Protein kinase CK2 phosphorylates Sec63p to stimulate the assembly of the endoplasmic reticulum protein translocation apparatus

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Summary

The heterotetrameric Sec62/63 complex associates with the heterotrimeric Sec61 complex to form the heptameric Sec complex. This complex is necessary and sufficient for post-translational protein translocation across the membrane of the endoplasmic reticulum. We show that Sec63p is phosphorylated at its C-terminal domain by the protein kinase CK2 and that this phosphorylation strengthens the interaction between the cytosolic domains of Sec63p and Sec62p. Exchanging either threonine 652 or threonine 654 against the nonphosphorylatable alanines in Sec63p impairs the binding to Sec62p and interferes with the efficient translocation of proteins across the membrane of the endoplasmic reticulum. These findings show that phosphorylation of Sec63p is required for tightly recruiting the putative signal-sequence-binding subunit Sec62p to the Sec complex.

Key words: Sec complex, Split-ubiquitin, Signal sequence, Protein interaction, *Saccharomyces cerevisiae*

Introduction

Protein translocation across the membrane of the endoplasmic reticulum (ER) constitutes the first step in correctly localizing membrane proteins and soluble proteins into the different compartments of the secretory pathway of each eukaryotic cell (Schnell and Hebert, 2003). The transfer of a signal-sequence-bearing protein across the ER-membrane is accomplished by the channel-forming protein Sec61p (Simon and Blobel, 1991; Görlich et al., 1992; Crowley et al., 1994; Beckmann et al., 1997; Plath et al., 1998; Menetret et al., 2000; Wirth et al., 2003; Van den Berg et al., 2004). The heterotrimeric Sec61 complex consists of the pore-forming α subunit as well as a β and γ chain (Sbh1p and Sss1p in yeast) (Johnson and van Waes, 1999). In the yeast *Saccharomyces cerevisiae* the targeting of the signal-sequence-bearing proteins to the Sec61 complex is achieved either co- or post-translationally (Hann and Walter, 1991). The choice between the two pathways is determined by the composition of the signal sequence of the translocating protein (Ng et al., 1996). Very hydrophobic signal sequences including transmembrane segments are preferentially recognized by a ribonucleoprotein particle (signal-sequence-recognition particle, SRP) as soon as they emanate from the ribosome. The complex of ribosome, nascent chain and SRP is then captured by the SRP receptor at the ER membrane where the nascent chain with the ribosome still attached is transferred to the trimeric Sec61 complex (Keenan et al., 2001). The translocation of proteins with more hydrophilic signal sequences is less strictly coupled to their synthesis (Johnsson and Varshavsky, 1994a). Here, the recruitment to the ER membrane is achieved post-translationally by the membrane-bound heterotetrameric Sec62/63 complex that contains the two nonessential members Sec71p and Sec72p, in addition to Sec62p and Sec63p (Deshaies et al., 1991; Lyman and Schekman, 1997). The Sec62/63 complex and the Sec61 complex assemble into the functional unit of post-translational protein translocation, the heptameric Sec complex (Deshaies et al., 1991; Panzner et al., 1995).

The isolation of the heptameric Sec complex and the reconstitution of protein translocation in otherwise protein-free liposomes allowed a detailed functional and structural characterization of its components (Panzner et al., 1995). Sec62p consists of two large cytosolic domains at its N- and C-terminus that are connected via two transmembrane elements (Deshaies and Schekman, 1989). Sec62p is actively involved in signal-sequence-recognition during the targeting of the nascent chain to the translocation channel (Wittke et al., 2000; Plath et al., 2004). The N-terminal domain of Sec62p directly interacts with an acidic stretch located at the C-terminal end of Sec63p (Wittke et al., 2000; Willer et al., 2003). The C-terminal domain of Sec62p harbors an additional binding site whose corresponding partner in the Sec complex is not yet mapped (Wittke et al., 2000). Sec63p is a member of the family of DnaJ proteins and has three membrane-spanning regions (Feldheim et al., 1992). Its J-domain is located between the transmembrane regions two and three in the lumen of the ER, whereas its large C-terminal domain protrudes into the cytosol. The J-domain of Sec63p interacts with the ER-lumenal Hsp70 (Kar2p) to stimulate its ATPase and polypeptide binding activity (Brodsky and Schekman, 1993; Matlack et al., 1999). By transiently binding to the incoming nascent chain, Kar2p keeps the translocating polypeptide from sliding back into the

cytosol (Sanders et al., 1992; Matlack et al., 1999). In contrast to the other members of the tetrameric Sec62/63 complex, Sec63p together with Kar2p fulfills additional functions during the cotranslational mode of protein translocation (Young et al., 2001). Whether Sec63p performs this role in the context of the tetrameric Sec62/63 complex or in combination with other partner proteins is unknown.

Modulating the activity or changing the binding partners of a protein is often achieved by post-translational modifications. Indeed, the phosphorylation of some of the members of the mammalian trimeric Sec61 complex and a subsequent stimulation of cotranslational protein import into microsomes has been observed in vitro (Gruss et al., 1999). Here, we describe our finding that in yeast recruitment of Sec62p to the Sec complex is stimulated by protein kinase CK2 via phosphorylation of the C-terminal domain of Sec63p.

