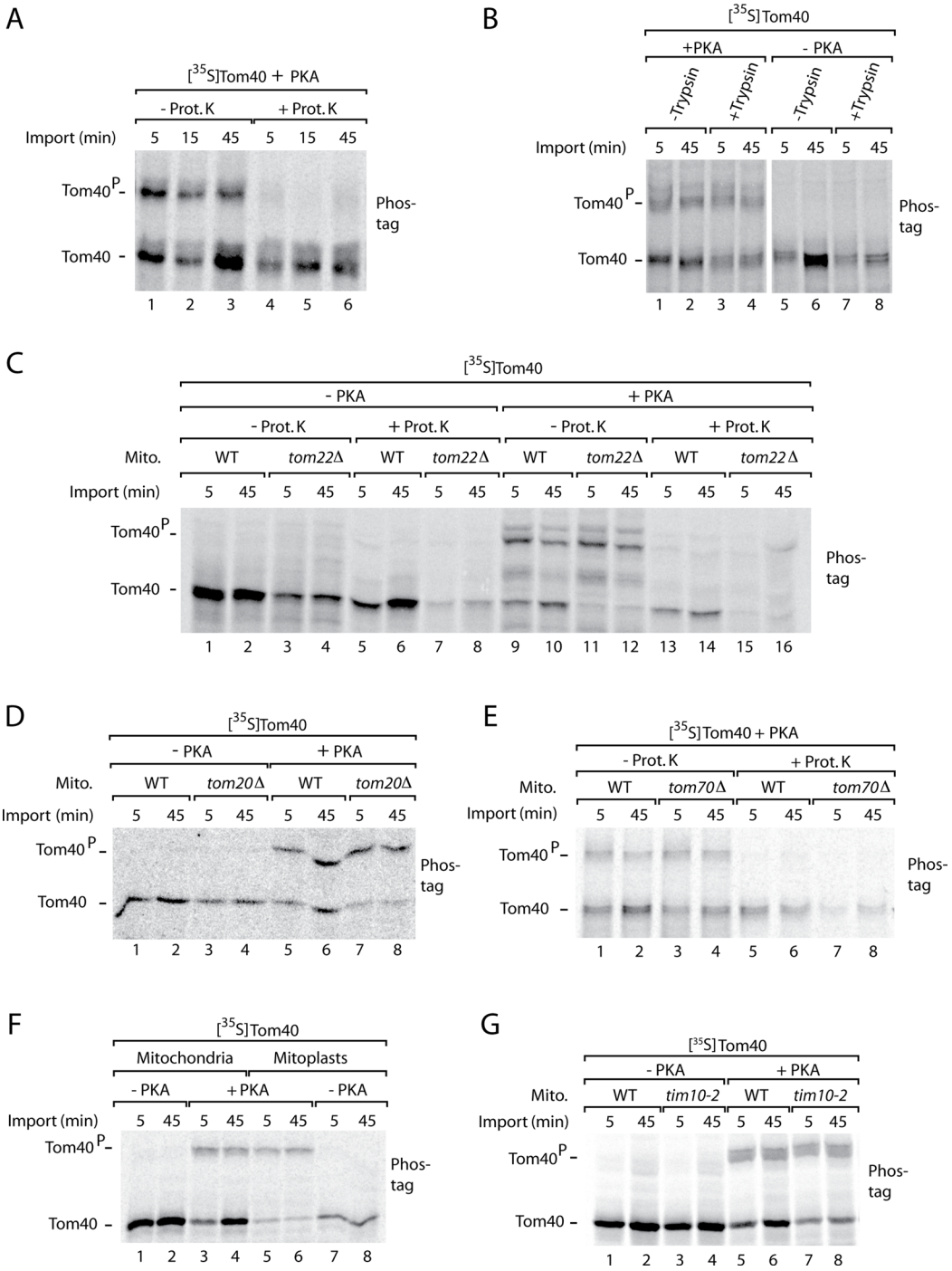

FIGURE 5: Mitochondria import the nonphosphorylated precursor of Tom40, whereas phosphorylated Tom40 remains on the mitochondrial surface. (A) [^{35}S]Tom40 was synthesized in the presence of PKA (New England BioLabs) and incubated with isolated wild-type mitochondria for the indicated periods at 25°C. The mitochondria were treated with proteinase K (Prot. K; Stojanovski *et al.*, 2007) where indicated and analyzed by Phos-tag SDS–PAGE and digital autoradiography. (B) [^{35}S]Tom40 was synthesized in the presence or absence of PKA and imported into mitochondria that had been pretreated with trypsin (Ryan *et al.*, 2001) as indicated. The mitochondria were analyzed by Phos-tag SDS–PAGE. (C) [^{35}S]Tom40 was imported into mitochondria, which were isolated from *tom22*Δ yeast or the corresponding wild-type (WT) strain, in the presence or absence of PKA. The mitochondria were treated with proteinase K where indicated and analyzed by Phos-tag SDS–PAGE. (D) [^{35}S]Tom40 was imported into mitochondria, which were isolated from *tom20*Δ and wild-type yeast, in the presence or absence of PKA. The mitochondria were analyzed by Phos-tag SDS–PAGE. (E) [^{35}S]Tom40 was imported into mitochondria, which were isolated from *tom70*Δ and wild-type yeast, in the presence of PKA. The mitochondria were treated with proteinase K where indicated and analyzed by Phos-tag SDS–PAGE. (F) Mitochondria were preincubated in isotonic or hypotonic (swelling) buffer for 30 min on ice (Stojanovski *et al.