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Regulation of Mitochondrial Protein Import by Cytosolic Kinases

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SUMMARY

Mitochondria import a large number of nuclear-encoded proteins via membrane-bound transport machineries; however, little is known about regulation of the preprotein translocases. We report that the main protein entry gate of mitochondria, the translocase of the outer membrane (TOM complex), is phosphorylated by cytosolic kinases—in particular, casein kinase 2 (CK2) and protein kinase A (PKA). CK2 promotes biogenesis of the TOM complex by phosphorylation of two key components, the receptor Tom22 and the import protein Mim1, which in turn are required for import of further Tom proteins. Inactivation of CK2 decreases the levels of the TOM complex and thus mitochondrial protein import. PKA phosphorylates Tom70 under nonrespiring conditions, thereby inhibiting its receptor activity and the import of mitochondrial metabolite carriers. We conclude that cytosolic kinases exert stimulatory and inhibitory effects on biogenesis and function of the TOM complex and thus regulate protein import into mitochondria.

INTRODUCTION

Mitochondria play crucial roles in cellular energy conversion, numerous metabolic pathways, maintenance of ion concentrations, and regulation of apoptosis. Proteomic studies indicate that mitochondria contain ~1000 (yeast) to 1500 (human) different proteins, 99% of which are being encoded by nuclear genes and synthesized as precursors on cytosolic ribosomes (Mootha et al., 2003; Neupert and Herrmann, 2007; Pagliarini et al., 2008; Chacinska et al., 2009). The central entry gate for virtually all nuclear-encoded mitochondrial proteins is the preprotein translocase of the outer membrane (TOM complex). The receptors Tom20 and Tom70 initially recognize the precursor proteins: Tom20 preferentially preproteins with N-terminal presequences, and Tom70 hydrophobic precursors with internal targeting signals (Young et al., 2003; Dolezal et al., 2006; Neupert and Herrmann, 2007). Upon interaction with the initial receptors, the precursors are transferred to the central receptor Tom22 and from here to the import channel Tom40 (Neupert and Herrmann, 2007; Chacinska et al., 2009). Three small Tom proteins modulate the assembly and stability of the TOM complex. After passing through the TOM complex, the precursor proteins use different machineries to reach their functional destination in the four mitochondrial subcompartments: outer membrane, intermembrane space, inner membrane, and matrix (Dolezal et al., 2006; Neupert and Herrmann, 2007; Chacinska et al., 2009).

Little is known about regulation of the translocases that mediate preprotein import into mitochondria. Reversible phosphorylation of proteins is a major means of regulation of cellular processes. Studies in recent years indicated that the number of mitochondrial phosphoproteins is larger than expected (Chi et al., 2007; Li et al., 2007; Reinders et al., 2007; Albuquerque et al., 2008; Gnad et al., 2009; Holt et al., 2009). The functional consequences of phosphorylation, however, have only been investigated for a limited set of mitochondrial proteins, including regulation of apoptotic processes, mitochondrial morphology, pyruvate dehydrogenase, and respiratory complexes; phosphorylation of a few preproteins was shown to modulate their interaction with Hsp70 chaperones and transfer into mitochondria (Desagher et al., 2001; Robin et al., 2003; Pagliarini and Dixon, 2006; de Rasmo et al., 2008; Soubannier and McBride, 2009). Large-scale studies of phosphoproteins in baker's yeast indicated the existence of phosphorylation sites on the translocases of the mitochondrial membranes (Chi et al., 2007; Li et al., 2007; Albuquerque et al., 2008; Gnad et al., 2009; Holt et al., 2009); however, it has not been reported so far whether

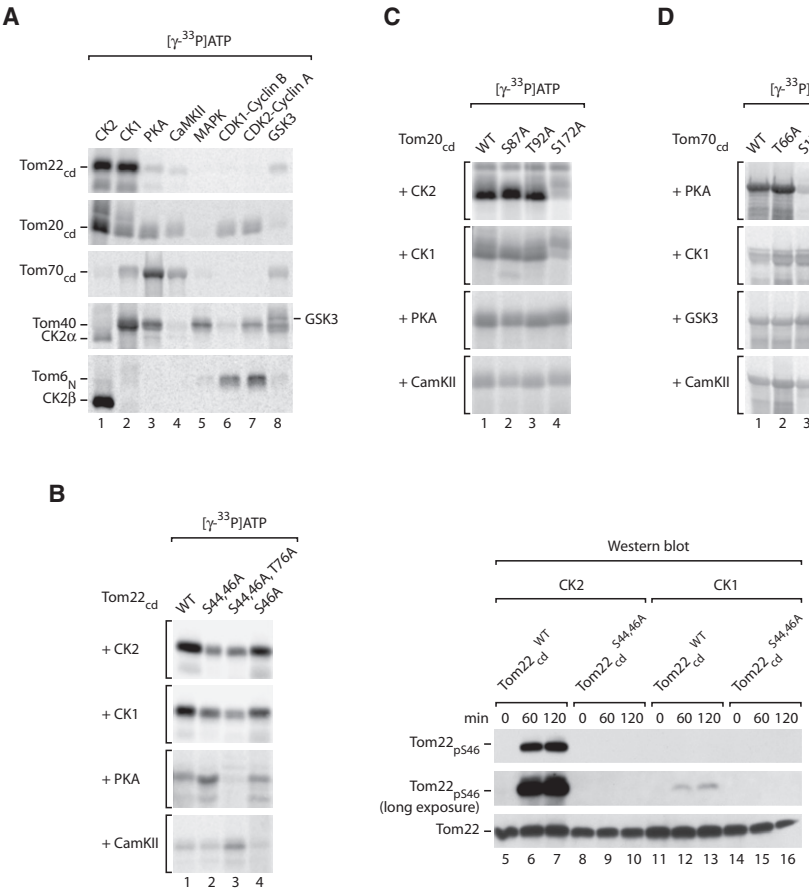

Figure 1. Phosphorylation of TOM Receptors by CK2 and PKA

(A) Phosphorylation of purified TOM subunits with recombinant kinases, analyzed by SDS-PAGE and autoradiography. Kinase autophosphorylation bands are indicated (CK2α, CK2β, and GSK3).

(B) Phosphorylation of $Tom22_{cd}$ phosphosite mutants. In samples 5–16, the applied kinase activity represented 50% for CK1 and 10% for CK2 of the one applied in (A) and samples 1–4. WT, wild-type.

(C) In vitro phosphorylation of $Tom20_{cd}$ phosphosite mutants.

(D) In vitro phosphorylation of $Tom70_{cd}$ phosphosite mutants.

See also Table S1 and Figure S1.

any of these phosphosites are of functional relevance. Thus, despite our detailed knowledge about composition and function of the preprotein translocases, posttranslational regulatory mechanisms acting at the membrane translocases of mitochondria have not been identified.

Here, we report that biogenesis and function of the TOM complex are regulated by protein kinases. Yeast mutant cells of casein kinase 2 (protein kinase CK2) show severe defects in the levels of the TOM complex. CK2 phosphorylates the receptor Tom22 and the mitochondrial import protein Mim1 that are critical for the biogenesis of further TOM subunits. Whereas CK2 plays a stimulatory role for mitochondrial biogenesis, protein kinase A (PKA) plays an inhibitory role. PKA phosphorylates the receptor Tom70 and thus impairs its activity for the import of metabolite carriers of the inner-mitochondrial membrane. Our study reveals that the main protein entry gate of mitochondria is not a static complex but is regulated by cytosolic kinases.

RESULTS

Phosphorylation of Tom Proteins by Cytosolic Kinases In Vitro

To map phosphorylation sites on the TOM complex, we purified *Saccharomyces cerevisiae* mitochondria, outer-membrane vesicles, and TOM complex (Figure S1A available online). Phosphopeptides were enriched and analyzed by tandem mass spectrometry (MS/MS). We identified 30 phosphosites in Tom proteins. Ten sites agreed with previously determined sites (Chi et al., 2007; Li et al., 2007; Albuquerque et al., 2008; Gnad et al., 2009; Holt et al., 2009), and we identify 20 additional sites, leading to a total of 31 TOM phosphosites (Table S1 and Figure S1B). All Tom proteins were phosphorylated (including Tom71, a low-abundant isoform of Tom70). Most phosphosites were located on the cytosolic side of the outer membrane, particularly in the cytosolic domains of the receptors Tom20, Tom22, Tom70, and Tom71 (Figure S1B).

Prediction of potential kinases for the TOM phosphosites yielded several cytosolic kinases—in particular, CK2 and PKA (Figure S1B). We performed an in vitro screen using recombinantly expressed TOM subunits, [γ-^{33}P]ATP, and the purified kinases CK2, CK1, PKA, calmodulin-dependent kinase II (CamKII), p42 mitogen-activated protein kinase (MAPK), cyclin-dependent kinase 1 (CDK1)-cyclin B complex, CDK2-cyclin A complex, and glycogen synthase kinase 3 (GSK3) (Figure 1A). Purified TOM subunits included the cytosolic domains of the receptors $Tom22_{cd}$, $Tom20_{cd}$, and $Tom70_{cd}$; Tom40 renatured from inclusion bodies; and the cytosolic domain of Tom6 fused to glutathione S-transferase (GST).