Materials and Methods

Construction of fusion proteins, mutations

Mutants of *F-FPR1-63*$_{C14}$ and *F-FPR1-63*$_{C47}$ were created by PCR using *F-FPR1-63*$_{C14}$ as a template and oligos annealing to the DNA coding for the last 14 residues of Sec63p but containing the desired nucleotide exchanges and an oligo annealing to the 3′ untranslated DNA of *SEC63* (Wittke et al., 2000). PCR products were cut with *Cla*I and *Acc*651 and inserted into the pRS314 vector. *P*$_{CUP1}$*-F-FPR1-63*$_{C14}$ and *P*$_{CUP1}$*-F-FPR1-63*$_{C47}$ were both cut with *Eag*I and *Cla*I, and, using the common *Cla*I site, inserted together with the P$_{CUP1}$-promoter in front of the mutated sequence to obtain *P*$_{CUP1}$*-F-FPR1-63*$_{C14}$*T652A* and *P*$_{CUP1}$*-F-FPR1-63*$_{C47}$*T654A*, as well as the other mutations at positions 652, 654 and 661 of Sec63p (Wittke et al., 2000). To insert the same mutations into the chromosomal *SEC63* a PCR product of *SEC63* spanning the last 448 bp of the *SEC63* ORF was cut with *Bam*HI and *Cla*I and inserted in front of the mutated *SEC63* sequence on the pRS304 or pRS305 vector. The vector was cut with *Pst*I in the *SEC63* sequence and transformed into the yeast JD53 or NJY126 essentially as described (Table 1) (Wittke et al., 1999). Successful recombination was confirmed by diagnostic PCR and sequencing. *P*$_{MET17}$*-SEC63-C*$_{ub}$*-RURA3*, all *N*$_{ub}$ fusions, *P*$_{CUP1}$*-SEC62-DHA,* *P*$_{CUP1}$*-SEC62*$_{\Delta C125}$*-DHA*, *P*$_{CUP1}$*-SEC62*$_{\Delta N144}$*-DHA,* *P*$_{CUP1}$*-SEC62*$_{\Delta C35}$*-DHA,* and all signal-sequence-containing *C*$_{ub}$*-URA3* constructs were as described (Dünnwald et al., 1999; Wittke et al., 1999; Wittke et al., 2002). The C-terminal mutations of *SEC63* were introduced into the *P*$_{MET17}$*-SEC63-C*$_{ub}$*-RURA3* vector by replacing the *Cla*I-*Sal*I fragment spanning the 3′ end of *SEC63* and the sequence connecting *SEC63* with *C*$_{ub}$ by a *Cla*I, *Sal*I cut PCR fragment containing the corresponding sequence but harboring the desired mutations. Genomic insertions of the sequence coding for protein A (ProA) behind *SEC63* or *CKA1* were performed as described (Knop et al., 1999) (Table 1). All insertions were tested by diagnostic PCR and western blots of protein extracts obtained from the transformed strains. Functionality of the *SEC63-ProA* allele was confirmed by the Ura3p-based translocation assay (Wittke et al., 2002). The ProA fusions were detected by consecutive incubations with horseradish peroxidase coupled rabbit anti-goat and goat anti-rabbit antibodies (Biorad, Hercules, USA). Oligos were obtained by Metabion (Munich, Germany). Sequencing was performed by Seqlab (Göttingen, Germany).

Media, synthetic lethality, translocation and split-ubiquitin assays

Yeast rich (YPD) and synthetic minimal media with 2% dextrose (SD) followed standard recipes. Split-ubiquitin interaction assays and translocation assays were performed as described previously (Wittke et al., 2002). Interaction assays were performed on media containing 10 mM methionine to reduce the expression of *P*$_{MET17}$*-SEC63-C*$_{ub}$*-RURA3*. The tests for establishing a synthetic lethality between the different *sec63* and *sec62* alleles were performed as described (Wittke et al., 2000).

[^{35}S]-Pulse and carboxypeptidase Y (CPY) immunoprecipitation

Cells were grown at 30°C or 37.5°C to an OD_{600} of 1, and 2 OD units were spun down, washed with water and resuspended in 1 ml of SD medium without methionine. Cells were kept at the appropriate temperature for 20 minutes and labeled with 250 μCi of [^{35}S]-Pro-mix labeling solution (Amersham Biosciences, Uppsala, Sweden). Cells were transferred after 5 minutes onto ice and the reaction was stopped by the addition of 0.25 ml 50% TCA. Cell extraction and immunoprecipitation with an anti-carboxypeptidase Y (CPY) monoclonal antibody (Molecular Probes, Portland, USA) was done as described previously (Bonifacino and Dell'Angelica, 1998).

In vivo phosphorylation

JD53 cells expressing F-Fpr1-63$_{C14}$ or F-Fpr1-63$_{C14T652;\ 654A}$ were cultured in 5 ml SD-trp to high cell densities, washed once in low phosphate medium (50 μM KH_2PO_4 in phosphate-free medium) (Qbiogene, Heidelberg, Germany) and diluted 1:700 into 4 ml of the same medium. Cells were grown to an OD_{600} of 0.7 and $CuSO_4$ was added to 100 μM. After 1 hour of further incubation at 30°C the cells were transferred into phosphate-free medium containing 100 μM copper and 50 μCi of [^{32}P]O_4 (Amersham Biosciences) per OD_{600}. After 2 hours at 30°C the reaction was stopped by the addition of 50% TCA to 12.5%. Cells were washed once in acetone and prepared for immunoprecipitation with the anti-Flag antibody (Sigma, Deisenhofen, Germany) as described (Bonifacino and Dell'Angelica, 1998).

Kinase assay

One hundred milliliters of cells expressing Cka1-ProA were grown at 30°C in YPD to an OD_{600} of approximately 1, washed once in water and then shock frozen in liquid nitrogen and either stored at –80°C or immediately processed for protein purification. Frozen cells from a 100 ml culture were ground under liquid nitrogen and the cell paste was extracted during thawing with 2 ml extraction buffer 1 (50 mM Hepes pH 7.5, 200 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) containing phosphatase inhibitor cocktail 1 and 2 (Sigma, Deisenhofen, Germany) and a protease inhibitor cocktail (Roche, Penzberg, Germany). The cell suspension was vortexed and centrifuged for 20 minutes at 16,000 ***g*** at 4°C. The supernatant was incubated with 40 μl IgG sepharose (Amersham Biosciences) for 4 hours at 4°C, the beads were washed five times with 10 ml of extraction buffer 1 and twice with kinase buffer (20 mM Hepes pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$). The procedure was repeated with JD53 cells not expressing a ProA fusion for the control reaction.

Cells expressing F-Fpr1-63$_{C14}$ or F-Fpr1-63$_{C14T652;\ 654A}$ under the P$_{CUP1}$-promoter were grown in SD-trp containing 100 μM copper at 30°C to an OD_{600} of approximately 1. Cells were extracted as described but in extraction buffer 2 (50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) containing phosphatase and protease inhibitors. Proteins were precipitated by incubation with anti-Flag antibody for 1 hour followed by incubation with 40 μl of Protein G sepharose (Amersham Biosciences) for 2 hours. Beads were washed four times with extraction buffer 2 and treated with phosphatase as described. After phosphatase treatment the beads were washed four times with extraction buffer 2 and the protein was recovered by incubating the beads with 50 μl of a 100 μg/ml solution of Flag

peptide (Sigma) in 10 mM Tris pH 7.5, 100 mM NaCl for 1 hour at room temperature.

The kinase assay contained 20 µl of Cka1-ProA beads, 20 µM ATP, 1 µCi of [γ-^{32}P]-GTP (Amersham) and 20 µl of Flag peptide eluate in a total volume of 50 µl kinase buffer. Inhibition of the kinase was achieved by 10 µg/ml Heparin (Sigma). The reaction was stopped after 30 minutes at 30°C by mixing the supernatant with an equal amount of twofold SDS sample buffer (SB) and boiling for 3 minutes.