*, 2007). The mitochondria/mitoplasts were reisolated and incubated with [^{35}S]Tom40 in the presence or absence of PKA and analyzed by Phos-tag SDS–PAGE. (G) [^{35}S]Tom40 was imported into mitochondria, which were isolated from *tim10-2* yeast or the corresponding wild-type strain, in the presence or absence of PKA. The mitochondria were analyzed by Phos-tag SDS–PAGE.

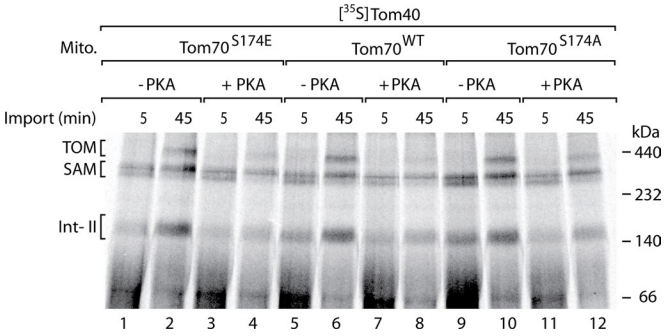

FIGURE 6: Inhibition of Tom40 import by PKA is not connected to the phosphorylation of Tom70 by PKA. ^{35}S-Labeled precursor of Tom40 was imported into mitochondria, which were isolated from Tom70^{S174E}, Tom70^{WT}, or Tom70^{S174A} yeast strains, at 25°C in the presence or absence of PKA (New England BioLabs) as indicated. The mitochondria were solubilized in digitonin-containing buffer and analyzed by blue native electrophoresis and digital autoradiography.

either to stimulate the biogenesis of Tom subunits (phosphorylation of Tom22 and Mim1 by CK2) or to inhibit the receptor activity of a Tom receptor without affecting its biogenesis (phosphorylation of Tom70 by PKA; Schmidt *et al.*, 2011). We found that the PKA effect on Tom40 biogenesis was independent of the phosphorylation status of Tom70, demonstrating that PKA exerts two different inhibitory effects on the TOM complex: inhibition of receptor activity (Tom70) and inhibition of precursor import (Tom40). In agreement with these observations, in a yeast strain lacking the inhibitory PKA subunit Bcy1, the steady-state level of Tom40, but not that of Tom70, was reduced.