$Tom22_{cd}$ was phosphorylated by the acidophilic kinases CK1 and CK2 (Figure 1A and Figure S1C). Ser44 and Ser46 of Tom22 match the CK2 consensus sequence (Meggio and Pinna, 2003). Replacement of Ser44 and Ser46 by alanines strongly reduced phosphorylation of $Tom22_{cd}$ by CK2 (Figure 1B). We generated an antiserum that selectively recognized phospho-Ser46 of Tom22 (Figure S1D). Comparison of the phosphorylation by CK1 and CK2 using lower kinase concentrations revealed that CK2 functions as major kinase for Tom22 (Figure 1B, right). In addition, $Tom22_{cd}$ was weakly phosphorylated by PKA at Thr76 (Figure 1B).

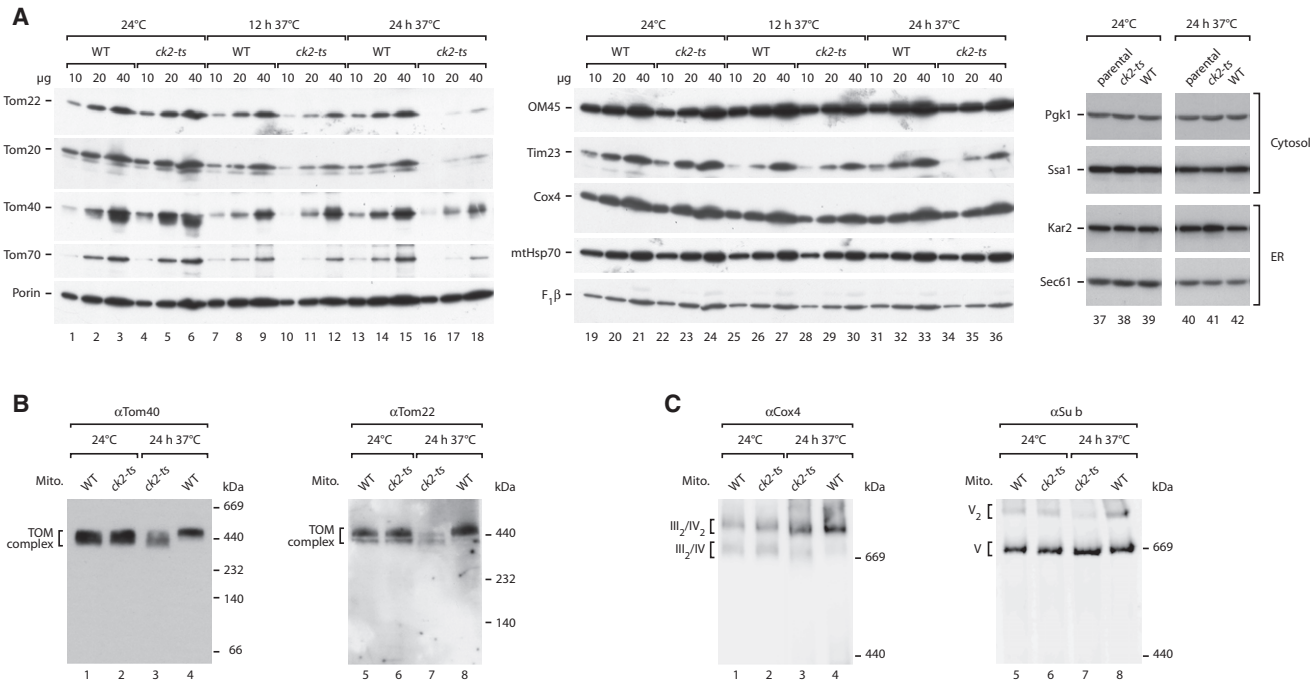

Figure 2. Inactivation of CK2 in Yeast Causes Depletion of Mitochondrial TOM Complexes

(A) WT and *ck2-ts* yeast were grown at 24°C and shifted to 37°C for 12–24 hr. Mitochondria or whole-cell extracts were analyzed by SDS-PAGE and immunoblotting. Parental, parental strain (YPH250) to *ck2-ts* and WT; μg, mitochondrial protein.

(B) Blue native electrophoresis and immunoblot analysis of the TOM complex from WT and *ck2-ts* mitochondria.

(C) Blue native electrophoresis and immunoblot analysis of the respiratory chain complexes III, IV, and V.

$Tom20_{cd}$ was mainly phosphorylated by CK2 and only weakly by several further kinases (Figure 1A and Figure S1C). Ser172 was the critical residue for the phosphorylation by CK2 (Figure 1C). Western blot analysis with an antiserum recognizing phospho-Ser172 confirmed phosphorylation of Tom20 by CK2 (Figure S1E).

$Tom70_{cd}$ was preferentially phosphorylated by PKA (Figure 1A). Mutant analysis revealed that Ser174 was the target residue for PKA, whereas the weak phosphorylation by CK1, GSK3, or CamKII was not altered in the mutant proteins analyzed (Figure 1D).

Tom40 was phosphorylated by several kinases in vitro, including CK1, PKA (Ser54), and MAPK (Figure 1A and Figure S1F). Tom6 was phosphorylated on Ser16 by the cyclin-dependent kinase complexes, CDK1-cyclin B and CDK2-cyclin A (Figure S1G).

These results indicate that purified Tom proteins can be phosphorylated by cytosolic kinases in vitro. The three receptors were found to be major targets for CK2 (Tom22 and Tom20) and PKA (Tom70), respectively.

Inactivation of CK2 Leads to Defects of the Mitochondrial TOM Complex In Vivo

To ask whether CK2 exerted an effect on the TOM complex in vivo, we used *ck2* mutant yeast cells. Yeast CK2 consists of two catalytic α subunits, Cka1 and Cka2, and two regulatory β subunits, Ckb1 and Ckb2 (Poole et al., 2005). Because the activity of CK2 is essential for yeast viability, we employed the temperature-sensitive mutant strain *ck2-ts*, in which *CKA1* and *CKA2* have been deleted and a conditional allele of *CKA2* is expressed from a plasmid (Hanna et al., 1995). When *ck2-ts* cells were grown at permissive temperature (24°C), the levels of mitochondrial proteins were in the wild-type (WT) range (Figure 2A). Upon shift to nonpermissive conditions (37°C), the levels of TOM subunits decreased. After a 12 hr shift, in particular, the levels of Tom22 and Tom20 were reduced, and after a 24 hr shift, the levels of all TOM subunits analyzed were severely reduced (Figure 2A). The levels of further mitochondrial proteins tested were not or were only partially reduced after the 24 hr shift. Proteins of the cytosol and endoplasmic reticulum (ER) analyzed were not reduced in *ck2-ts* cells compared to wild-type cells after the 24 hr shift, including phosphoglycerate kinase (Pgk1), the chaperones Ssa1 and Kar2, and the translocase Sec61 (Figure 2A).

The TOM complex was analyzed by blue native electrophoresis upon lysis of mitochondria with digitonin. The levels of the TOM complex were strongly reduced after the 24 hr shift of *ck2-ts* cells to nonpermissive conditions (Figure 2B). Inner-membrane complexes such as supercomplexes of the respiratory chain (complexes III and IV) and the ATP synthase (complex V) were not or were only moderately affected (Figure 2C). We conclude that inactivation of CK2 causes a reduction of the amount of TOM complexes.

Tom22 Is Quantitatively Phosphorylated by CK2 in Yeast

To analyze the phosphorylation of Tom22 in a homologous system, we incubated isolated yeast mitochondria with yeast cytosolic extract in the presence of [γ-^{33}P]ATP. Phosphorylated Tom22 was only observed in the autoradiography when mitochondria had been dephosphorylated by alkaline phosphatase