Phosphatase treatment

Twenty microliters of Protein G sepharose presenting the anti-Flag antibody bound protein were treated with 10 units of calf intestinal phosphatase (CIP) (New England Biolabs, Beverly, USA) or without enzyme for 1 hour at 37°C in phosphatase buffer (NEB 3, New England Biolabs). Proteins immobilized on nitrocellulose were incubated with 5 ml of phosphatase buffer containing 30 units of CIP or no enzyme at 37°C for 1 hour.

Overlay assay

Protein extracts were derived from 10 ml yeast cultures as described (Wittke et al., 2002) or were obtained by resuspending the washed cell pellets in 100 µl twofold SB and subjecting them to four consecutive cycles of boiling, vortexing and freezing in liquid nitrogen. Extracts were separated by 12.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose (Wittke et al., 1999). The membrane was incubated for 2 hours at 4°C with 4 ml of 1:2 diluted extracts from cells expressing Sec62$_{\Delta C125}$-Dha under the inducible P$_{CUP1}$-promoter. Bound Sec62$_{\Delta C125}$-Dha was detected with anti-Ha antibody (Hiss Diagnostics, Freiburg, Germany) essentially as described (Wittke et al., 2002). The cells expressing Sec62$_{\Delta C125}$-Dha were grown in 100 ml SD-leu, 100 µM copper to an OD$_{600}$ of 1-1.5. The extracts from these cells were prepared as described for the cells expressing F-Fpr1-63$_{C14}$.

Binding assays

F-Fpr1-63$_{C14}$ presenting beads were incubated with 2 ml of extract derived from cells expressing Sec62$_{\Delta C125}$-Dha. After incubation for 2 hours at 4°C, the beads were washed four times with extraction buffer 2 and resuspended in 40 µl of twofold SB. Extractions of Sec63-ProA or Sec63T654A-ProA expressing cells were as for F-Fpr1-63$_{C14}$-expressing cells but with buffer containing 0.2% Triton X-100. 1.5 ml of the extracts were incubated with 30 µl of IgG sepharose beads for 2 hours at 4°C. Beads were spun down and 20 µl of the supernatants were boiled directly with 20 µl of twofold SB. Beads were washed five times with extraction buffer 2 (plus 0.2% Triton) and boiled in 120 µl SB. Fifteen microliters of the supernatants and 40 µl of the bound fractions were used for western blot analysis. Yeast cells expressing F-Fpr1-63$_{C14}$ together with Sec62$_{\Delta C125}$-Dha were grown to an OD$_{600}$ of 1-1.5 and extracted as described for cells expressing F-Fpr1-63$_{C14}$. The extract was incubated with anti-Ha antibody agarose (Hiss Diagnostics), washed four times with 1 ml of extraction buffer 2 and resuspended in 80 µl of twofold SB.

Results

Phosphorylation of the C-terminal domain of Sec63p mediates interaction with the N-terminal domain of Sec62p in vitro

The interaction between the cytosolic domains of the two membrane proteins Sec62p and Sec63p can be reconstituted with the N-terminal domain of Sec62p (Sec62$_{\Delta C125}$) and the cytosolic last 14 residues of Sec63p (63$_{C14}$). To enable their detection 63$_{C14}$ was attached to a Flag epitope-labeled carrier protein Fpr1p (F-Fpr1-63$_{C14}$), and Sec62$_{\Delta C125}$ was linked to an Ha-tagged dihydrofolate reductase (Sec62$_{\Delta C125}$-Dha) (Fig. 1A)

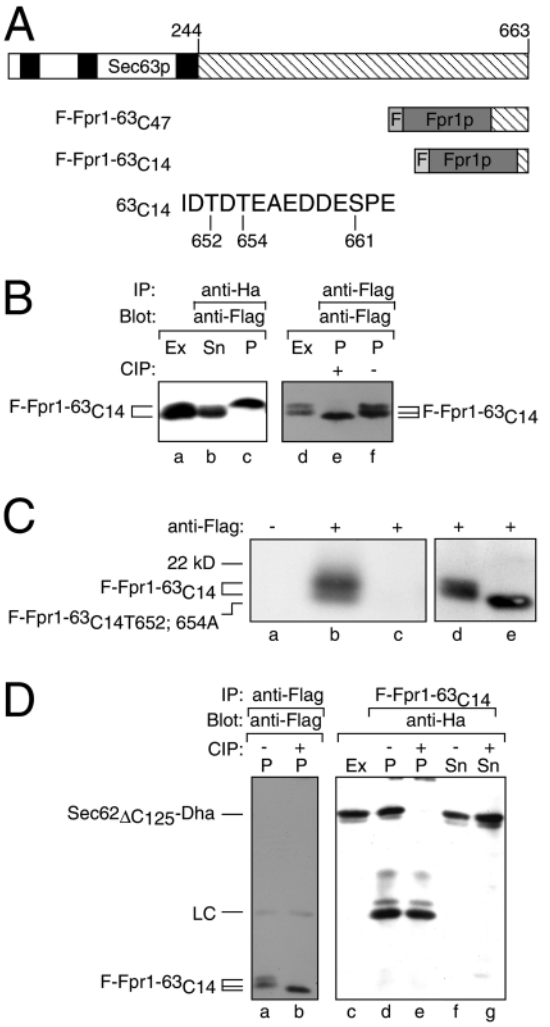

Fig. 1. The interaction between the C-terminus of Sec63p and the N-terminus of Sec62p is phosphorylation dependent.
(A) Representation of Sec63p and its C-terminal-derived fusion constructs F-Fpr1-63$_{C47}$ and F-Fpr1-63$_{C14}$. The sequence of the last 14 residues of Sec63p is given in the one-letter code. (B) Extracts of yeast cells expressing F-Fpr1-63$_{C14}$ together with Sec62$_{\Delta C-125}$-Dha (lanes a, b, c) or alone (lanes d, e, f) were incubated with anti-Ha or anti-Flag antibodies to precipitate Sec62$_{\Delta C-125}$-Dha (lanes b, c) or F-Fpr1-63$_{C14}$ (lanes e, f), respectively. Extracts (Ex), supernatant (Sn) and precipitates (P) were probed with anti-Flag antibody directly (lanes a-f) or after the treatment of the precipitate with calf intestinal phosphatase (CIP) (lane e). (C) In vivo phosphorylation of F-Fpr1-63$_{C14}$. Yeast cells expressing F-Fpr1-63$_{C14}$ (lanes a, b) or F-Fpr1-63$_{C14T652; 654A}$ (lane c) were labeled with [^{32}P]O$_4$. Extracts were incubated either with anti-Flag antibody (lanes b, c) or not (lane a) followed by the precipitation of the antibody, SDS-PAGE of the bound fraction, and autoradiography of the dried gel. Anti-Flag western blot of the immunoprecipitates of the unlabeled cells is shown in lanes d and e. (D) Sepharose-coupled F-Fpr1-63$_{C14}$ was incubated without (–) or with (+) phosphatase (lanes a, b) followed by incubation with Sec62$_{\Delta C125}$-Dha (lane c). Precipitates and supernatants were subjected to 12.5% SDS-PAGE and immunoblot detection with anti-Flag (lanes a, b) or anti-Ha antibody (lanes c-g). LC indicates the light chain of the antibody.