PKA is rapidly activated by addition of fermentable carbon sources such as glucose and sucrose that lead to increased intracellular cAMP levels in yeast cells (Broach, 1991; Thevelein, 1994; Santangelo, 2006; Tamaki, 2007; Zaman *et al.*, 2008; Smets *et al.*, 2010). PKA was shown to affect numerous cellular processes, including morphology of the mitochondrial membranes, programmed cell death, and oxidative metabolism. In addition, a few precursor proteins were found to be phosphorylated in a cAMP-dependent manner, affecting their interaction with molecular chaperones and translocation into mitochondria (Anandatheerthavarada *et al.*, 1999; Cho *et al.*, 2001; Robin *et al.*, 2002, 2003; Pagliarini and Dixon, 2006; Chang and Blackstone, 2007; Carlucci *et al.*, 2008; De Rasmo *et al.*, 2008; Santel and Frank, 2008; Soubannier and McBride, 2009). The inhibitory effects of PKA on TOM biogenesis (this study) and function (Schmidt *et al.*, 2011) provide a direct means to control the mitochondrial preprotein entry gate upon shift to fermentable growth conditions, under which a lower mitochondrial activity is required. Under nonfermentable, respiratory conditions the cAMP levels and PKA activity are low (Russell *et al.*, 1993; Thevelein, 1994; Zaman *et al.*, 2008; Smets *et al.*, 2010), and thus Tom40 is efficiently imported.

The biogenesis pathway of Tom40 can be dissected into several steps, involving transport by the TOM complex, intermembrane space chaperones, and the SAM complex (Model *et al.*, 2001; Wiedemann *et al.*, 2003; Waizenegger *et al.*, 2004; Habib *et al.*, 2005; Kutik *et al.*, 2008). Phosphorylation of the Tom40 precursor by PKA inhibits the initial stage of precursor translocation through the TOM complex to a protease-protected location. Whereas nonphosphorylated Tom40 precursor is efficiently imported, the phosphorylated form remains nonproductively bound to the mitochondrial surface and is not translocated. Numerous mutational studies have been performed with Tom40 (Rapaport and Neupert, 1999; Rapaport *et al.*, 2001; Taylor *et al.*, 2003, Humphries *et al.*, 2005; Sherman *et al.*, 2006; Kutik *et al.*, 2008). A linear sorting signal for binding of Tom40 to the SAM complex has been identified in the carboxy-terminal region of the precursor (Kutik *et al.*, 2008); however, a targeting signal that is responsible for the initial recognition of Tom40 by the TOM complex has not been found. It is conceivable that several elements of the precursor may contribute to the targeting process. Serine 54 is not essential for the targeting of Tom40, since this residue can be deleted (Rapaport and Neupert, 1999; Rapaport *et al.*, 2001; Taylor *et al.*, 2003; Humphries *et al.*, 2005; Sherman *et al.*, 2006; Kutik *et al.*, 2008) or replaced by alanine (this study) without blocking Tom40 targeting. Harsman *et al.* (2010) reconstituted purified Tom40^{WT} and Tom40^{S54E} into planar lipid bilayers and observed a similar gating behavior of the Tom40 channel of wild type and mutant, indicating that the replacement of Ser-54 by the phosphomimetic residue glutamate did not disturb the overall folding of Tom40. Of interest, the association rate of positively charged presequence peptides with Tom40 was altered when Ser-54 was replaced by glutamate (Harsman *et al.*, 2010), suggesting that modification of this residue can affect the interaction properties of Tom40 in vitro. We analyzed the biogenesis of Tom40^{S54E} in organello and observed an impairment of import into mitochondria. Using PKA, we showed that phosphorylation of Ser-54 inhibits the translocation of Tom40 via the TOM complex, demonstrating that phospho–Ser-54 interferes with the targeting process. Although the exact nature of the Tom40 targeting signal remains elusive, the strong inhibitory effect of a single phosphorylation event provides an efficient mechanism to control the import of Tom40.

Taking our results together with the findings by Schmidt *et al.*, (2011), we conclude that cytosolic kinases regulate biogenesis and function of the TOM complex at multiple levels. The main protein entry gate of mitochondria is not functioning autonomously but is tightly integrated into a network of regulatory kinases.