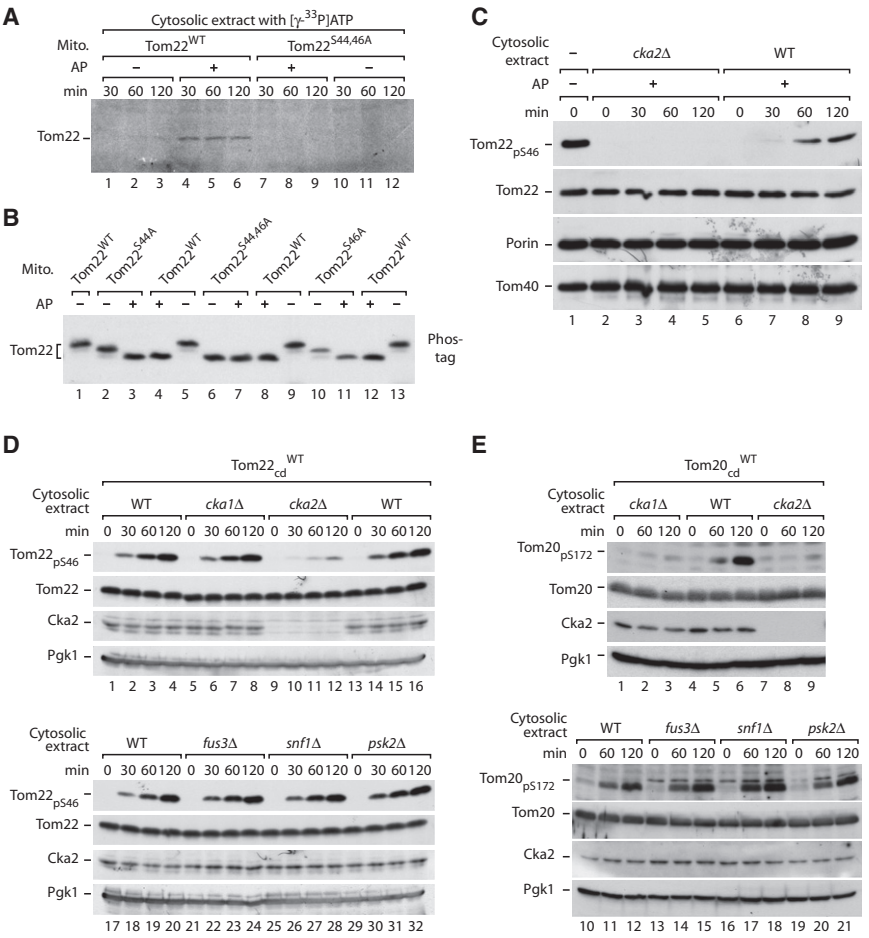

Figure 3. Tom22 Is Quantitatively Phosphorylated by CK2 in Yeast

(A) Yeast mitochondria were treated with alkaline phosphatase (AP) as indicated and were subjected to phosphorylation by yeast cytosolic extract, followed by SDS-PAGE and autoradiography.

(B) Mitochondria of Tom22 phosphomutant strains were treated with AP, lysed, and analyzed by Phos-tag SDS-PAGE and immunoblotting.

(C) Mitochondria were treated with AP, incubated with cytosolic extract, and analyzed by SDS-PAGE and immunoblotting.

(D) Phosphorylation of $Tom22_{cd}^{WT}$ with cytosolic extract.

(E) Phosphorylation of $Tom20_{cd}^{WT}$ with cytosolic extract.

See also Figure S2.

(AP) before the incubation with cytosolic extract (Figure 3A, lanes 4–6), suggesting that wild-type mitochondria contained Tom22 in the phosphorylated state. Phosphorylation of Tom22 was inhibited on mitochondria that were isolated from a yeast strain expressing the $Tom22^{S44,46A}$ mutant form (Figure 3A), demonstrating that Ser44/46 is the major site of Tom22 phosphorylation in organello. For an independent analysis, we used phosphate-affinity (Phos-tag) SDS-PAGE that retards the gel mobility of the phosphorylated forms of proteins (Kinoshita et al., 2006). Treatment of wild-type mitochondria with AP led to a quantitative mobility shift of Tom22 detected with a holo-Tom22 antibody (Figure 3B). In contrast, no shift was observed with $Tom22^{S44,46A}$ mutant mitochondria; the mutant Tom22 migrated at the position of dephosphorylated Tom22. The single-mutant forms $Tom22^{S44A}$ and $Tom22^{S46A}$ migrated between the phosphorylated and dephosphorylated wild-type species and were shifted to the faster-migrating form by AP treatment (Figure 3B). Together with the observation that the Ser44/46-containing peptides of Tom22 were consistently observed in the doubly phosphorylated form by MS/MS (Table S1 and Extended Experimental Procedures), these results demonstrate that mitochondrial Tom22 is quantitatively phosphorylated at Ser44 and Ser46.

A cytosolic extract prepared from a yeast strain lacking Cka2, the α′ catalytic subunit of CK2, was strongly impaired in the phosphorylation of Ser46 on both AP-treated mitochondria (Figure 3C) and purified $Tom22_{cd}$ (Figure 3D). A cytosolic extract prepared from a yeast strain that lacked the other catalytic subunit, Cka1, was able to phosphorylate $Tom22_{cd}$ (Figure 3D). For comparison, the phosphorylation of Ser172 of Tom20 was strongly impaired with *cka1Δ* as well as *cka2Δ* cytosolic extracts (Figure 3E), indicating a differential dependence of Tom22 and Tom20 phosphorylation on the catalytic subunits of CK2. As control, cytosolic extracts prepared from yeast strains lacking other cytosolic kinases like the MAPK Fus3, Snf1 (the yeast homolog of AMP-dependent protein kinase), or Psk2 (a PAS domain-containing kinase) were competent in phosphorylation of Tom22 and Tom20 (Figures 3D and 3E).

To probe for an interaction of Tom22 with CK2, we performed a pull-down assay with tagged $Tom22_{cd}$. Cka1 and Cka2 were found in the eluate when wild-type yeast cytosol was used (Figure S2A). However, both catalytic subunits were lacking in the eluate when *cka2Δ* cytosolic extract was used, although the extract contained wild-type levels of Cka1 (Figure S2A), indicating that the CK2 complex interacts with Tom22 via Cka2. The yield of Cka2 pull-down by Tom22 was influenced by the ability of Tom22 to be phosphorylated; the mutant form $Tom22_{cd}^{S44,46A}$ bound Cka2 with lower efficiency, and similarly, addition of ATP to the binding buffer to complete the phosphorylation reaction decreased the interaction of Tom22 with Cka2 (Figure S2B).

Phosphorylation of Tom22 Promotes Its Import and Association with Tom20

Yeast mutant cells expressing $Tom22^{S44,46A}$ instead of wild-type Tom22 from the endogenous promoter contained reduced

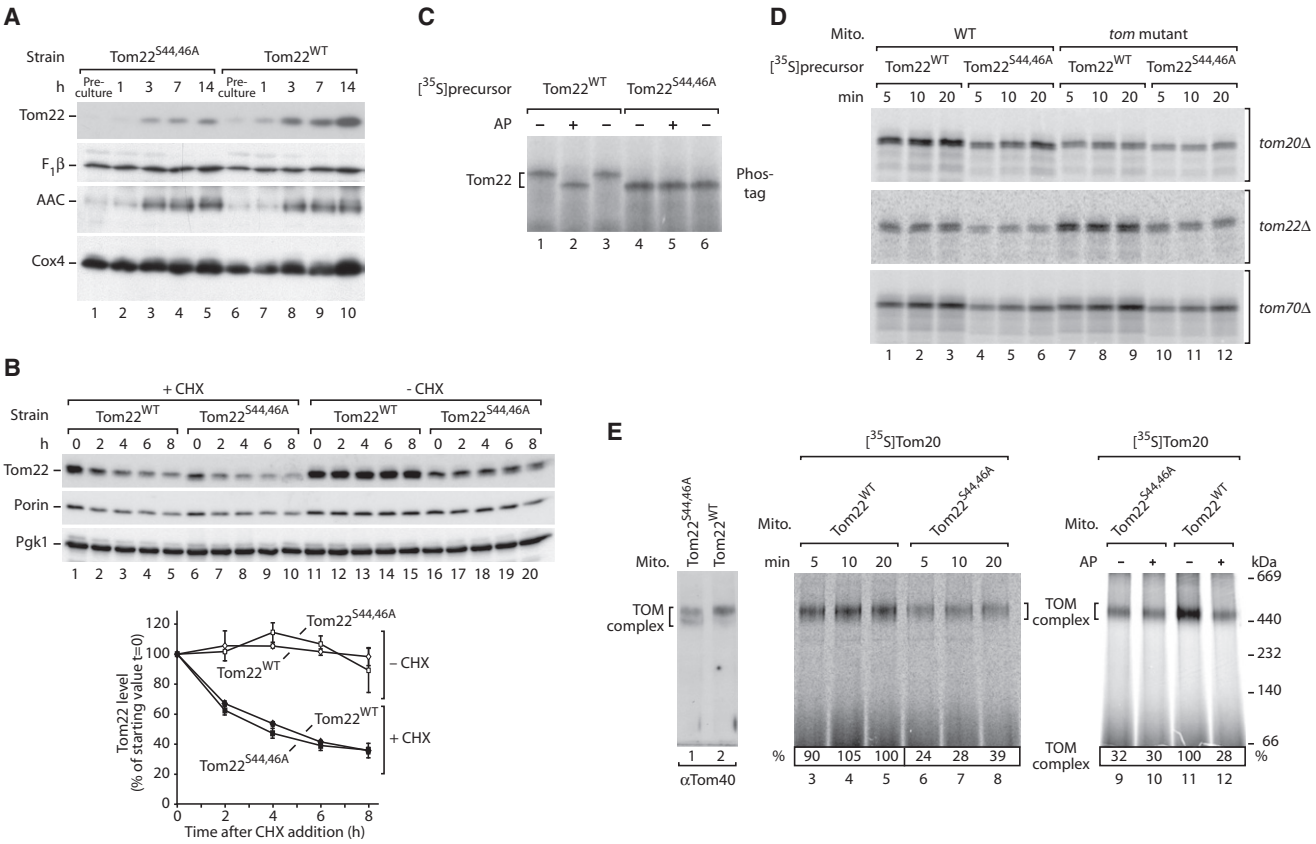

Figure 4. Phosphorylation of Tom22 by CK2 Promotes Import and Assembly

(A) Western blot of whole-yeast extract from Tom22^{WT} and Tom22^{S44,46A} strains grown from a stationary preculture to early/mid exponential growth phase at 30°C.