(Wittke et al., 2000). We noticed earlier that during SDS-PAGE F-Fpr1-63_{C14} separated into two forms of slightly different mobilities (Wittke et al., 2000). Even when great care was taken to prevent proteolysis, F-Fpr1-63_{C14} appeared during SDS-PAGE as a closely spaced doublet. Only the slower migrating species could be precipitated by $Sec62_{\Delta C125}$-Dha, whereas the faster migrating fraction remained in the supernatant and could not be detected in the bound fraction (Fig. 1B; note that the strong signal on this blot interfered with a clear separation of the two F-Fpr1-63_{C14} bands in lane a) (Wittke et al., 2000). To test whether the decreased mobility of the fusion protein was caused by the phosphorylation of the Sec63p derived peptide, we treated F-Fpr1-63_{C14} with an unspecific phosphatase. The shift to an apparent lower molecular weight during SDS-PAGE indicated that the fusion protein was indeed phosphorylated (Fig. 1B). The last 14 residues of Sec63p contain two threonines and one serine in positions 652, 654 and 661, respectively. Only the two threonines were strictly conserved in the *SEC63* sequences of other *Saccharomyces* species (Fig. 1A) (Cliften et al., 2003). To confirm the phosphorylation of the C-terminal peptide of Sec63p we labeled yeast cells with [^{32}P]O_4 to follow its incorporation into F-Fpr1-63_{C14} and F-Fpr1-$63_{C14T652;\ 654A}$. F-Fpr1-$63_{C14T652;\ 654A}$ contains two alanines instead of the conserved threonines at the position 652 and 654 of Sec63p. Two nearly overlapping bands can be detected on the autoradiograph of the gel at the running position of F-Fpr1-63_{C14}. No [^{32}P]O_4-incorporation could be observed for F-Fpr1-$63_{C14T652;\ 654A}$, although both F-Fpr1p fusion proteins were expressed at similar levels and could be immunoprecipitated from extracts with similar efficiencies (Fig. 1C; lanes d, e). Supported by the observation that phosphatase-treated F-Fpr1-63_{C14} migrated faster during SDS-PAGE than both bands of the untreated fusion protein, we propose a minimum of two phosphorylation sites in F-Fpr1-63_{C14} (Fig. 1B, d-f). As only the slower migrating form of F-Fpr1-63_{C14} was coprecipitated with $Sec62_{\Delta C125}$-Dha, phosphorylation seemed to be required for a strong binding to the N-terminal domain of Sec62p (Fig. 1B, lanes b, c). Accordingly, phosphatase treated F-Fpr1-63_{C14} failed to precipitate $Sec62_{\Delta C125}$-Dha from cell extracts proving that phosphorylation of the C-terminal domain of Sec63p is a prerequisite for its tight interaction with the N-terminal domain of Sec62p (Fig. 1D).

To conclusively map the phosphorylation sites in 63_{C14} we replaced the threonine residues individually (F-Fpr1-$63_{C14T652A}$; F-Fpr1-$63_{C14T654A}$) by alanines or exchanged the last three residues of Sec63p including Ser 661 from S-P-E to A-L-P (F-Fpr1-$63_{C14S661A}$). The mutated F-Fpr1p fusion proteins were analyzed before and after phosphatase treatment by SDS-PAGE (Fig. 2A). Similar to F-Fpr1-63_{C14}, untreated F-Fpr1-$63_{C14S661A}$ displayed two bands that merged into a single faster migrating band after phosphatase treatment (Fig. 2A; lanes a, b, and g, h). F-Fpr1-$63_{C14T652A}$ and the double mutation F-Fpr1-$63_{C14T652;\ 654A}$ were detected as single bands. The running positions of both mutants remained unaltered after phosphatase treatment and were identical to the dephosphorylated F-Fpr1-63_{C14} (Fig. 2A; lanes c, d, and i, j). Interestingly, F-Fpr1-$63_{C14T654A}$ migrated as a double band (Fig. 2A; lane e). After phosphatase treatment the upper band merged with the lower band, the mobility of which remained unchanged (Fig. 2A; lanes e, f), indicating a single phosphorylation in F-Fpr1-$63_{C14T654A}$. These findings were confirmed and extended by the different binding affinities of the mutants to $Sec62_{\Delta C125}$-Dha. The single and the double replacements of T652 and T654 by alanines drastically reduced their binding to the N-terminal domain of Sec62p, whereas the exchange of serine at position 661 had no detectable effect (Fig. 2B). Single exchanges of T652 and T654 against