MATERIALS AND METHODS

Yeast strains and cloning

Most *S. cerevisiae* strains used in this study are derived from the strain YPH499 (*MAT*a, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*; Sikorski and Hieter, 1989). YPH499-*tom40Δ*+pFL39-*TOM40* was made by transforming the shuffling strain *tom40Δ*+Yep-*TOM40* (Kutik *et al.*, 2008) with the plasmid pFL39-*TOM40* as described (Schmidt *et al.*, 2011). The mutations Tom40^{S54A} and Tom40^{S54E} were generated by site-directed mutagenesis. Clones were verified by sequencing. The Tom70^{S174E} and Tom70^{S174A} yeast strains, *tom20Δ* strain, *tom22Δ* strain, *tom70Δ* strain, *tim10-2* strain, and *bcy1Δ* strain have been described previously (Moczko *et al.*, 1994; van Wilpe *et al.*, 1999; Truscott *et al.*, 2002; Stojanovski *et al.*, 2007; Schmidt *et al.*, 2011). The Bcy1$_{ProtA}$ strain was constructed by amplifying the HIS3MX6-pNOP-ProtA-TEV plasmid (Meisinger *et al.*, 2007) with primers A1 (5′ ATTACAACAAGC AGATTATTTTCAAAAGACAA-CAGTAAGAATAAACGGGAATACGAATTCGAGCTC 3′) and A2 (5′ GTTCTGGAACAGTTGCAATTCGGCTTGCGATTCCTTG-GGCAAAGA AGATACCACGTCACTCATACCCTGA 3′) and integrating into the *BCY1* open reading frame by homologous recombination. The Tom70^{WT}/pET19 and Kemptide-GST/pETGEXct constructs were reported previously (Brix *et al.*, 1997; Schmidt *et al.*, 2011).

Isolation of mitochondria and preparation of yeast protein extracts

Yeast strains were typically grown on nonfermentable YPG medium (1% [wt/vol] yeast extract, 2% [wt/vol] bactopeptone, 3% [wt/vol]

glycerol, pH 5.0) at 23°C until an OD_{600} of 0.5–1.0 was reached. Mitochondria were isolated by differential centrifugation (Meisinger *et al.*, 2006a). Mitochondria were resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM 3-(*N*-morpholino)propanesulfonic acid–KOH, pH 7.2), adjusted to a protein concentration of 10 mg/ml, frozen in liquid nitrogen, and stored at −80°C. For preparation of yeast protein extracts, yeast cells were grown on fermentable YPS medium (1% [wt/vol] yeast extract, 2% [wt/vol] bactopeptone, 2% [wt/vol] sucrose), and extracts corresponding to the same OD_{600} were prepared by postalkaline lysis.

Purification of yeast PKA

The yeast strain expressing $Bcy1_{ProtA}$ and the corresponding wild-type strain (YPH499) were grown in YPG medium at 30°C to an OD_{600} of 2.0, harvested, and lysed as described for the preparation of mitochondria (Meisinger *et al.*, 2006a) but using homogenization buffer with 2 mg/ml bovine serum albumin, 2 mM phenylmethylsulfonyl fluoride (PMSF), and complete EDTA-free protease inhibitors (Roche, Indianapolis, IN). The lysate was cleared by centrifugation (30,000 × *g*) and was added to human immunoglobulin G–coupled Sepharose beads. Unbound material was removed by washing with excess homogenization buffer. The Bcy1-Tpk complexes were eluted by cleavage with TEV protease in homogenization buffer. After addition of 10–20% (vol/vol) glycerol and 2 mM dithiothreitol (DTT), the eluates were stored at −80°C. Alternatively, a yeast strain expressing $Tpk1_{TAP}$ was grown on fermentable YPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] bactopeptone, 2% [wt/vol] glucose) until an OD_{600} of 1.0, and extracts were purified as described (Mah *et al.*, 2005). The kinase was eluted by cleavage with TEV protease in kinase buffer (100 mM Tris, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 20% glycerol, 1 mM PMSF, 1× PhosStop [Roche]). Eluates were stored at −80°C.

