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Short Article

Glucose-Induced Regulation of Protein Import Receptor Tom22 by Cytosolic and Mitochondria-Bound Kinases

Carolin Gerbeth,[1,2,3,7] Oliver Schmidt,[1,3,4,7,8] Sanjana Rao,[1,3,5,9] Angelika B. Harbauer,[1,2,3,4] Despina Mikropoulou,[1,2,3] Magdalena Opalińska,[1] Bernard Guiard,[6] Nikolaus Pfanner,[1,4,*] and Chris Meisinger[1,4,*]
[1]Institut für Biochemie und Molekularbiologie, ZBMZ
[2]Trinationales Graduiertenkolleg 1478
[3]Faculty of Biology
[4]BIOSS Centre for Biological Signalling Studies
[5]Spemann Graduate School of Biology and Medicine
Universität Freiburg, 79104 Freiburg, Germany
[6]Centre de Génétique Moléculaire, CNRS, 91190 Gif-sur-Yvette, France
[7]These authors contributed equally to this work
[8]Present address: Division of Cell Biology, Biocenter, Medical University Innsbruck, 6020 Innsbruck, Austria
[9]Present address: Institute of Biochemistry, ETH Zürich, 8093 Zürich, Switzerland
*Correspondence: nikolaus.pfanner@biochemie.uni-freiburg.de (N.P.), chris.meisinger@biochemie.uni-freiburg.de (C.M.)
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SUMMARY

Most mitochondrial proteins are imported by the translocase of the outer mitochondrial membrane (TOM). Tom22 functions as central receptor and transfers preproteins to the import pore. Casein kinase 2 (CK2) constitutively phosphorylates the cytosolic precursor of Tom22 at Ser44 and Ser46 and, thus, promotes its import. It is unknown whether Tom22 is regulated under different metabolic conditions. We report that CK1, which is involved in glucose-induced signal transduction, is bound to mitochondria. CK1 phosphorylates Tom22 at Thr57 and stimulates the assembly of Tom22 and Tom20. In contrast, protein kinase A (PKA), which is also activated by the addition of glucose, phosphorylates the precursor of Tom22 at Thr76 and impairs its import. Thus, PKA functions in an opposite manner to CK1 and CK2. Our results reveal that three kinases regulate the import and assembly of Tom22, demonstrating that the central receptor is a major target for the posttranslational regulation of mitochondrial protein import.

INTRODUCTION

Mitochondria play crucial roles in cellular energy conversion, metabolic pathways, and the regulation of apoptosis. They import more than 1,000 different precursor proteins from the cytosol (Dolezal et al., 2006; Neupert and Herrmann, 2007; Chacinska et al., 2009; Endo and Yamano, 2010). Most proteins are imported by the main protein entry gate of mitochondria, the translocase of the outer membrane (TOM), and use distinct sorting pathways to their intramitochondrial destinations. The two main sorting pathways are the presequence pathway and the carrier pathway. Precursors with N-terminal cleavable targeting signals (presequences) are transferred from the TOM complex to the presequence translocase of the inner membrane (TIM23 complex), whereas noncleavable hydrophobic precursors with internal targeting signals use the carrier translocase of the inner membrane (TIM22 complex).

The TOM complex consists of the channel-forming protein Tom40, three receptor proteins that expose domains to the cytosol, and three small proteins involved in the stability of the complex (Meisinger et al., 2001; Neupert and Herrmann, 2007; Endo and Yamano, 2010; Dukanovic and Rapaport, 2011). The precursor proteins are initially recognized by the receptors Tom20 and Tom70, transferred to the central receptor Tom22, and translocated across the outer membrane by Tom40.

Little is known about the regulation of the mitochondrial protein import machinery. The functional implications of the phosphorylation of mitochondrial proteins have only been studied for a subset of proteins, including regulatory processes in mitochondrial energy metabolism, membrane morphology, preprotein transfer, turnover, and apoptosis (Antico Arciuch et al., 2009; Soubannier and McBride, 2009; Avadhani et al., 2011; Narendra and Youle, 2011). Recent studies showed that the TOM complex is regulated by two kinases, casein kinase 2 (CK2) and protein kinase A (PKA). CK2 phosphorylates the cytosolic precursor of Tom22 and stimulates its import into mitochondria (Schmidt et al., 2011; Rao et al., 2011). PKA is activated under fermentable growth conditions (Zaman et al., 2008). The cytosolic form of PKA exerts an inhibitory effect on the TOM machinery by impairing the receptor activity of Tom70 and diminishing the import of Tom40 into mitochondria (Schmidt et al., 2011; Rao et al., 2012).

Here, we asked whether the regulation of the TOM machinery is limited to cytosolic phosphorylation events. We report that CK1 is bound not only to the plasma membrane (Zaman et al., 2008; Roth et al., 2011) but also to the outer mitochondrial membrane. CK1 phosphorylates mitochondria-bound Tom22

and promotes its assembly. Moreover, PKA phosphorylates the precursor of Tom22 and impairs its import into mitochondria. Thus, Tom22 is regulated by stimulatory kinases as well as inhibitory kinases.

RESULTS AND DISCUSSION

CK1 Is Bound to the Mitochondrial Outer Membrane and Phosphorylates Tom22

A large-scale proteomics analysis of purified yeast mitochondria yielded peptides corresponding to Yck1 (Reinders et al., 2006). Yck1 and its homolog Yck2 are redundant isoforms of yeast CK1, are involved in glucose-induced signal transduction, and have been shown to be localized to the plasma membrane by palmitoylation (Moriya and Johnston, 2004; Zaman et al., 2008; Roth et al., 2011; Reddi and Culotta, 2013). We detected full-length Yck1 and Yck2 in highly purified mitochondrial preparations, whereas subunits of PKA (Tpk1 and Bcy1) and CK2 (Cka1 and Cka2) were not found in the mitochondrial fraction (Figure 1A). Yck1 and Yck2 were accessible to protease added to mitochondria (Figure 1B), yet neither released from the membranes at increasing salt concentrations nor alkaline pH (Figure 1C), indicating that they are stably anchored to the outer membrane and exposed to the cytosol.