(B) Western blot analysis of whole-yeast extract from Tom22^{WT} and Tom22^{S44,46A} strains grown in the presence or absence of cycloheximide (CHX). Quantification: the Tom22 level at CHX addition (t = 0 hr) was set to 100% for each growth condition. Data are represented as mean ± SEM (n = 3).

(C) Tom22^{WT} and Tom22^{S44,46A} precursors were synthesized in reticulocyte lysate, treated with AP, and analyzed by Phos-tag SDS-PAGE and autoradiography.

(D) Transport of Tom22^{WT} and Tom22^{S44,46A} to mitochondria from WT, *tom20Δ*, *tom22Δ*, and *tom70Δ* yeast strains. Analysis by SDS-PAGE and autoradiography.

(E) (Left) Blue native electrophoresis and immunoblot analysis of Tom22^{WT} and Tom22^{S44,46A} mitochondria. (Middle and right) Assembly of [^{35}S]Tom20 into the TOM complex of Tom22^{WT} and Tom22^{S44,46A} mitochondria, followed by treatment with AP as indicated. Analysis by blue native electrophoresis and digital autoradiography (quantification: control WT values set to 100%).

See also Figure S3.

steady-state levels of the receptor (Figure 4A) (the antibody used recognized wild-type and mutant Tom22 with the same efficiency; Figure S3A). Possible explanations for reduced levels of Tom22 may be an impaired biogenesis or a faster turnover of the mutant protein. To test the second possibility, protein synthesis in the yeast cells was blocked by cycloheximide, and the half-life of Tom22^{WT} and Tom22^{S44,46A} was compared. Despite the lower protein level of Tom22^{S44,46A}, the rate of degradation was indistinguishable between mutant and wild-type protein (Figure 4B).

Tom22 is synthesized on cytosolic ribosomes and posttranslationally imported into mitochondria. We synthesized and ^{35}S labeled the precursor in reticulocyte lysate (lacking mitochondria). Treatment with AP quantitatively shifted Tom22^{WT} on Phos-tag SDS-PAGE, whereas the mobility of Tom22^{S44,46A} was not altered by AP yet the mutant protein migrated like AP-treated Tom22^{WT} (Figure 4C). We conclude that the precursor of Tom22 is quantitatively phosphorylated at Ser44/46 in the cytosol. We incubated radiolabeled Tom22 precursor with isolated mitochondria and observed that the association of Tom22^{S44,46A} with mitochondria was of lower efficiency compared to Tom22^{WT} (Figure 4D). To test which Tom receptor was critical for the import of phosphorylated Tom22 precursor into mitochondria, we used mitochondria from yeast strains lacking Tom20, Tom22, or Tom70. *tom20Δ* mitochondria were impaired in import of phosphorylated Tom22^{WT}, and the difference in import efficiency of Tom22^{WT} and Tom22^{S44,46A} became much smaller than with wild-type mitochondria (Figure 4D, top). In contrast, *tom22Δ* and *tom70Δ* mitochondria showed the same difference in the efficiency of importing Tom22^{WT} and Tom22^{S44,46A} as wild-type mitochondria (Figure 4D), indicating that these mutant mitochondria discriminated between phosphorylated and nonphosphorylated Tom22 precursors. We conclude that the receptor Tom20 is required for recognition and efficient import of phosphorylated Tom22.

Thus, phosphorylation of the Tom22 precursor stimulates its targeting to mitochondria.

As the levels of Tom20 were reduced in *ck2-ts* mutants (Figure 2A), we asked whether the phosphorylation of Tom20 was required for its biogenesis. Phos-tag gel analysis of yeast mitochondria, however, revealed that only a very minor fraction of Tom20 molecules was phosphorylated in contrast to Tom22 (Figure S3B, lane 1). Only upon incubation with CK2 was a larger fraction of mitochondrial Tom20 phosphorylated at residue Ser172 (Figure S3B). We generated yeast mutant strains that expressed $Tom20^{S172A}$ or $Tom20^{S172E}$ (phosphomimetic glutamate) instead of wild-type Tom20 yet did not observe any difference in the protein composition of the resulting mitochondria (Figure S3C). Import of the precursors of $Tom20^{S172A}$ and $Tom20^{S172E}$ into mitochondria was indistinguishable from that of wild-type Tom20, as analyzed by binding to mitochondria and insertion into the membranes (treatment with Na_2CO_3 at pH 11.5) (Figure S3D) and assembly into the TOM complex (Figure S3E) (in lanes 4–6 of Figure S3E, the wild-type precursor of Tom20 was treated with CK2, yet the assembly of Tom20 was not affected). Mutant mitochondria carrying $Tom20^{S172A}$ or $Tom20^{S172E}$ imported precursor proteins with wild-type efficiency both via the presequence pathway and via the carrier pathway (Figures S3F and S3G). Thus, mitochondrial Tom20 is phosphorylated by CK2 only to a low level, and replacement of the phosphorylated residue neither affects biogenesis of Tom20 nor import of precursor proteins.

Because the phosphorylation status of Tom20 itself was not responsible for the reduced levels of Tom20 in *ck2-ts* cells, we asked whether the phosphorylation status of Tom22 affected the biogenesis of Tom20. The interaction between Tom22 and Tom20 is involved at two different stages of biogenesis of the TOM complex: (1) mature Tom20 functions as receptor for the precursor of Tom22, and (2) though targeting of the precursor of Tom20 to mitochondria does not require surface receptors, the subsequent assembly of Tom20 into the TOM complex depends on the interaction with Tom22 (Meisinger et al., 2001). Blue native analysis of the TOM complex from a yeast strain containing $Tom22^{S44,46A}$ revealed a double band (Figure 4E, lane 1), the lower band migrating like a TOM complex that lacked Tom20 (Meisinger et al., 2001). We synthesized the precursor of Tom20 and imported it into mitochondria. Mitochondria from the $Tom22^{S44,46A}$ yeast strain were indeed impaired in the assembly of Tom20 into the TOM complex (Figure 4E). Remarkably, when wild-type mitochondria with assembled Tom20 were treated with AP after the import reaction, the association of Tom20 with the TOM complex was strongly reduced (Figure 4E, lane 12). In $Tom22^{S44,46A}$ mitochondria, the amount of Tom20 found on the TOM complex was already at the low level that was not further affected by dephosphorylation (Figure 4E).

Taken together, these results show that phosphorylation of Tom22 at Ser44/46 plays a dual role: it not only stimulates targeting of Tom22 to mitochondria, but also the association of Tom20 with the TOM complex.

Phosphorylation of Mim1 by CK2 Is Required for the Biogenesis of Tom Proteins

Import and assembly of the third import receptor, Tom70, was not affected in $Tom22^{S44,46A}$ mitochondria (Figure 5A), although the levels of Tom70 were reduced in *ck2-ts* yeast mutants (Figure 2A). Mitochondria isolated from *ck2-ts* yeast were impaired in the assembly of the Tom70 precursor (Figure 5B, lanes 7–9) (Tom70 is only loosely associated with the TOM complex and migrates as dimer on blue native gels; Becker et al., 2008). Import of the precursor of Tom70 does not require TOM receptors but depends on the outer-membrane protein Mim1 (Becker et al., 2008). We thus asked whether the levels of Mim1 were affected in *ck2-ts* yeast cells. Upon a short 8 hr shift of *ck2-ts* cells to nonpermissive temperature, the levels of Mim1 were partially reduced, similar to the levels of Tom22 (Figure 5C). After a 16 hr shift, the levels of Mim1 were strongly reduced (Figure 5C).

Two high-confidence phosphorylation sites were predicted in Mim1, Ser12 and Ser16, both closely matching the CK2 consensus sequence (Meggio and Pinna, 2003). On Phos-tag gels, mitochondrial Mim1 migrated in two distinct bands that shifted to a single faster-migrating band upon treatment with AP (Figure 5D). We generated a yeast strain in which both Ser12 and Ser16 were replaced by alanine; the resulting $Mim1^{S12,16A}$ migrated as a single band and was not further affected by AP. Replacement of either Ser12 or Ser16 by alanine led to bands with intermediate mobility that were sensitive to treatment with AP (Figure 5D). We conclude that mitochondrial Mim1 is phosphorylated at Ser12 and Ser16.