In vitro phosphorylation assays

Tom40 was purified from inclusion bodies as described (Hill *et al.*, 1998). The inclusion bodies were solubilized in urea buffer (8 M urea, 50 mM Tris/HCl pH 8.0, 1 mM EDTA, and 100 mM DTT). Kemptide-GST and $Tom70_{cd}$ were prepared as described (Schmidt *et al.*, 2011).

In vitro phosphorylation reactions with recombinant mouse PKA (New England BioLabs, Ipswich, MA) and [γ-^{33}P]ATP were performed as described (Schmidt *et al.*, 2011). For analysis by Phos-tag SDS–PAGE and immunoblotting, 5–10 mM ATP and 50 U/µl PKA (New England BioLabs) were used. Tom40 in vitro phosphorylation was performed in a buffer with 25 mM tricine, 7.5 mM BisTris, and 0.5% (vol/vol) heptyl-thio-glucopyranoside, pH 7.0.

Bcy1-Tpk complexes were supplemented with 1× PKA assay buffer (New England BioLabs), 1× PhosStop (Roche), 2 mM PMSF, 0.2 mM ATP, 5 mCi of [γ-^{33}P]ATP (PerkinElmer, Waltham, MA), 50–100 µM 8Br-cAMP (Sigma-Aldrich, St. Louis, MO; or Biaffin, Kassel, Germany) where indicated, Kemptide-GST, or $Tom70_{cd}^{WT}$ in a total volume of 18 µl and incubated for 30 min at 25°C under constant shaking. The reactions were stopped by addition of Laemmli buffer and heating to 95°C.

For Tom40, the Bcy1-Tpk complexes or the Tpk1 eluate were incubated with 1 µg of $Tom40^{WT}$, $Tom40^{S54E}$, or $Tom40^{S54A}$, 50–100 µM 8Br-cAMP (where indicated), 0.2 mM ATP, and 5 mCi [γ-^{33}P]ATP (PerkinElmer) in a total volume of 20 µl for 90 min at 25°C under constant shaking. The reactions were stopped by addition of Laemmli buffer and heating to 95°C.

Isolated mitochondria were supplemented with 1× PKA assay buffer, 1× PhosStop, 5–10 mM ATP, and either 50 U/µl recombinant PKA in SEM buffer or Bcy1-Tpk complexes with 50–100 µM 8Br-cAMP and incubated at 25°C. For Phos-tag and immunoblot analysis, 5–10 mM ATP and recombinant $Tom40^{WT}$ or $Tom40^{S54A}$ were used in a total volume of 15 µl.

For Phos-tag SDS–PAGE, standard discontinuous 8–12.5% polyacrylamide gels were prepared with the modification that 50 µM Phos-tag reagent (Kinoshita *et al.*, 2006) and 100 µM $MnCl_2$ were added to the separation gel mix prior to polymerization.

Protein import assays

^{35}S-Labeled precursor proteins were generated by in vitro translation and incubated with isolated mitochondria in import buffer as described (Ryan *et al.*, 2001; Stojanovski *et al.*, 2007). Where indicated, the reactions were supplemented with 50 U/µl recombinant PKA (New England BioLabs) during translation or import. The import efficiency of different precursor forms was directly compared by adjusting the levels of the radiolabeled proteins added to the import reactions.

The import reactions were stopped on ice, and mitochondria were reisolated, washed in SEM buffer, and either lysed directly in sample buffer or treated with 100 mM Na_2CO_3 (pH 11.5) and centrifuged at 100,000 × *g* for 1 h before lysis in sample buffer to test for membrane integration (Fujiki *et al.*, 1982; Stojanovski *et al.*, 2007). SDS–PAGE or Phos-tag SDS–PAGE was followed by digital autoradiography (GE Healthcare, Piscataway, NJ). To analyze protein complex formation, mitochondria were lysed in 1% digitonin-containing buffer and analyzed by blue native electrophoresis (Ryan *et al.*, 2001; Wittig *et al.*, 2006; Stojanovski *et al.*, 2007) and digital autoradiography.

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