We used a conditional *ck1-ts* yeast mutant, which is defective in the chromosomal *YCK1* and *YCK2* genes and expresses a temperature-sensitive allele of *YCK2* from a plasmid (Vancura et al., 1994). Highly pure mitochondria were isolated from cells grown under permissive conditions and incubated with [γ-^{33}P]ATP. Two labeled bands in the 20–25 kDa range were weaker in *ck1-ts* mitochondria and virtually absent when the mitochondria were subjected to a heat shock before the labeling assay (Figure 1D). In vitro screening assays with recombinant kinases raised the possibility that Tom20 and Tom22 are targets not only of CK2 but also CK1 (Schmidt et al., 2011). Thus, we tested whether Tom20 and Tom22 were candidates for the *ck1-ts*-sensitive bands.

We incubated full-length Tom22 with isolated mitochondria and [γ-^{33}P]ATP. With *ck1-ts* mutant mitochondria, the phosphorylation of wild-type (WT) Tom22 was partially reduced, and that of Tom22^{S44,46A}, which lacks the CK2 target residues Ser44 and Ser46 (Schmidt et al., 2011), was strongly inhibited (Figure 1E, left). To identify the CK1 target residue, we generated Tom22 triple mutants in which individual serines or threonines were replaced by alanine in a Ser44,46Ala background. The replacement of Thr57 blocked the phosphorylation by mitochondria, whereas the replacement of other residues did not affect the *ck1*-sensitive phosphorylation (Figure 1E; Figure S1A available online). We analyzed *tom22* mutant mitochondria using phosphate affinity (Phos-tag) SDS-PAGE that retards the gel mobility of phosphorylated proteins (Figure S1B) (Kinoshita et al., 2006). The phosphorylation of Thr57 was blocked in *ck1-ts* mitochondria (Figure 1F).

The phosphorylation of Tom22-Thr57 was considerably increased upon cell growth on glucose-containing medium in comparison to growth on nonfermentable medium (Figure 1G). The half-life of TOM subunits was similar upon growth on glucose and glycerol (~4 hr; Supplemental Experimental Procedures) (Schmidt et al., 2011), indicating that the degradation of TOM subunits was not altered. The mitochondrial levels of Yck1 and Yck2 were increased upon cell growth on glucose (Figure 1H). Yeast mutants lacking the palmitoyl transferase Akr1 (Figure S1C) (Roth et al., 2011; Reddi and Culotta, 2013) were strongly inhibited in the recruitment of Yck1 and Yck2 to mitochondria and the phosphorylation of Thr57 (Figure 1I), indicating that palmitoylation is required for the interaction of CK1 with mitochondria. We conclude that mitochondrial CK1 phosphorylates the cytosolic receptor domain of Tom22 at Thr57. Under fermentative growth conditions, the mitochondria-bound levels of CK1 and Thr57 phosphorylation of Tom22 are increased.

Ser172 of the cytosolic domain of Tom20 is phosphorylated by CK2 and possibly also CK1 (Schmidt et al., 2011). We analyzed the CK1-mediated phosphorylation of Tom20-Ser172 by a gel mobility shift and recognition by an antiserum specific for phospho-Ser172; phosphorylation was blocked when Ser172 was replaced by alanine (Figure S1D). Only a small fraction of Tom20 was phosphorylated by CK1. Given that yeast mutants containing Tom20^{S172A} or Tom20^{S172E} (phospho-mimetic glutamate) show a normal mitochondrial protein composition, TOM assembly and protein import (Schmidt et al., 2011), neither the phosphorylation of Tom20 by CK2 nor by CK1 reveals an effect on mitochondrial biogenesis. Thus, we focused our analysis on the phosphorylation of Tom22.

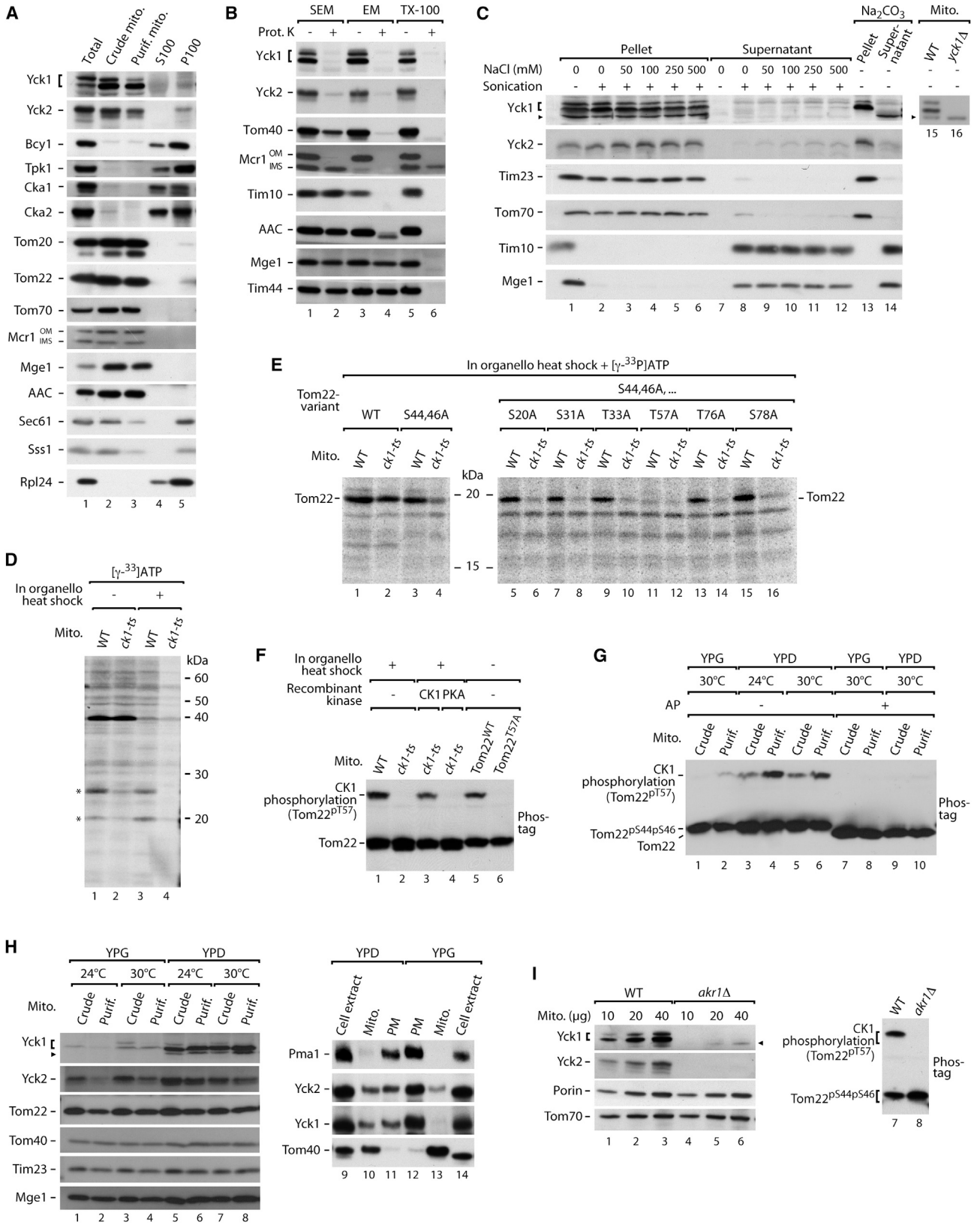
Phosphorylation by CK1 Promotes Assembly of Tom22