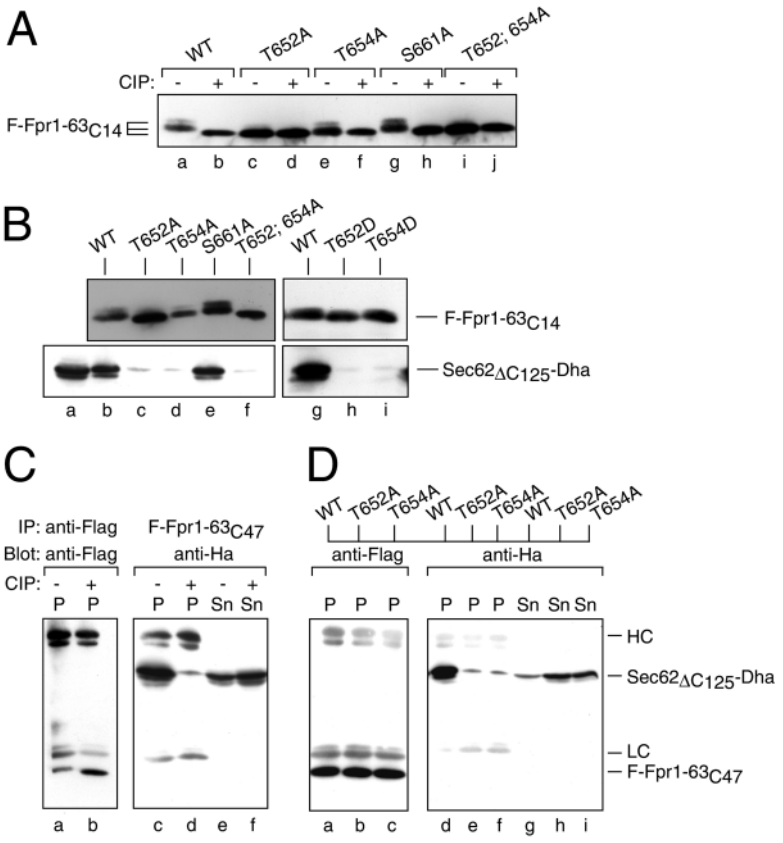

Fig. 2. The phosphorylatable threonines in position 652 and 654 of Sec63p are required for binding to the N-terminal domain of Sec63p. (A) Immunoprecipitation of F-Fpr1-63_{C14} (WT) and the mutants carrying single alanine exchanges in position 652 (T652A), 654 (T654A), 661 (S661A) or the exchange at position 652 and 654 (T652; 654A). The precipitates were either mock (–) or phosphatase (+) treated and subjected to anti-Flag detection after 12.5% SDS-PAGE. (B) Extracts of cells expressing F-Fpr1-63_{C14} (WT) or its mutants were anti-Flag immunoprecipitated and the precipitates were subsequently incubated with extracts containing $Sec62_{\Delta C125}$-Dha. Bound fractions were probed with anti-Flag (upper panel) or anti-Ha antibodies (lower panel) after 12.5% SDS-PAGE. An extract of cells expressing $Sec62_{\Delta C125}$-Dha is shown in lane a. (C) Sepharose-coupled F-Fpr1-63_{C47} was treated without or with phosphatase (– or +) and incubated with extracts containing $Sec62_{\Delta C125}$-Dha. The bound (lanes a-d) and unbound fractions (lanes e, f) were probed with anti-Flag (a, b) and anti-Ha antibodies (lanes c-f). (D) Same as C but with F-Fpr1-63_{C47} (WT), and its T/A mutants in position 652 and 654. LC and HC indicate the light and heavy chains of the antibody, respectively.

either aspartate or glutamate in F-Fpr1-63_{C14} or the replacement of both threonines by aspartates did not recover the binding to Sec$62_{\Delta C125}$-Dha (Fig. 2B; lanes h, i, and our unpublished observation).

The borders of the C-terminal binding site of Sec63p have not yet been precisely defined. As the predominance of negative charges extends well beyond the last 14 residues of Sec63p we repeated the binding experiments with a fusion protein containing the last 47 residues of Sec63p (F-Fpr1-63_{C47}). Fig. 2C,D shows that the binding of the complete C-terminal acidic domain of Sec63p to Sec$62_{\Delta C125}$-Dha is drastically impaired after phosphatase treatment or through single alanine exchanges at positions 652 or 654. The residual affinities of the mutated or phosphatase-treated F-Fpr1-63_{C47} for the N-terminal domain of Sec62p are above the background and therefore significant (Fig. 2C,D, and our unpublished observation).

Sec63p is phosphorylated in vivo

Phosphatase treatment of the full-length Sec63p did not change the mobility of the protein during SDS-PAGE. We therefore exploited the phosphorylation-dependent binding of the N-terminal domain of Sec62p to the C-terminus of Sec63p to measure the phosphorylation status of Sec63p. Protein extracts of yeast cells were separated by SDS-PAGE and transferred onto nitrocellulose. Treatment of the membrane with an extract from yeast cells overexpressing Sec$62_{\Delta C125}$-Dha was followed by incubations with an anti-Ha and a peroxidase-coupled anti-mouse antibody. A specific signal appeared at the running position of Sec63p, indicating the binding of Sec$62_{\Delta C125}$-Dha to the immobilized Sec63p (Fig. 3A,B; lane a). Phosphatase treatment of the membrane before the incubation with Sec$62_{\Delta C125}$-Dha or incubation with extracts from yeast cells not expressing Sec$62_{\Delta C125}$-Dha abolished the signal (Fig. 3A; lanes b, c). Further evidence that the signal reflects the phosphorylation-dependent binding of Sec62p to Sec63p was obtained by integrating the sequence of protein A (ProA) in frame to the sequence of the chromosomal *SEC63*. Extracts of these cells yielded a signal in the overlay assay that was shifted relative to Sec63p by the approximate size of ProA (Fig. 6D;

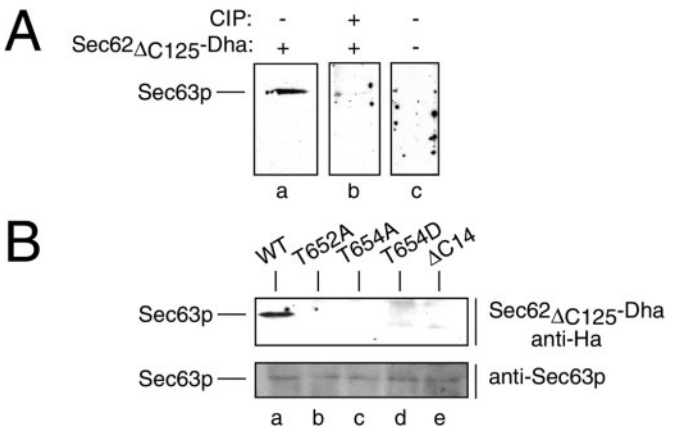

Fig. 3. Full-length Sec63p is phosphorylated at its C-terminal domain in vivo. (A) A protein extract from wild-type cells was separated by 12.5% SDS-PAGE and transferred onto nitrocellulose. The blot was treated either with (+) or without (–) CIP and subsequently incubated with extracts from yeasts either expressing Sec$62_{\Delta C125}$-Dha (+) or not (–). Bound Sec$62_{\Delta C125}$-Dha was detected with anti-Ha antibody. (B) Extracts of yeast cells expressing Sec63p or its alleles were subjected to the Sec$62_{\Delta C125}$-Dha overlay assay (top), or detection with anti-Sec63p antibody (bottom). ΔC14 indicates a mutant of Sec63p lacking the last 14 amino acids.