We tested the in vitro phosphorylation of the N-terminal domain of Mim1 fused to GST using the purified kinases CK1, CK2, PKA and CamKII. CK2 efficiently phosphorylated the Mim1 fusion protein in a site-specific manner (Ser12/Ser16) (Figure 5E). In a homologous yeast system, wild-type cytosolic extract phosphorylated the Mim1 fusion protein on Ser12/16 (Figure 5F). A cytosolic extract lacking Cka2 did not phosphorylate Mim1, whereas *cka1Δ* cytosol and mutants of further kinases phosphorylated Mim1 with wild-type efficiency (Figure 5F). Phos-tag gel analysis revealed that the precursor of Mim1 synthesized in reticulocyte lysate was partially phosphorylated at Ser12/16 (Figure 5G), indicating that full phosphorylation of Mim1 occurred at mitochondria. Taken together, we conclude that Mim1 is phosphorylated by CK2, mainly by the catalytic subunit Cka2.

We analyzed the biogenesis of Tom receptors in $Mim1^{S12,16A}$ mutant mitochondria. The assembly of the precursor of Tom70 was impaired (Figure 5H). Assembly of Tom20 into the TOM complex was also reduced in $Mim1^{S12,16A}$ mitochondria (Figure 5H). In contrast, the assembly of Tom22 into the TOM complex was not affected in the $Mim1^{S12,16A}$ mitochondria (Figure 5H), in agreement with the Mim1-independent biogenesis of Tom22 (Becker et al., 2008). Thus, replacement of the CK2 target sites Ser12/16 of Mim1 impairs biogenesis of the receptors Tom70 and Tom20.

Inactivation of CK2 Causes Defects of the Main Mitochondrial Protein Import Pathways

Do the defects of the TOM complex in *ck2-ts* mutant cells lead to an impairment of the main mitochondrial protein import pathways? To directly determine the capacity of mitochondria for importing precursor proteins, we used the in organello import assay with radiolabeled precursor proteins (Stojanovski et al., 2007). Precursor proteins destined for the two major mitochondrial import pathways were analyzed: (1) presequence pathway into the

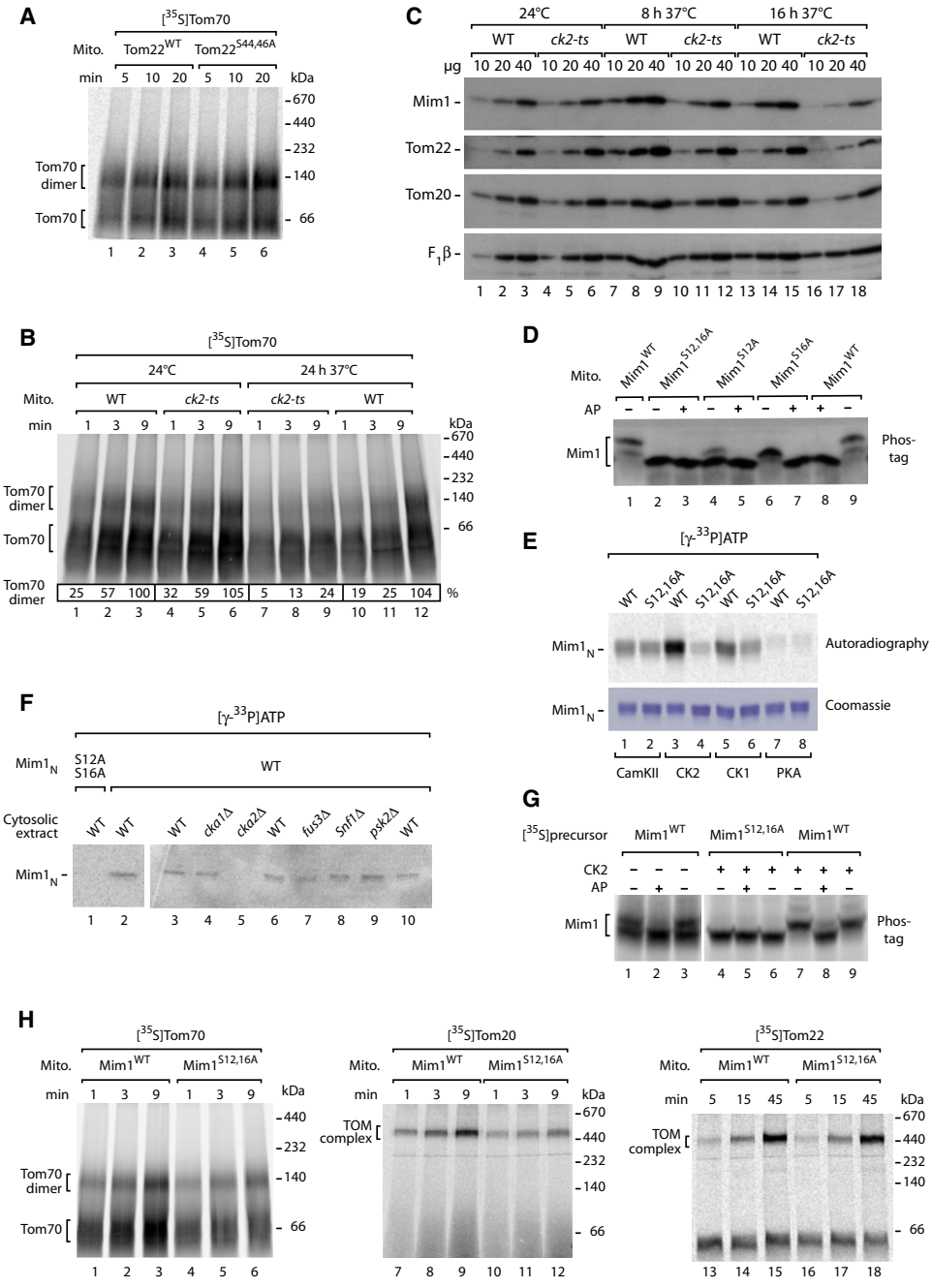

Figure 5. Phosphorylation of Mim1 by CK2 Promotes Import of Tom70 and Tom20

(A) Assembly of [^{35}S]Tom70 into Tom22^{WT} and Tom22^{S44,46A} mitochondria analyzed by blue native electrophoresis.

(B) Import and assembly of [^{35}S]Tom70 into WT and *ck2-ts* mitochondria. Quantification by digital autoradiography (control WT value [lane 3] set to 100%).

(C) Levels of mitochondrial proteins of WT and *ck2-ts* yeast cells grown at 24°C or shifted to 37°C for 8–16 hr. Analysis by SDS-PAGE and immunoblotting.

(D) Mitochondria were treated with AP and analyzed by Phos-tag SDS-PAGE and immunoblotting.

(E) Phosphorylation of Mim1$_N^{WT}$-GST and Mim1$_N^{S12,16A}$-GST with recombinant kinases.

(F) Phosphorylation of Mim1$_N^{WT}$-GST and Mim1$_N^{S12,16A}$-GST with yeast cytosolic extracts.

(G) [^{35}S]Mim1^{WT} and Mim1^{S12,16A} were synthesized in reticulocyte lysate in the presence or absence of CK2 (10 U/µl), treated with AP, and analyzed by Phos-tag SDS-PAGE.

(H) Assembly of [^{35}S]Tom70, Tom20, and Tom22 into Mim1^{WT} and Mim1^{S12,16A} mitochondria analyzed by blue native electrophoresis and autoradiography.