The TOM proteins are synthesized as precursors in the cytosol and imported into mitochondria (Chacinska et al., 2009; Dukanovic and Rapaport, 2011). We synthesized [^{35}S]Tom22 precursor in an import-competent form in reticulocyte lysate (Stojanovski et al., 2007); however, we did not detect a Thr57-dependent phosphorylation of the precursor, nor did we detect it after the addition of CK1 to the reticulocyte lysate (Figure 2A). However, upon import into mitochondria, Thr57 phosphorylation of the [^{35}S]Tom22 precursor was observed (Figure 2B), indicating that Tom22 is not phosphorylated by CK1 in the cytosol but is phosphorylated after import into mitochondria.

The assembly of Tom22 into the TOM complex of ~450 kDa can be monitored by blue native electrophoresis of digitonin-lysed mitochondria. The prevention of Thr57 phosphorylation (Tom22^{T57A}) significantly inhibited the assembly of Tom22 (Figure 2C), although only a fraction of Tom22 molecules were observed in the Thr57-phospho form (Figure 2B). We found that mitochondrial Tom22^{pT57} was dephosphorylated by incubation with cytosolic extract (Figure S2A), indicating that a cytosolic phosphatase activity dephosphorylates Thr57.

The phosphorylation of Thr57 of imported Tom22 and the assembly of Tom22 into the TOM complex were increased in mitochondria from glucose-grown cells in comparison to mitochondria from glycerol-grown cells (Figures 2D and S2B). Given that, for both mitochondria, the same [^{35}S]Tom22 WT precursor synthesized in reticulocyte lysate was used, these findings support the conclusion that mitochondria-bound factors are critical for Thr57 phosphorylation of Tom22 (whereas cytosolic factors promote its dephosphorylation).

To directly demonstrate that CK1 was required for the assembly of Tom22, we used mitochondria isolated from *ck1-ts* cells and observed an impaired assembly (Figure 2E). In order to



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analyze the effects in vivo, the protein levels were analyzed. After shifting *ck1-ts* cells to nonpermissive conditions (37°C) for 12 hr, mainly the protein levels of Tom22 and Tom20 were reduced. After a longer shift (22 hr), the levels of additional TOM proteins, Tom40 and small Tom proteins, were also considerably reduced (Figure 2F). Altogether, we conclude that the phosphorylation of Thr57 by mitochondria-bound CK1 promotes the efficient assembly of Tom22 into the TOM complex.

Phosphorylation of Tom22 by CK1 Promotes Assembly of Tom20 and Protein Import into Mitochondria

We asked whether the CK1-mediated phosphorylation of Tom22-Thr57 was involved in the biogenesis of Tom20. In order to monitor integration into the lipid phase of the membrane, the translocation of [^{35}S]Tom20 into the outer membrane was analyzed by several approaches (Figure 3A): (1) binding to mitochondria, (2) formation of characteristic fragments of imported Tom20 by added protease, and (3) resistance to alkaline extraction (Schmidt et al., 2011). None of the assays revealed a defect in the translocation of Tom20 into Tom22^{T57A} mitochondria. Tom22 is required for the assembly of Tom20 into the TOM complex (Meisinger et al., 2001; Endo and Yamano, 2010; Schmidt et al., 2011). The assembly of Tom20 was impaired in Tom22^{T57A} mitochondria (Figure 3B; [^{35}S]Tom20 and western blot analysis). The phosphorylation of Tom22 at Ser44,46 by CK2 also promotes the assembly of Tom20 (Schmidt et al., 2011). Tom22^{S44,46A,T57A} triple mutant mitochondria were blocked in the blue native-stable assembly of Tom20 (Figure 3B). We conclude that both the CK1 target Thr57 and the CK2 target Ser44,46 of Tom22 are important for the recruitment and assembly of Tom20 into the TOM complex.

We asked whether the inhibition of Tom22-Thr57 phosphorylation impaired protein import into mitochondria. To analyze Thr57-specific effects, we used a yeast strain in which the constitutively phosphorylated Ser44 and Ser46 were replaced by alanine (Figure 3C). We accumulated a presequence-carrying preprotein (Oxa1) and a carrier precursor (AAC carrying a folded passenger protein) (Meisinger et al., 2001) in the TOM complex. The yield of precursor accumulation was considerably decreased when Thr57 was replaced by alanine. In agreement with these findings, the import of presequence-carrying matrix-targeted preproteins (F_1-ATPase subunit β and Su9-DHFR) and AAC into *ck1-ts* mitochondria was strongly inhibited (Figures 3D and 3E), demonstrating that the main mitochondrial protein import pathways were impaired. Upon the shifting of *ck1-ts* cells to nonpermissive conditions, the mitochondrial morphology was also altered, leading to an increased fragmentation of mitochondria (Figure S3). These findings indicate that CK1 plays a major role in the biogenesis and dynamics of mitochondria.