Table 1. Yeast strains

Strain	Relevant genotype	Source/comment
JD53	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52*	Dohmen et al., 1995
NJY145	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 SEC63-PROA-kanMX4*	This work
NJY146	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 CKA1-PROA-kanMX4*	This work
NJY147	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 sec63T654A-PROA-kanMX4*	This work
NJY148	*MATα his3-Δ200 leu2-3,112 lys252-801 trp1-Δ63 ura3-52* sec63T652A::pRS304	This work
NJY149	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 sec63T654A::pRS304*	This work
NJY150	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 sec63T654D::pRS304*	This work
NJY151	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 sec63$_{\Delta C14}$::pRS304*	This work
YDH6	*MATa his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 cka1-Δ1::HIS3 cka2-Δ1::TRP1 CKA2:CEN6/ARSH4 LEU2*	Hanna et al., 1995
YDH8	MATa his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 *cka1-Δ1::HIS3 cka2-Δ1::TRP1 cka2-8ts:CEN6/ARSH4 LEU2*	Hanna et al., 1995
YDH6/63	*MATa his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 cka1-Δ1::HIS3 cka2-Δ1::TRP1 CKA2:CEN6/ARSH4 LEU2 SEC63-PROA-kanMX4*	This work
YDH8/63	*MATa his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 cka1-Δ1::HIS3 cka2-Δ1::TRP1 cka2-8ts:CEN6/ARSH4 LEU2 SEC63-PROA-kanMX4*	This work
NYJ126/63A	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 SEC62::kanMX4 sec63T654A::pRS304 P_{CUP1}SEC62:pRS316*	This work
NYJ126ΔN144	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 SEC62::kanMX4 P_{CUP1}sec62$_{\Delta N144}$:pRS314*	Wittke et al., 2000

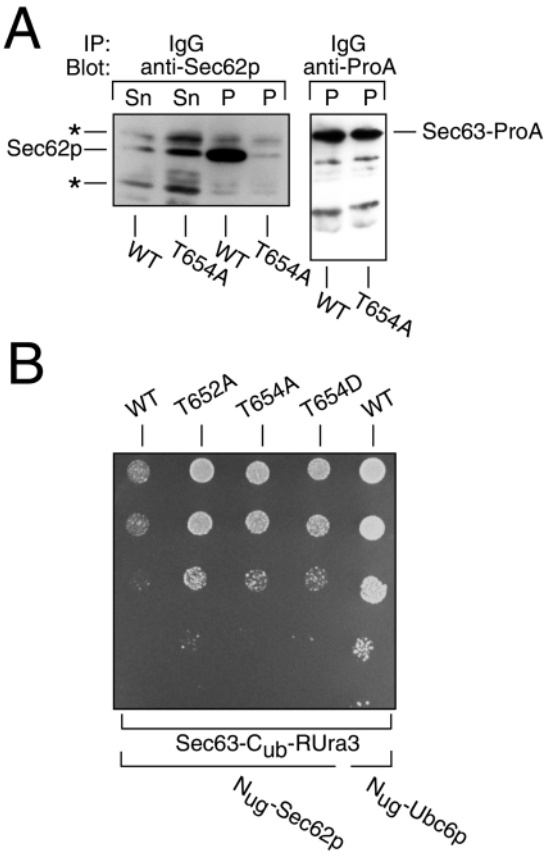

Fig. 4. Monitoring the effects of phosphorylation on the Sec63p-Sec62p interaction in vivo. (A) Extracts of yeast cells expressing Sec63-ProA or Sec63T654A-ProA were precipitated with IgG-sepharose and the supernatants (Sn) and the precipitates (P) were probed with anti-Sec62p serum. The precipitate was additionally probed with rabbit anti-goat/goat anti-rabbit antibodies. The asterisk (*) indicates cross-reacting bands of the anti-Sec62p serum. (B) Split-ubiquitin assay. Yeast cells coexpressing Sec63-C_{ub}-RUra3p or its mutants together with N_{ug}-Sec62p or N_{ug}-Ubc6p were grown to an OD_{600} of 1. Four millilitres of these cultures and tenfold dilutions thereof were spotted on plates lacking uracil, tryptophan and histidine to select for the presence of the plasmids. To check for cell numbers dilutions were also spotted on SD+ura plates (not shown).

lanes k, l). A mutant lacking the last 14 residues of Sec63p ($Sec63_{\Delta C14}$) was not detected by the overlay assay (Fig. 3B; lane e). To show the contributions of the phosphorylatable threonines to this binding we engineered alanine exchanges at position 652 or 654 or an aspartate exchange at position 654 into the chromosomal *SEC63*. All mutations abolished the binding to the N-terminal domain of Sec62p in the overlay assay (Fig. 3B; lanes b, c, d). We conclude that full-length Sec63p is phosphorylated at its C-terminus in vivo.

Sec62/63 complex stability depends on phosphorylation in vivo

To confirm the influence of Sec63p phosphorylation on its interaction with Sec62p in living cells we replaced *SEC63* by *SEC63-PROA* or *Sec63T654A-PROA*. The $Sec62_{\Delta C125}$-Dha overlay assay with extracts from yeasts expressing Sec63-ProA or Sec63T654A-ProA confirmed the functionality of the ProA fusions and the contribution of the phosphorylatable threonine on the binding of Sec63p to the N-terminal domain of Sec62p (our unpublished observation). Precipitation of Sec63-ProA from cell extracts and the analysis of the bound material by an anti-Sec62p antibody revealed the interaction of the wild-type Sec63p with Sec62p and its strong reduction due to the T654A mutation (Fig. 4A). Additional evidence on the influence of the phosphorylation on the Sec62/63 complex stability in living cells was obtained by the split-ubiquitin assay (Johnsson and Varshavsky, 1994b). Sec63p or its mutants were fused to the C-terminal half of ubiquitin that was extended by RUra3p (Sec63-C_{ub}-RUra3p), whereas Sec62p was fused behind the N-terminal half of ubiquitin (N_{ug}-Sec62p). In this configuration of the assay, interactions are detected by the nongrowth of the N_{ub}/C_{ub}- cotransformants on SD-ura (Wittke et al., 1999). The split-Ub assay confirmed that wild-type Sec63p binds stronger to the full-length Sec62p than the mutants bearing a nonphosphorylatable residue at position 652 or 654 (Fig. 4B). Residual interactions between Sec62p and the mutants of Sec63p were still detectable in this assay as the cells that co-express N_{ug}-Ubc6p together with Sec63-C_{ub}-RUra3p grow better on SD-ura than the cells co-expressing N_{ug}-Sec62p together with the mutants of Sec63-C_{ub}-RUra3p (Fig. 4B). Ubc6p is a membrane protein of the ER that does not bind to Sec63p (Wittke et al., 2002).