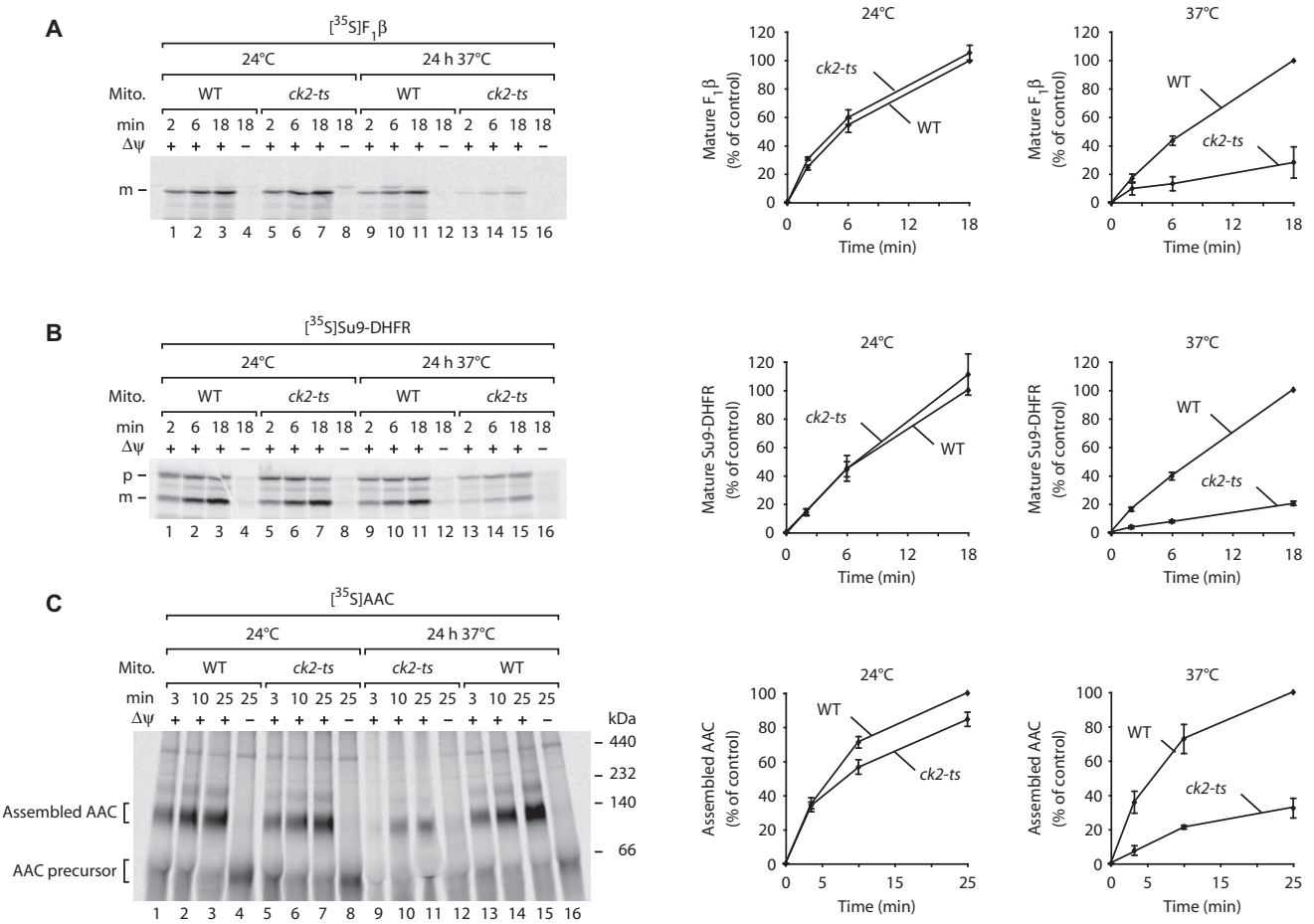

Figure 6. Inactivation of CK2 Causes Defects in Mitochondrial Protein Import

(A) WT and *ck2-ts* yeast cells were grown at 24°C and shifted to 37°C for 24 hr. [^{35}S]$F_1\beta$ was imported into mitochondria ($\Delta\psi$, membrane potential). Mitochondria were treated with proteinase K and analyzed by SDS-PAGE. p, precursor; m, mature. Quantifications are shown as mean ± SEM (n = 3). Import into WT mitochondria after the longest import time was set to 100% (control).

(B) Import of [^{35}S]Su9-DHFR was performed as described in (A).

(C) Import of [^{35}S]AAC was analyzed by blue native electrophoresis; quantification as in (A).

matrix: F_1-ATPase subunit β and the model precursor Su9-DHFR, consisting of a mitochondrial presequence and the passenger protein dihydrofolate reductase (Stojanovski et al., 2007) and (2) carrier pathway into the inner membrane with the ADP/ATP carrier (AAC) as major substrate (Wiedemann et al., 2001; Dolezal et al., 2006; Neupert and Herrmann, 2007). All three precursor proteins were efficiently imported in a membrane potential ($\Delta\psi$)-dependent manner in *ck2-ts* mitochondria under permissive conditions (Figures 6A–6C). Upon shift of the mutant cells to nonpermissive temperature for 24 hr, the resulting mitochondria were strongly impaired in import of the different precursor proteins (Figures 6A–6C). We conclude that the inactivation of CK2 leads to major defects in the biogenesis of mitochondrial proteins, both for the presequence pathway and the carrier pathway.

PKA Phosphorylates Mitochondrial Tom70 under Fermentable Conditions

To study the phosphorylation of Tom70 in vivo, we generated an antiserum that selectively recognized phospho-Ser174 and observed a specific signal of mitochondrial Tom70 from wild-type yeast, but not from mutant yeast in which Ser174 was replaced by alanine (Figure 7A, lanes 1 and 2).

PKA is a heterotetramer composed of two catalytic subunits and two regulatory subunits. In yeast, the closely related genes *TPK1*, *TPK2*, and *TPK3* encode the catalytic subunits, and *BCY1* encodes the regulatory (inhibitory) subunit (Zaman et al., 2008). Binding of cAMP to Bcy1 releases the active catalytic subunits. Yeast cytosolic extract phosphorylated the cytosolic receptor domain of yeast Tom70 at Ser174; H89 that inhibits the catalytic subunits of PKA blocked the phosphorylation (Figure 7B, top). When cytosolic extract with a low phosphorylating activity was used, the PKA activator 8Br-cAMP stimulated the phosphorylation of $Tom70_{cd}$ (Figure 7B, middle). Cytosolic extract from a yeast mutant in which the catalytic subunits were inactivated (mutant form of *TPK3* and deletion of *TPK1* and *TPK2*) was strongly impaired in phosphorylation of Tom70 (Figure 7B, bottom). Yeast PKA is activated on fermentable medium, whereas the PKA activity is low under nonfermentable

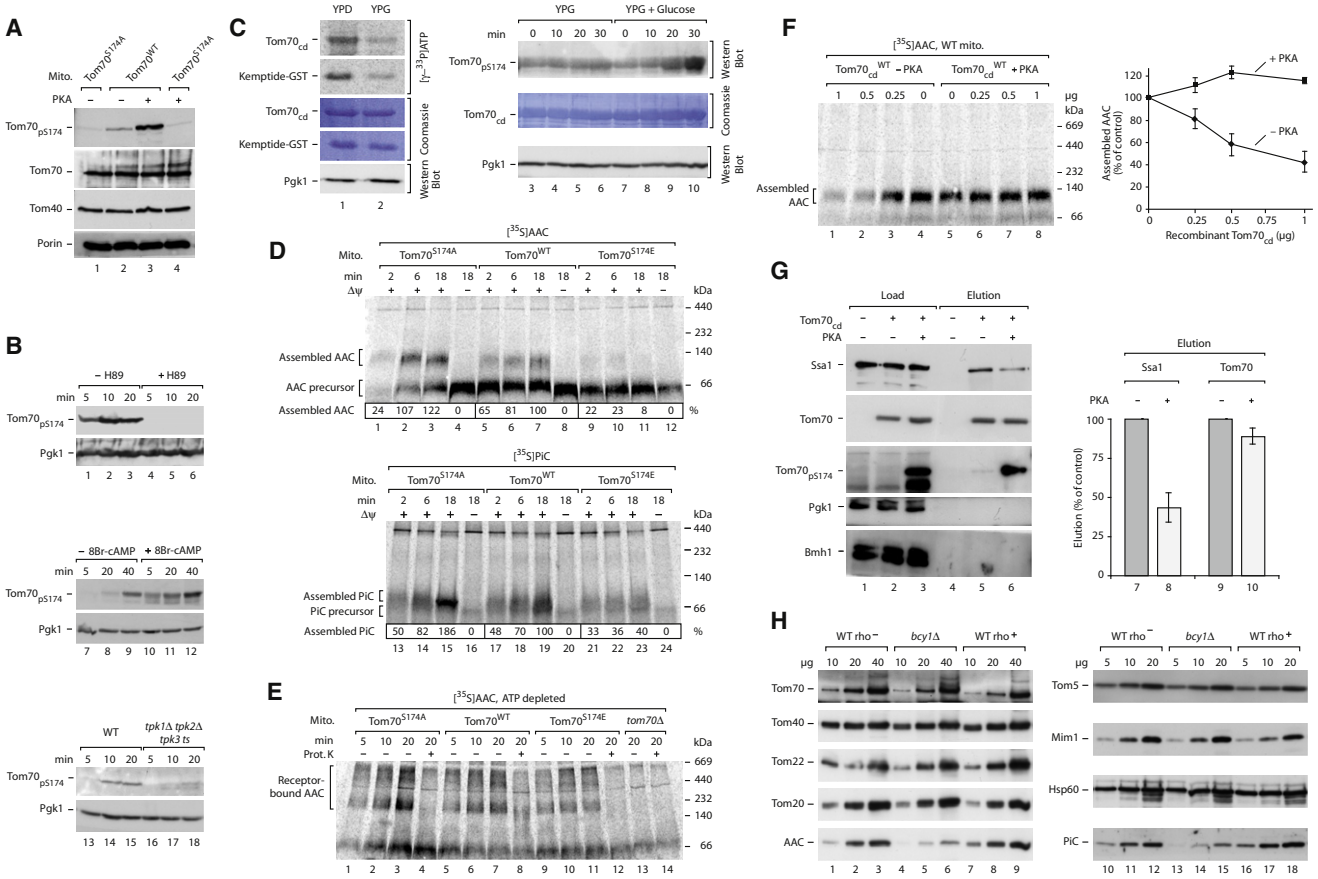

Figure 7. Phosphorylation of Tom70 by Protein Kinase A Impairs Carrier Import

(A) WT and mutant yeast strains were grown on fermentable medium, and mitochondria were isolated and treated with PKA as indicated. Analysis by SDS-PAGE and western blotting.