Phosphorylation by PKA Impairs Import of Tom22 into Mitochondria

PKA has been observed as a soluble cytosolic form and in association with cell organelles and can affect mitochondrial energy metabolism, morphology, protein transfer, and apoptosis (Antico Arciuch et al., 2009; Soubannier and McBride, 2009; Avadhani et al., 2011; Schmidt et al., 2011; Rao et al., 2012). In vitro screening suggested Thr76 of Tom22 as a potential target of PKA (Schmidt et al., 2011). We demonstrated the phosphorylation of Tom22-Thr76 by mammalian PKA (Figure 4A), purified yeast PKA (activated by 8Br-cAMP), and yeast cytosolic extract (Figure 4B). In yeast mutants lacking the inhibitory subunit Bcy1, PKA is constitutively active (Zaman et al., 2008). We grew the cells on sucrose-containing medium and compared them to rho$^+$ and rho$^-$ WT cells. We observed not only reduced levels of the PKA target Tom40 in *bcy1*Δ cells (Rao et al., 2012) but also a considerable reduction of the levels of Tom22 (Figure 4C). The levels of the TOM complex were similarly reduced (Figure 4C, right). To directly analyze whether PKA-mediated phosphorylation of Tom22 affected its biogenesis, we synthesized mutant forms of the Tom22 precursor. The assembly of Tom22^{T76A} into the TOM complex proceeded with similar or slightly better efficiency than that of the WT precursor, whereas the assembly

Figure 1. CK1 Is Bound to the Mitochondrial Outer Membrane and Phosphorylates Tom22 at Thr57

(A) Subcellular fractionation of wild-type (WT) yeast cells with differential centrifugation analyzed by SDS-PAGE and immunoblotting. Purif. mito., sucrose-gradient-purified mitochondria; S100, cytosol-containing fraction; P100, microsomal fraction. Mcr1 (outer membrane and intermembrane space forms), Mge1 (matrix), and AAC (inner membrane) are shown as mitochondrial marker proteins. Sec61 and Sss1, ER proteins; Rpl24, ribosomal subunit.

(B) WT mitochondria were treated with iso-osmotic (SEM) or hypo-osmotic (EM) buffers or lysed with Triton X-100 (TX-100). Samples were treated with proteinase K (Prot. K) as indicated and separated by SDS-PAGE.

(C) Mitochondria were sonicated in the presence of NaCl or treated with Na_2CO_3. Pellet and supernatant fractions were separated by SDS-PAGE. Arrowhead, nonspecific band.

(D) In organello phosphorylation assay. WT and *ck1-ts* mitochondria were preincubated for 15 min at 37°C where indicated (+ heat shock). After incubation with [γ-^{33}P]ATP at 25°C, phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. *, *ck1-ts*-sensitive bands in the 20–25 kDa range.

(E) Phosphorylation of cell-free synthesized Tom22 variants (wheat germ lysate) by incubation with isolated WT or *ck1-ts* mitochondria and [γ-^{33}P]ATP after 15 min heat shock at 37°C. Supernatants were separated from the mitochondrial pellets by centrifugation and subjected to SDS-PAGE and autoradiography (the intensity of the left panel is reduced for a better comparison of mutant forms).

(F) Mitochondria were incubated with ATP in the presence or absence of purified mammalian CK1 or PKA. Where indicated, mitochondria were subjected to a prior heat shock at 37°C for 15 min. Samples were subjected to Phos-tag SDS-PAGE followed by immunoblotting with Tom22-specific antibody.

(G) Mitochondria isolated from WT yeast, grown on YPG or YPD at indicated temperatures were incubated with ATP and treated with alkaline phosphatase (AP) where indicated. The phosphorylation of Tom22 at Thr57, analyzed as in (F), was ~2.5-fold increased in YPD versus YPG.

(H) Crude and sucrose-gradient-purified mitochondria (left) as well as cell extracts, mitochondria, and plasma membrane fractions (PM) obtained from sucrose gradient preparations of cryogenic grinded WT yeast (right) grown on YPG or YPD were analyzed by SDS-PAGE and immunoblotting. Cell extract, 0.25% of total extract; Pma1, plasma membrane ATPase; arrowhead, nonspecific band.

(I) Mitochondria isolated from WT, and *akr1*Δ yeast were subjected to SDS-PAGE (left) or treated with ATP and analyzed by Phos-tag SDS-PAGE (right) and immunoblotting. Arrowhead, nonspecific band.

See also Figure S1.