Nonphosphorylatable Sec63p causes a protein translocation defect

Deletions of the last C-terminal 27 or 28 residues of Sec63p including the phosphorylatable threonines abolish post-translational protein translocation but leave the cotranslational translocation pathway largely intact (Ng et al., 1996; Young et al., 2001). To monitor the influence of the phosphorylation of Sec63p on protein translocation we followed the maturation of the post-translational translocation substrate carboxypeptidase Y (CPY) (Hann and Walter, 1991). CPY was immunoprecipitated from [^{35}S]-methionine-labeled cells carrying the alleles *sec63T652A* or *sec63T654A*, and the ratio of translocated CPY versus cytosolic preproCPY was compared with the corresponding ratio measured in wild-type cells and cells carrying the *sec62-1* allele (Fig. 5A). *Sec62-1* cells display a strong translocation defect for post-translational translocation substrates (Deshaies and Schekman, 1989). Both threonine exchanges cause a significant accumulation of preproCPY although clearly less severe than the *sec62-1* allele (Fig. 5A). We made use of a sensitive growth assay to follow the influence of the threonine exchanges on the translocation of other signal-sequence-bearing proteins. A selection of signal sequences that at least partially rely on the post-translational translocation pathway were fused in front of the N-terminus of Ura3p. Cells expressing these fusion proteins and displaying a translocation defect grow on plates lacking uracil because of the accumulation of Ura3p activity in the cytosol, whereas nongrowth of the cells reflects the efficient import of translocation substrates into the lumen of the ER (Wittke et al., 2002). According to this assay, cells expressing $Sec63_{T652A}$, $Sec63_{T654A}$, $Sec63_{T654D}$ or $Sec63_{\Delta C14}$ only incompletely translocate the fusion proteins containing the signal sequences of the α-factor, invertase, CPY and Kar2p (Fig. 5B). A similar

phenotype was observed for the cells expressing a mutant of Sec62p ($Sec62_{\Delta N144}$) that lacks the N-terminal binding site for the C-terminus of Sec63p (Fig. 5B) (Wittke et al., 2000). Fig. 5B also confirms that neither the deletion of the C-terminal binding site on Sec63p including the two threonines 652 and 654 nor the deletion of the corresponding N-terminal domain in Sec62p is deleterious for the cells (Ng and Walter, 1996; Wittke et al., 2000; Young et al., 2001). As Sec62p contains an additional binding site for the Sec complex in its C-terminal domain, we assumed that the phosphorylatable threonines at positions 652 and 654 become only essential for the formation of the Sec complex once this alternative binding site has been removed. The synthetic lethality between the alleles *sec62Δ$_{C35}$-DHA* and *sec63T654A* supports this hypothesis (Fig. 5C).

Sec63p is constitutively phosphorylated by protein kinase CK2

The sequence environments of T652 and T654 both fulfill the requirements of the consensus protein kinase CK2 phosphorylation site (Pinna, 2003). In *S. cerevisiae* two CK2 isoforms are encoded by an essential gene pair, *CKA1* and *CKA2* (Glover, 1998). To test the effect of CK2 on the phosphorylation of Sec63p and its C-terminal peptides we used the strain YDH8 that contains a temperature sensitive *cka2* allele as its sole CK2 encoding copy (Hanna et al., 1995). Because the phosphorylation of F-Fpr1-63_{C14} can be easily followed by the phosphorylation-induced shift of its apparent molecular weight during SDS-PAGE we first introduced F-Fpr1-63_{C14} into YDH8 and the isogenic wild-type strain YDH6. The expression of F-Fpr1-63_{C14} was immediately induced after the transfer to the restrictive temperature. The appearance of the indicative double band in the wild-type but not in the *cka2*ts strain clearly shows that the in vivo phosphorylation of the last 14 residues of Sec63p depends on CK2 (Fig. 6A). Treatment of F-Fpr1-63_{C14} with phosphatase confirmed that no phosphorylation had occurred in the *cka2*ts strain, whereas F-Fpr1-63_{C14} was very probably phosphorylated at both sites in the wild-type strain (Fig. 6B).

To verify that the C-terminal domain of Sec63p can be directly phosphorylated by CK2 we performed an in vitro phosphorylation assay with the isolated proteins. Phosphatase-treated F-Fpr1-63_{C14} and F-Fpr1-$63_{C14T652;\,654A}$ were incubated with Cka1p purified from yeast in the presence of [γ-^{32}P]-GTP. Cka1p significantly phosphorylated only F-Fpr1-63_{C14} but not the mutant (Fig. 6C; lanes a, c). The phosphorylation was blocked by the CK2-inhibitor heparin (Fig. 6C; lane b).

Using the $Sec62_{\Delta C125}$-Dha overlay assay we failed to observe a rapid dephosphorylation of the endogenous full-length Sec63p in the YDH8 strain after the shift to the nonpermissive temperature (our unpublished observation). This observation raises the question whether full-length Sec63p is indeed a substrate for CK2. Alternatively, Sec63p is phosphorylated by CK2 but turned over only slowly. To distinguish between these two interpretations we expressed Sec63p ectopically from the inducible P_{MET17}-promoter to selectively follow the modification of the newly made Sec63p in cells without CK2 activity (Fig. 6D). To separate the induced from the naturally expressed Sec63p we tagged the chromosomal *SEC63* with