(B) Phosphorylation of $Tom70_{cd}$ with yeast cytosolic extract, analyzed by immunoblotting. (Lanes 1–6) Cytosolic extract of WT yeast (grown on YPD) treated with the PKA inhibitor H89 as indicated. (Lanes 7–12) Cytosolic extract of WT yeast (grown on YPG) supplemented with 8Br-cAMP as indicated. (Lanes 13–18) Cytosolic extracts of WT and PKA mutant yeast strains (grown on YPD), supplemented with 8Br-cAMP.

(C) (Left) Cytosolic extract of WT yeast grown on YPD or YPG was incubated with $Tom70_{cd}$ or Kemptide-GST in presence of [γ-^{33}P]ATP. (Right) WT yeast cells grown on YPG were incubated with glucose for 15 min as indicated before preparation of cytosolic extract, which was used for phosphorylation of $Tom70_{cd}$.

(D) [^{35}S]AAC and PiC were incubated with $Tom70^{WT}$, $Tom70^{S174A}$, and $Tom70^{S174E}$ mitochondria. Mitochondria were treated with proteinase K, lysed with digitonin, and analyzed by blue native electrophoresis (quantification: control WT values [lanes 7 and 19] set to 100%).

(E) [^{35}S]AAC was incubated with isolated mitochondria under ATP-depleted conditions, followed by treatment with proteinase K (Prot. K) as indicated. The samples were analyzed by blue native electrophoresis.

(F) Assembly of [^{35}S]AAC into WT mitochondria in the presence of soluble $Tom70_{cd}^{WT}$ (μg) that was phosphorylated by PKA prior to the import reaction as indicated. Data are represented as mean ± SEM (n = 4). Assembly in the absence of $Tom70_{cd}$ was set to 100%.

(G) Pull-down from cytosolic extract of WT yeast cells with His_{10}-tagged $Tom70_{cd}^{WT}$ that was pretreated with PKA as indicated. Ssa1, Pgk1, and Bmh1: 5% of load, 100% of ATP-elution; Tom70 and $Tom70_{pS174}$: 5% of load, 5% of imidazole-elution. Data are represented as mean ± SEM (n = 5); values without PKA were set to 100%.

(H) WT (rho$^+$), *bcy1Δ*, and WT rho$^-$ yeast strains were grown on fermentable medium, and mitochondria were analyzed by SDS-PAGE and immunoblotting. μg, mitochondrial protein loaded. See also Figure S4.

conditions (Zaman et al., 2008), shown in Figure 7C for the phosphorylation of $Tom70_{cd}$ and the PKA model substrate Kemptide. MS analyses detected phospho-Ser174 containing peptides of mitochondrial Tom70 only from cells grown on fermentable medium, but not from cells grown on nonfermentable medium (Chi et al., 2007; Albuquerque et al., 2008; Extended Experimental Procedures).

The phospho-Ser174 signal observed after growth of yeast on glucose (Figure 7A, lane 2) was strongly increased when the isolated mitochondria were treated with PKA (Figure 7A, lane 3), indicating that only a fraction of the Tom70 molecules had been phosphorylated at Ser174 in vivo. The total mitochondrial levels of Tom70, detected with Tom70-holo antiserum, were not altered by its phosphorylation status (Figure 7A). Thus, also

on a fermentable medium, only a fraction of Tom70 molecules were phosphorylated.

Phosphorylation of Tom70 Impairs Import of Mitochondrial Metabolite Carriers

For a functional analysis, we first asked whether phosphorylation of Tom70 influenced its targeting to mitochondria. We synthesized the Tom70 precursor in reticulocyte lysate and analyzed its targeting and membrane integration (resistance to extraction at pH 11.5). When Ser174 was replaced by alanine or the phosphomimetic residue glutamate, targeting and membrane insertion of Tom70 were not affected (Figure S4A); the blue native mobility of the Tom70 dimer and the TOM complex were also not altered (Figure S4B). These results indicate that neither targeting of Tom70 to mitochondria nor the oligomeric state of the TOM machinery was altered by the replacement of Ser174.

We thus asked whether the replacement of Tom70-Ser174 influenced the import of precursor proteins into mitochondria. Tom70 functions as a main receptor for noncleavable hydrophobic precursors, including the AAC, phosphate carrier (PiC), and dicarboxylate carrier (DIC) of the inner membrane (Neupert and Herrmann, 2007; Chacinska et al., 2009). Import and assembly of the ^{35}S-labeled carrier proteins to the mature dimers can be monitored by blue native electrophoresis (Wiedemann et al., 2001). Mutant mitochondria containing a glutamate instead of Tom70-Ser174 were impaired in import of the carrier proteins, whereas the replacement of Ser174 by alanine enhanced their import (Figure 7D and Figure S4C). To exclude indirect effects of the Tom70 mutants on mitochondrial protein biogenesis, we used two precursor proteins that are not imported by the Tom70-carrier pathway (Chacinska et al., 2009): the presequence-carrying model preprotein b_2-DHFR that is transported to the inner membrane in a $\Delta\psi$-dependent manner and the precursor of Tim9 that is transported into the intermembrane space. Both precursor proteins were efficiently imported into the Tom70-Ser174 mutant mitochondria (Figures S4D and S4E). These results indicate that replacement of Tom70-Ser174 by a phosphomimetic residue diminishes the efficiency of protein import via the carrier pathway, whereas replacement by a nonphosphorylatable residue enhances the import efficiency. In wild-type mitochondria, a fraction of Tom70 molecules is phosphorylated, leading to an intermediate import efficiency.

The in organello binding of carrier precursors to Tom70 can be visualized by blue native electrophoresis when [^{35}S]AAC is accumulated at the outer membrane of mitochondria upon depletion of ATP (Wiedemann et al., 2001). The precursor proteins are observed in high molecular mass complexes in a Tom70-dependent manner and are accessible to externally added protease (Figure 7E). Replacement of Tom70-Ser174 by alanine enhanced the binding, whereas the replacement by glutamate diminished the binding (Figure 7E). To obtain further evidence that the phosphorylation status of Tom70 is important for precursor binding, we used a competition assay with the soluble cytosolic receptor domain. $Tom70_{cd}$ added to in organello import assays has been shown to bind AAC precursors and thus to compete with their import into mitochondria (Brix et al., 2000). The wild-type form of recombinant $Tom70_{cd}$, which is not phosphorylated upon synthesis in *E. coli*, competed with AAC import into mitochondria, as did recombinant $Tom70_{cd}^{S174A}$ (Figure S4F). The phosphomimetic mutant form $Tom70_{cd}^{S174E}$ did not affect the import of AAC (Figure S4F). To obtain direct evidence that phosphorylation of Tom70 by PKA impairs precursor binding, we phosphorylated $Tom70_{cd}$ by PKA before the competition assay. Indeed, PKA-treated $Tom70_{cd}$ did not compete for the import of AAC, in contrast to nonphosphorylated $Tom70_{cd}$ (Figure 7F).

Carrier proteins are delivered to Tom70 in a complex with the cytosolic chaperone Hsp70 that specifically binds to Tom70 (Young et al., 2003). Ser174 is located in close proximity to the Hsp70-binding pocket of Tom70 (Li et al., 2009). As the negatively charged C-terminal EEVD motif of Hsp70 is involved in binding to Tom70 (Li et al., 2009), we hypothesized that introduction of negative charge by phosphorylation might interfere with Hsp70 binding. We incubated yeast cytosol with tagged $Tom70_{cd}$ and observed binding of Hsp70 (Ssa1) to the receptor, but not of control proteins like Pgk1 or Bmh1 (Figure 7G). Phosphorylation of $Tom70_{cd}$ by PKA diminished the binding of Ssa1 to Tom70 (Figure 7G). We conclude that PKA-mediated phosphorylation of Tom70 at Ser174 impairs the activity of the receptor for binding of Hsp70 and import of carrier precursors.

In yeast lacking the regulatory subunit Bcy1, the activity of PKA is not controlled anymore by cAMP levels. *bcy1*Δ cells are unable to grow on nonfermentable medium (Zaman et al., 2008). We compared the levels of mitochondrial proteins from *bcy1*Δ cells to that of wild-type cells. The levels of TOM proteins, as well as of Mim1 and the control matrix protein Hsp60, were not or were only moderately affected in *bcy1*Δ cells (Figure 7H). However, the levels of AAC and PiC were considerably decreased in *bcy1*Δ cells (Figure 7H). To exclude that decreased levels of carrier proteins were a general phenotype of strains that cannot grow on nonfermentable medium, we also analyzed mitochondrial protein levels of a rho^- strain that lacks functional mitochondrial DNA (but contains the subunits of PKA). The levels of the carrier proteins in the rho^- cells were close to that of the rho^+ cells (Figure 7H). In agreement with the in organello studies, the in vivo results indicate that activation of PKA exerts a negative effect on the levels of mitochondrial metabolite carriers.