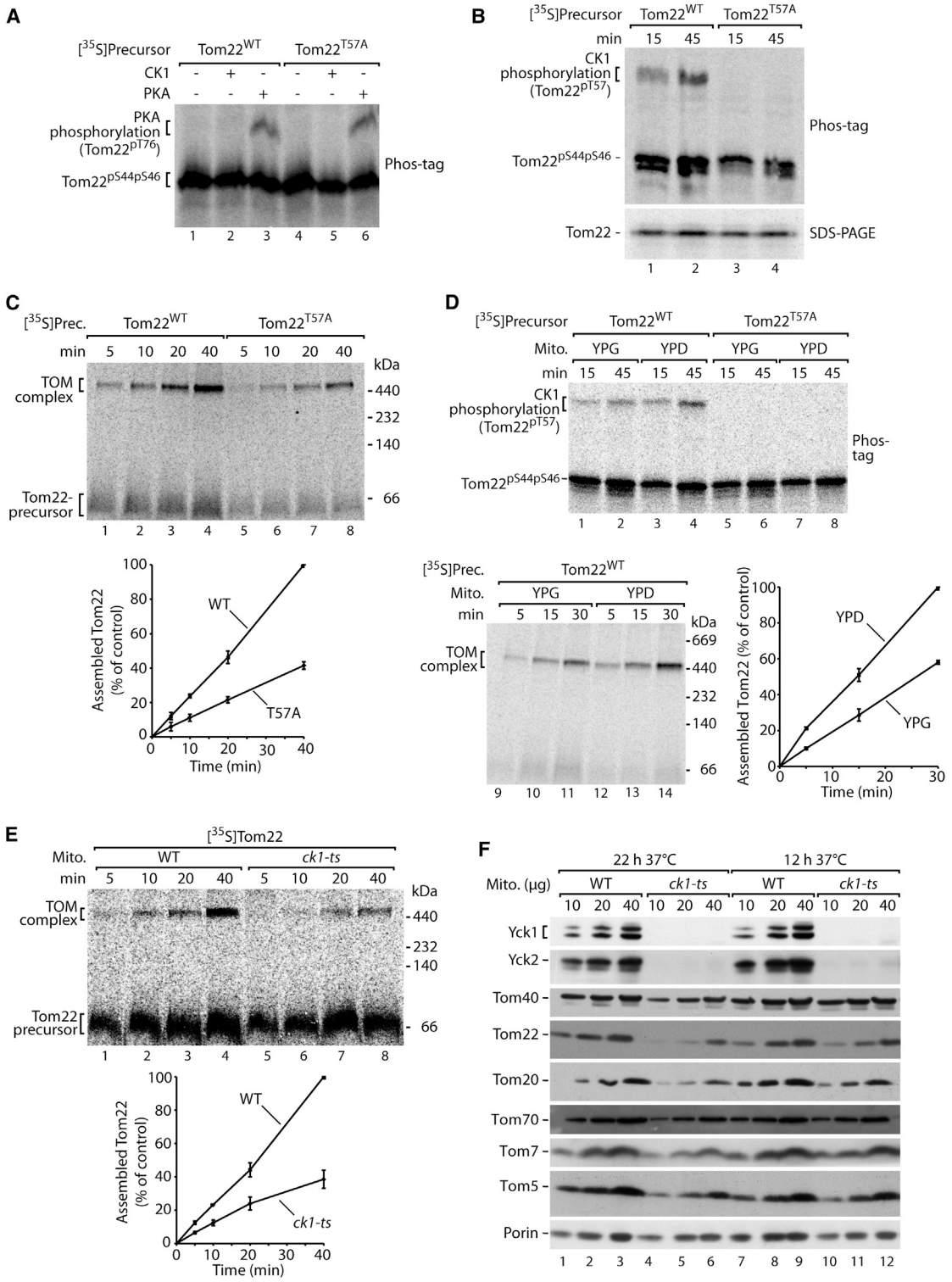

Figure 2. Phosphorylation of Tom22 by CK1 Promotes Assembly of Tom22

(A) $Tom22^{WT}$ and $Tom22^{T57A}$ were synthesized in reticulocyte lysate in presence of CK1 or PKA and analyzed by Phos-tag SDS-PAGE and autoradiography.

(B) $Tom22^{WT}$ and $Tom22^{T57A}$ precursors were incubated with WT mitochondria for the indicated periods at 25°C and analyzed by Phos-tag SDS-PAGE or standard SDS-PAGE and autoradiography.

(C) $Tom22^{WT}$ and $Tom22^{T57A}$ precursors were incubated with WT mitochondria and analyzed by lysis with digitonin, blue native electrophoresis, and autoradiography. Data are represented as mean ± SEM (n = 3). Assembled $Tom22^{WT}$ after 40 min import was set to 100% (control).

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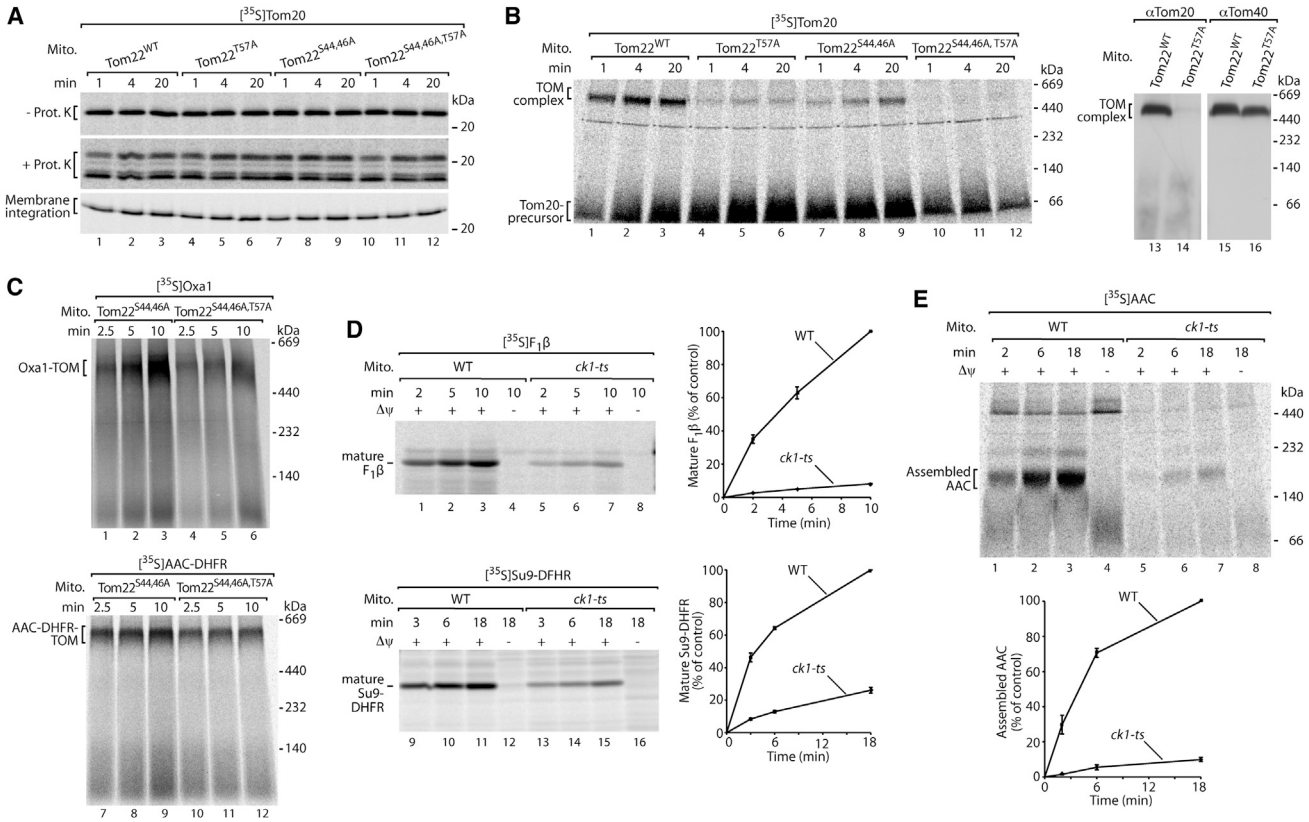


Figure 3. Phosphorylation of Tom22 by CK1 Promotes Assembly of Tom20 and Regulates TOM Complex Function

(A) Import of [^{35}S]Tom20 into mitochondria. Where indicated, samples were treated with proteinase K or Na_2CO_3 (membrane integration) and subjected to SDS-PAGE and autoradiography.

(B) Assembly of [^{35}S]Tom20. Mitochondria were lysed with 0.4% digitonin and analyzed by blue native electrophoresis and autoradiography (left). Right, analysis of mitochondria by blue native electrophoresis and immunoblotting.

(C) Accumulation of Oxa1 (dissipation of membrane potential $\Delta\psi$) and AAC-DHFR (presence of methotrexate) in the TOM complex. Mitochondria were analyzed by blue native electrophoresis.

(D) Mitochondria (isolated from cells that had been shifted to 37°C for 22 hr) were incubated with [^{35}S]F$_1\beta$ and [^{35}S]Su9-DHFR, treated with proteinase K, and analyzed by SDS-PAGE. Data are represented as mean ± SEM (n = 3). Import into WT mitochondria after the longest import time was set to 100% (control).

(E) Import of [^{35}S]AAC was analyzed by blue native electrophoresis and quantified as described in (D).

See also Figure S3.

of phospho-mimetic Tom22^{T76E} was considerably impaired (Figure 4D). We conclude that mimicking Thr76 phosphorylation impairs the biogenesis of Tom22.

A fraction of Tom22 precursors synthesized in reticulocyte lysate were phosphorylated by PKA in a Thr76-dependent manner (Figure 4E). Additionally, the Phos-tag analysis showed the phosphorylation of Ser44,46 by CK2 (Schmidt et al., 2011). Replacing Ser44,46 with alanines did not impair the PKA-dependent phosphorylation of Thr76. Thus, PKA phosphorylated the precursor of Tom22 in the cytosol independently of the activity of CK2. Figure 1F shows that, in contrast to CK1, PKA was not able to phosphorylate Tom22 in mitochondria. Our results indicate that PKA and CK1 phosphorylate Tom22 in different intracellular locations—PKA in the cytosol and CK1 at mitochondria.

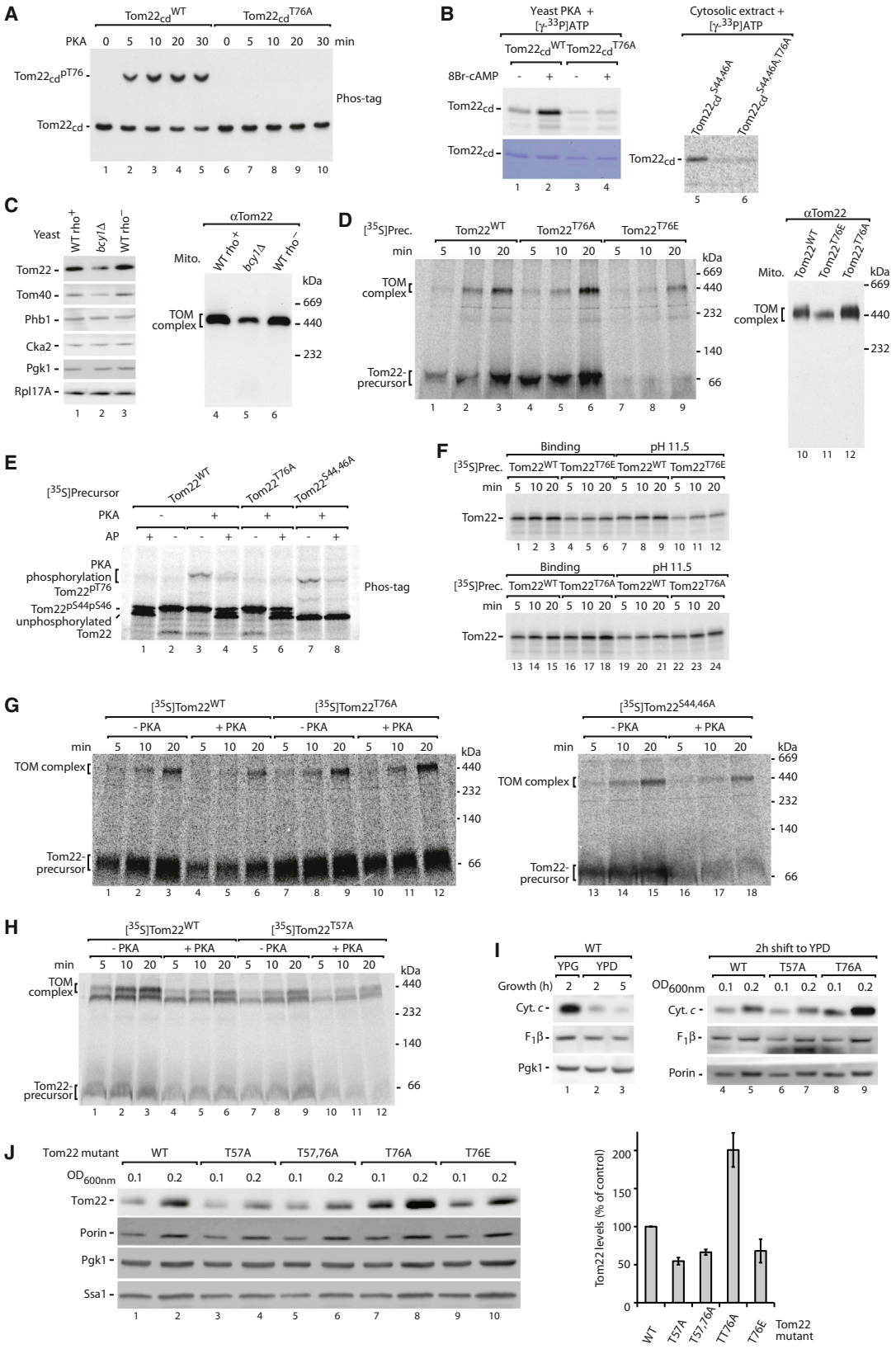
We asked whether PKA already impaired the biogenesis of Tom22 in the early phase of translocation from the cytosol into the outer membrane or only in the later phase of assembly into the TOM complex and analyzed the binding of the precursor to mitochondria and integration into the lipid phase of the membrane. The binding of phospho-mimetic [^{35}S]Tom22^{T76E} to

(D) Import of Tom22^{WT} and Tom22^{T57A} precursors into WT mitochondria isolated from yeast grown on YPG or YPD. The phosphorylation of Tom22 and integration into TOM were analyzed as described in (B) and (C), respectively. Data are represented as mean ± SEM (n = 3). Assembled Tom22 after 30 min import (YPD) was set to 100% (control).