DISCUSSION

The TOM complex is essential for the import of most mitochondrial proteins from the cytosol. Though numerous studies have investigated the cytosolic-mitochondrial network in apoptosis and information on the regulation of mitochondrial morphology is increasing (Desagher et al., 2001; Pagliarini and Dixon, 2006; Soubannier and McBride, 2009), little has been known about the regulation of mitochondrial protein import, and posttranslational regulatory mechanisms acting at the preprotein translocases of the mitochondrial membranes have been unknown. We report that the TOM complex is targeted by cytosolic kinases. CK2 and PKA differentially regulate biogenesis and function of the TOM complex and thus control mitochondrial protein homeostasis.

CK2 plays important roles in the regulation of numerous cellular processes, including gene expression, cell-cycle progression, cell polarity, and ion homeostasis; roles of CK2 in protection from apoptosis and phosphorylation of enzymes

involved in mitochondrial lipid metabolism have also been reported (Hanna et al., 1995; Desagher et al., 2001; Meggio and Pinna, 2003; Onorato et al., 2005; Poole et al., 2005; Tripodi et al., 2010). We found that CK2 phosphorylates two key components required for the biogenesis of the TOM complex, Tom22 and Mim1, and thus *ck2* mutant yeast cells contain strongly reduced levels of the TOM complex. (1) The precursor of Tom22 is quantitatively phosphorylated by CK2 in the cytosol. Phosphorylation of Ser44/46 stimulates targeting of the precursor to mitochondria. The mechanistic basis is that the interaction of Tom22 with its import receptor Tom20 is enhanced by the phosphorylation. The phosphorylation-stimulated interaction of Tom22 and Tom20 serves a dual function in TOM biogenesis: it is not only required for importing the precursor of Tom22, but also for the assembly of imported Tom20 into the TOM complex. (2) The outer-membrane protein Mim1 is not a structural part of the TOM complex but transiently interacts with the complex and is required for the import of several Tom proteins, including the precursors of Tom70, Tom20, and small Tom proteins, but not for the import of Tom22 (Chacinska et al., 2009). CK2 efficiently phosphorylates Mim1, and the levels of Mim1 are reduced in *ck2* mutant yeast. Mitochondria containing a mutant form of Mim1 that cannot be phosphorylated by CK2 are impaired in the import of Tom70 and Tom20.

Taken together, CK2 controls the biogenesis of all TOM receptors: Tom22 by direct phosphorylation, Tom70 via phosphorylation of Mim1, and Tom20 via Mim1 and Tom22. The strong reduction in the levels of all three TOM receptors, in turn, leads to further defects, of particular importance being the biogenesis of the import channel Tom40 that involves all three receptors, Tom20, Tom22, and Tom70 (Chacinska et al., 2009). The low TOM levels then cause major defects in mitochondrial biogenesis, including the main protein import pathways, presequence pathway and carrier pathway, that strictly depend on the TOM complex. CK2 is constitutively active, though its activity for specific substrates may be modulated by a regulation of copy number, subcellular localization, conformational changes, and interaction partners (Poole et al., 2005). It has been observed that the activity of CK2 is higher in rapidly growing cells, both in mammals and yeast, i.e., under conditions in which more mitochondria are needed (Meggio and Pinna, 2003; Tripodi et al., 2010). By phosphorylation of Tom22 and Mim1, CK2 plays an important stimulatory role in the biogenesis of the TOM complex. CK2 thus controls the levels of the main mitochondrial entry gate, suggesting a role of CK2 in promoting biogenesis of mitochondria.

In contrast to the stimulatory role of CK2 on mitochondrial biogenesis, PKA impairs the receptor activity of Tom70. Addition of glucose to yeast cells leads to an activation of this cAMP-dependent protein kinase (Zaman et al., 2008). PKA has been found in association with different cell organelles and has been found to affect mitochondrial morphology, oxidative metabolism, and apoptosis; cAMP-dependent phosphorylation of a few nuclear-encoded proteins was shown to modulate their interaction with Hsp70 chaperones and transfer into mitochondria (Robin et al., 2003; Pagliarini and Dixon, 2006; de Rasmo et al., 2008; Soubannier and McBride, 2009). The identification of Tom70 as a target of PKA reveals that PKA not only affects mitochondrial import via phosphorylation of a small set of precursor proteins, but also exerts a direct regulatory effect on the TOM machinery. Phosphorylation of Tom70 by PKA impairs its receptor activity by diminishing the interaction with cytosolic Hsp70 and the import of hydrophobic precursors such as the inner-membrane metabolite carriers. On nonfermentable medium, cells depend on respiration, and thus a high activity of mitochondria is required, including an increased need for exchange of metabolites between mitochondria and the rest of the cell. Under these conditions, Tom70 is not phosphorylated by PKA and can exert its full receptor activity. When shifting to growth on glucose (fermentable medium), metabolite exchange between mitochondria and the rest of the cell is diminished, and fewer metabolite carrier proteins are needed; a fraction of Tom70 molecules becomes phosphorylated and is impaired in the import of metabolite carriers. The phosphorylation by PKA thus serves as a regulatory means to adjust the import rate of metabolite carriers to the metabolic state of the cell.

In summary, the protein import machinery of the mitochondrial outer membrane is regulated by cytosolic kinases at several levels, TOM biogenesis and receptor activity, and under different growth conditions. The regulatory effects of CK2 and PKA on the TOM receptors demonstrate that the main mitochondrial entry gate for preproteins is not functioning autonomously but is embedded into cellular signaling networks. These findings reveal a posttranslational mechanism for controlling mitochondrial preprotein translocases.

EXPERIMENTAL PROCEDURES

Analysis of Yeast Strains

S. cerevisiae cells were grown on YPD (2% glucose), YPG (3% glycerol), or synthetic medium (−Trp; 3% glycerol) at 23°C –37°C. Mitochondria were isolated by differential and gradient centrifugation. Yeast strains expressing $Tom22^{WT}$ or $Tom22^{S44,46A}$ from the authentic promoter were inoculated from an overnight preculture to an OD_{600nm} of 0.1 in YPG and were grown at 30°C. For analysis of protein turnover, cycloheximide (50 μg/ml) was added 3 hr after inoculation. YPH250 (parental strain), YDH6 (WT, *cka1::HIS3 cka2::TRP1* + p*(CEN6/ARSH4 LEU2 CKA2)*), and YDH13 (*ck2-ts*, *cka1::HIS3 cka2::TRP1* + p*(CEN6/ARSH4 LEU2 cka2–13)*) (Hanna et al., 1995) were inoculated to an OD_{600nm} of 0.2 in YPG and were grown for 2 hr at 24°C, followed by an incubation at 24°C or 37°C for 24 hr. Samples were taken, and whole-cell extracts were generated by precipitation with trichloroacetic acid or postalkaline extraction and analyzed by SDS-PAGE and western blotting. See Extended Experimental Procedures.

In Vitro and In Organello Phosphorylation

Substrate proteins were expressed in *E. coli* and were purified. In vitro phosphorylation with recombinant protein kinases (CK2, CK1, PKA, CamKII, p42 MAPK, CDK1-cyclinB, CDK2-cyclinA, and GSK3; New England Biolabs) was done in the presence of [γ-^{33}P]ATP (Perkin Elmer). For generation of cytosolic extract, yeast cells were treated with zymolyase 20T and lysed with glass beads; cell debris was removed by centrifugation, and the protein content was adjusted by absorbance at 280 nm. The cytosolic extract was supplemented with ATP and mixed with recombinant protein or isolated mitochondria. Phosphorylation was detected by digital autoradiography or western blotting with phosphosite-specific antibodies.

Alkaline Phosphatase Treatment and Phosphate-Affinity SDS-PAGE

Mitochondria were resuspended in ST buffer (10 mM Tris/HCl [pH 7.2], 250 mM sucrose, 2 mM $MgCl_2$, and 2 mM PMSF) to a protein concentration of 1 mg/ml, and 1 U AP per 5 μg mitochondrial protein was added. Samples

were incubated for 30 min at 25°C and mild shaking. Mitochondria were washed, lysed, and subjected to SDS-PAGE or Phos-tag electrophoresis (Kinoshita et al., 2006) on 12.5%–15% acrylamide gels containing 50–100 μM Phos-tag and 100–200 μM $MnCl_2$.

Protein Import Assays

[^{35}S]Methionine-labeled precursor proteins were synthesized in rabbit reticulocyte lysate, and levels were adjusted as necessary. Precursors were incubated with isolated mitochondria in import buffer (250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM methionine, 3% (w/v) fatty acid-free bovine serum albumin, and 10 mM MOPS/KOH [pH 7.2]) with 2 mM ATP and 2–3 mM NADH (Stojanovski et al., 2007). Mitochondria were washed, treated with proteinase K or Na_2CO_3, and analyzed by SDS-PAGE or blue native electrophoresis and digital autoradiography. For competition assays (Brix et al., 2000), recombinant $Tom70_{cd}$ was phosphorylated with PKA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and one table and can be found with this article online at doi:10.1016/j.cell.2010.12.015.

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