(E) WT and *ck1-ts* mitochondria (isolated from cells that had been shifted to 37°C for 12 hr) were incubated with Tom22 precursor and analyzed by blue native electrophoresis and autoradiography. Data are represented as mean ± SEM (n = 3). Assembled Tom22 after 40 min import (WT) was set to 100% (control).

(F) Mitochondria were isolated from WT or *ck1-ts* yeast cultures that had been shifted to 37°C for 12 or 22 hr. Proteins were analyzed by SDS-PAGE and immunoblotting.

See also Figure S2.



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mitochondria was decreased, and its membrane integration was considerably reduced (Figure 4F). In contrast, the binding and membrane integration of [^{35}S]Tom22^{T76A} proceeded slightly faster than that of the WT precursor. Altogether, PKA phosphorylates the precursor of Tom22 in the cytosol and causes an inhibitory effect on the early biogenesis phase of the translocation of the precursor into mitochondria.

Regulation of Tom22 Biogenesis by Stimulatory and Inhibitory Kinases

We asked whether the inhibitory effect of PKA on Tom22 biogenesis was influenced by the CK2-mediated phosphorylation of Ser44,46 (Figure 4E) (Schmidt et al., 2011). PKA diminished the assembly of both Tom22^{WT} and Tom22^{S44,46A} into the TOM complex (Figure 4G), demonstrating that PKA inhibits Tom22 biogenesis independently of its phosphorylation by CK2.

Both PKA and CK1 function under fermentative growth conditions (Zaman et al., 2008) yet exert opposite effects on the biogenesis of Tom22. Given that PKA phosphorylates Tom22 in the cytosol and, thus, acts before mitochondria-bound CK1, we wondered whether inhibition by PKA was dominant over stimulation by CK1. We directly compared the effect of PKA on WT Tom22 and Tom22^{T57A}, which cannot be phosphorylated by CK1. PKA impaired the assembly of both precursors (Figure 4H). Remarkably, the prevention of CK1 action by the T57A replacement was as efficient in the reduction of Tom22 assembly as phosphorylation by PKA. A combination of both, prevention of CK1 action, and activation of PKA led to the strongest inhibition of Tom22 assembly. Thus, CK1 also exerts a stimulatory effect on the assembly of Tom22 in the presence of active PKA.

First, to analyze the in vivo role of Tom22 phosphorylation during the shift of yeast to fermentation, we studied the downregulation of cytochrome *c*. When WT cells were shifted to fermentable medium, the levels of cytochrome *c* were considerably decreased and the efficiency of downregulation was reduced in Tom22^{T76A} mutant cells (Figure 4I), underscoring the importance of PKA-mediated Tom22 phosphorylation for downregulation. Second, we determined the levels of Tom22 upon cell growth under fermentable conditions. Preventing Tom22 phosphorylation by CK1 (Tom22^{T57A}) decreased the levels of Tom22, whereas preventing the phosphorylation by PKA (Tom22^{T76A}) increased the levels (Figure 4J). Mimicking PKA phosphorylation with the Tom22^{T76E} mutant decreased the Tom22 levels, fully supporting the findings obtained in organello and in vitro. Cells with a double mutant Tom22 form (Tom22^{T57,76A}), which was neither phosphorylated by CK1 nor PKA, contained moderately decreased Tom22 levels, indicating the importance of CK1 for maintaining the levels of Tom22 even when the inhibitory activity of PKA is prevented.

CONCLUSIONS

Our findings indicate a high complexity of regulation of the mitochondrial protein import machinery. So far, three translocase components have been found to be regulated by phosphorylation, each one being targeted by one kinase—Tom22 by CK2 and Tom40 and Tom70 by PKA (Schmidt et al., 2011; Rao et al., 2012). We show that the biogenesis of the central receptor Tom22 is regulated by three kinases. In addition to the cytosolic kinases CK2 and PKA, Tom22 is also regulated by membrane-bound CK1.

The kinases either phosphorylate the cytosolic precursor of Tom22 (CK2 and PKA) or membrane-bound Tom22 (CK1). In a site-specific manner, the phosphorylated residues exert stimulatory or inhibitory effects on the import and assembly of Tom22. Though both CK2 and PKA phosphorylate the precursor of Tom22 in the cytosol, they lead to opposite consequences. CK2 stimulates the translocation of Tom22 into the outer membrane (Schmidt et al., 2011), whereas PKA inhibits the import of Tom22. The activity of CK2 is increased in rapidly growing cells, which require a higher activity of mitochondria (Tripodi et al., 2010). PKA is activated when glucose is added to yeast cells. Under fermentable conditions, the mitochondrial activity is decreased (Zaman et al., 2008), and the inhibition of Tom22 biogenesis by PKA provides a rapid means of decreasing protein import via the general import receptor.

The role of CK1 in phosphorylating Tom22 revealed two unexpected findings. First, the CK1 subunits Yck1 and Yck2 have been known to be located at the plasma membrane (Vancura et al., 1994; Roth et al., 2011). We observed a dual localization

Figure 4. PKA Phosphorylates the Cytosolic Tom22 Precursor and Impairs Import into Mitochondria

(A) Purified cytosolic domains (cd) of Tom22^{WT} or Tom22^{T76A} were incubated with PKA in the presence of ATP and analyzed by Phos-tag SDS-PAGE and immunoblotting with a Tom22-specific antibody.

(B) Left, yeast PKA was purified from a Bcy1$_{ProtA}$ strain (Rao et al., 2012) and incubated with Tom22$_{cd}^{WT}$ and Tom22$_{cd}^{T76A}$ in the presence of [γ-^{33}P]ATP and 8Br-cAMP as indicated. Samples were analyzed by SDS-PAGE and autoradiography or Coomassie staining. Right, Tom22$_{cd}^{S44,46A}$ and Tom22$_{cd}^{S44,46A,T76A}$ were incubated with cytosolic extract from WT yeast grown on fermentable medium in the presence of [γ-^{33}P]ATP.

(C) Yeast cell extracts or mitochondria from strains grown on fermentable medium were analyzed by SDS-PAGE (left) or blue native electrophoresis (right) and immunodecoration.

(D) Left, [^{35}S]Tom22 precursors were imported into WT mitochondria and analyzed by blue native electrophoresis and autoradiography. Right, mitochondria from the indicated yeast strains were analyzed by blue native electrophoresis and immunoblotting.

(E) [^{35}S]Tom22 precursors were synthesized in reticulocyte lysate in the presence or absence of PKA, treated with AP where indicated, and analyzed by Phos-tag SDS-PAGE and autoradiography.

(F) [^{35}S]Tom22 precursors were incubated with WT mitochondria. pH 11.5 samples were treated with Na_2CO_3 and membrane pellets obtained by ultracentrifugation. All samples were analyzed by SDS-PAGE.

(G) Import of [^{35}S]Tom22 precursors into WT mitochondria in the presence or absence of PKA. Samples were analyzed by blue native electrophoresis.

(H) Import of [^{35}S]Tom22 precursors into WT mitochondria in the presence or absence of PKA analyzed as in (G).

(I) Left, WT yeast cells grown in YPG were reinoculated in YPG or YPD for the indicated periods. Right, WT or Tom22 mutant yeast grown in YPG were shifted to YPD for 2 hr. Cell extracts were analyzed by SDS-PAGE and western blotting.

(J) Western blot analysis of whole-yeast extracts from WT and Tom22 mutants grown on fermentable medium. Data are represented as mean ± SEM (n = 3). The Tom22 level in WT yeast was set to 100% (control).

of CK1. Fractions of both subunits are bound to the mitochondrial outer membrane and are active in protein phosphorylation. Second, the growth of cells on glucose promoted the recruitment of CK1 to mitochondria in line with the involvement of CK1 in glucose-induced signal transduction pathways (Moriya and Johnston, 2004; Zaman et al., 2008). However, the phosphorylation of Tom22 by CK1 stimulated its assembly into the TOM complex and the interaction with Tom20. Mitochondria-bound CK1 acts after cytosolic PKA in Tom22 biogenesis. Therefore, CK1 does not reverse the PKA-induced diminished translocation of the Tom22 precursor into the outer membrane but stimulates the subsequent assembly into the TOM complex. In consequence, CK1 mitigates the inhibitory effect of PKA on the formation of mature TOM complex. Our results show that stimulatory and inhibitory phosphorylation steps regulate Tom22 biogenesis during fermentative growth.

Upon switching from respiratory to fermentative growth conditions, mitochondria have to undergo rapid changes in their composition and activity (Zaman et al., 2008). This involves major changes in gene expression (glucose repression and glucose induction). TOM phosphorylation via glucose-induced pathways provides a rapid and direct means of regulating mitochondrial biogenesis. The TOM complex is an ideal target, given that it is responsible for importing most mitochondrial proteins and is readily accessible to cytosolic signaling pathways. Though the respiratory mitochondrial activity is decreased under fermentable conditions, other mitochondrial functions have to be maintained, including the biogenesis of Fe-S clusters and transport of various metabolites (Lill, 2009). The activation of PKA leads to inhibitory effects on three major Tom components, the receptors Tom22, Tom70, and the channel protein Tom40 (Schmidt et al., 2011; Rao et al., 2012; this study). We propose that the stimulatory effect of CK1 on Tom22 assembly and increased mitochondrial levels of CK1 under fermentable conditions are part of a regulatory network that mitigates strong inhibition by PKA. Thus, the receptor Tom22 is a target of three signaling pathways, leading to a differential metabolic regulation of the mitochondrial protein import gate.

EXPERIMENTAL PROCEDURES

Yeast Growth, Isolation, and Fractionation of Mitochondria

Saccharomyces cerevisiae cells were grown in yeast extract peptone glycerol (YPG; 3%), yeast extract peptone glucose (YPD; 2%), yeast extract peptone sucrose (YPS; 2%), or synthetic (with 2% glucose) medium. Whole-cell lysates were obtained by postalkaline extraction and subsequently analyzed by SDS-PAGE and immunoblotting. Crude mitochondria were isolated by differential centrifugation and further purified by sucrose-gradient centrifugation. For analysis of submitochondrial protein localization, mitochondria were either incubated in hypo-osmotic buffer in order to rupture the outer membrane or solubilized with Triton X-100 and digested with proteinase K (Stojanovski et al., 2007). Samples were subjected to SDS-PAGE and immunoblotting.

In Organello and In Vitro Phosphorylation Assay

Isolated mitochondria were incubated with 5 mM ATP or 5–10 μCi [γ-^{33}P]ATP (PerkinElmer), and recombinant kinases were added. Mitochondria were reisolated, separated by (Phos-tag) SDS-PAGE, and analyzed by immunoblotting or autoradiography. For in vitro experiments, purified substrates were incubated with [γ-^{33}P]ATP in the presence of either mitochondria, yeast cytosolic extracts, or purified mammalian kinases (CK1, PKA, or CK2; New England BioLabs). Samples were subjected to SDS-PAGE, and phosphorylation was detected by autoradiography. For additional information, see the Supplemental Experimental Procedures.

Phos-Tag SDS-PAGE

The separation of phosphorylated proteins with Phos-tag SDS-PAGE was described by Kinoshita et al. (2006) and Schmidt et al. (2011). We added 50 μM Phos-tag reagent and 100 μM $MnCl_2$ to the separation gel mixture of a standard 15% SDS polyacrylamide gel prior to polymerization.

Protein Import into Mitochondria

[^{35}S]-labeled precursor proteins for import reactions were generated with a cell-free in vitro transcription and translation system (Stojanovski et al., 2007). Precursor proteins were incubated with isolated mitochondria in import buffer (250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 5 mM methionine, 2 mM KH_2PO_4, 3% [w/v] BSA, and 10 mM MOPS/KOH [pH 7.2]) in the presence of 2–4 mM ATP and 2 mM NADH. Where indicated, samples were treated with proteinase K after import. Mitochondria were reisolated by centrifugation and analyzed by SDS-PAGE or blue native electrophoresis. Membrane integration of precursors was tested by Na_2CO_3 extraction (Stojanovski et al., 2007).

Quantification and Statistics

For quantitative analysis of results, digital autoradiography (Storm 820), ImageQuant 5.2 software (GE Healthcare), ScanMaker 1000XL, SilverFast SDK XRay 6.6.2r1 (Microtek), luminescent image analyzer 4000 (LAS 4000), and MultiGauge 3.2 (Fujifilm) were used. All data values were obtained from at least three independent experiments and are shown as mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.09.006.

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