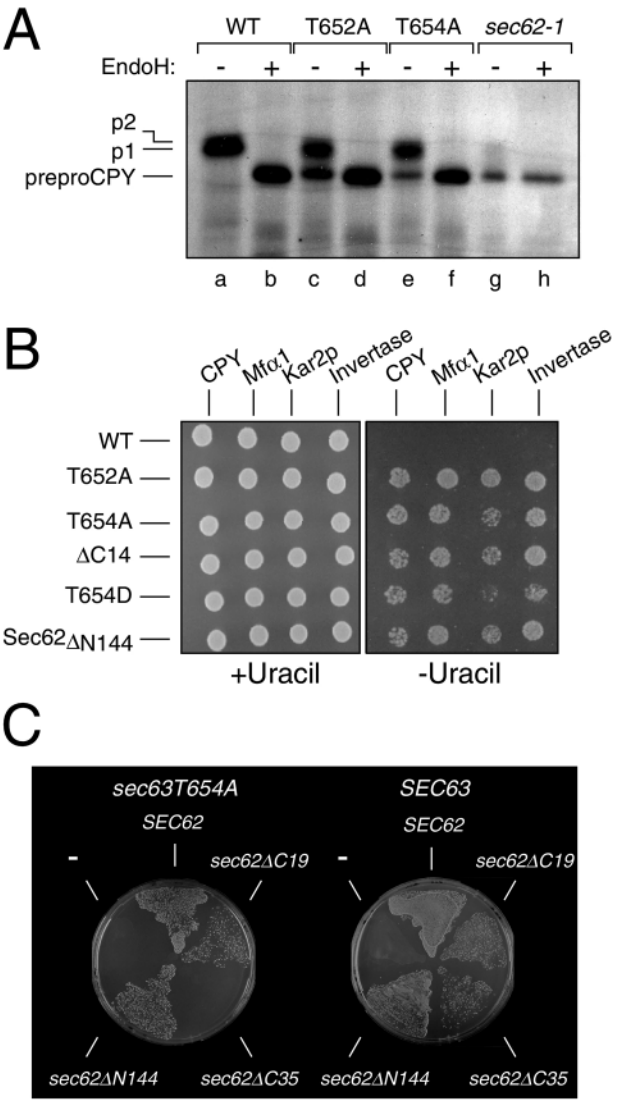

Fig. 5. Yeast cells carrying alanine replacements of the phosphorylatable threonines in Sec63p display protein translocation defects. (A) Pulse analysis of CPY translocation. Wild-type cells (WT), cells carrying the alanine replacements in position 652 (T652A) or 654 (T654A) of Sec63p, or *sec62-1* ts cells (*sec62-1*) were labeled with [^{35}S]-methionine for 5 minutes and subjected to a CPY immunoprecipitation. P1 and P2 indicate the positions of the translocated and preproCPY the position of the nontranslocated fraction of CPY on the autoradiogram of the gel (Pilon et al., 1997). The shift in the apparent molecular weight after EndoH treatment (+) confirmed the localization of P1 and P2 in the secretory pathway. (B) Steady-state analysis. Yeast cells carrying the indicated alleles of *SEC63* or *SEC62* and expressing signal-sequence-bearing C_{ub}-Ura3p constructs were spotted on plates without histidine and with or without uracil (+Uracil or –Uracil, respectively) to select for the presence of the plasmids. Growth of cells on SD-ura plates indicates a translocation defect for the respective signal sequence. (C) Synthetic lethality. Yeast cells containing *SEC63* or *sec63T654A* and expressing Sec62p from a *URA3* plasmid were transformed with the different *TRP1* plasmids containing the indicated alleles of *SEC62* carrying the Dha module at their C-termini (Wittke et al., 2000). Cells were plated on 5-FOA-containing media. Cells that contain the *sec63T654A* and the *sec62ΔC35-DHA* allele do not growth on this media, indicating a synthetic lethality between the two alleles.

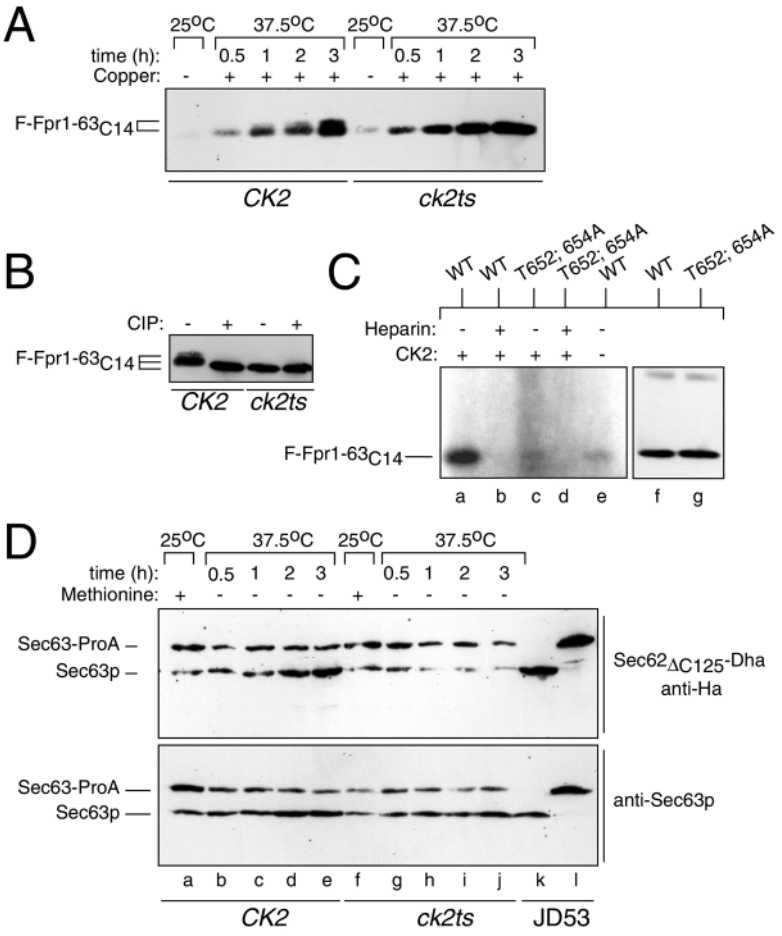

Fig. 6. Sec63p is phosphorylated by protein kinase CK2 in vivo and in vitro. (A) Yeast cells carrying a deletion of *CKA1* and the indicated alleles of *CKA2* were shifted to 37.5°C and the expression of F-Fpr1-63_{C14} was induced. Cell extracts were prepared at the indicated times and probed with anti-Flag antibody after transfer onto nitrocellulose. (B) As A but F-Fpr1-63_{C14} of cells grown for 3 hours at 37.5°C was precipitated by anti-Flag antibody and either mock treated (–) or treated with an unspecific phosphatase (+). (C) Autoradiogram (lanes a-e) of CIP-treated F-Fpr1-63_{C14} (WT) or F-Fpr1-$63_{C14T652;\,654A}$ (T652; 654A) incubated in the presence (+) of [γ-^{32}P] GTP with Cka1-ProA attached to IgG-sepharose (CK2) or with mock-treated IgG-beads (–), or with (+) or without (–) heparin. Anti-Flag western blot of the substrates for the kinase assays (lanes f, g). (D) As A but with a *CK2* and a *ck2ts* strain, that contain *SEC63-ProA* and expressing Sec63p ectopically from the P_{MET17}-promotor. Cells were grown at 25°C in medium containing 10 mM methionine (+) and shifted to 37.5°C and methionine-free medium (–). Extracts were probed with the Sec$62_{\Delta C125}$-Dha overlay assay (upper panel) or with anti-Sec63p antibody (lower panel). (Lanes k and l) JD53 cells containing *SEC63* (k) or *SEC63-ProA* (l) were probed with the Sec$62_{\Delta C125}$-Dha overlay assay (upper panel) or with anti-Sec63p antibody (lower panel).

PROA. The Sec$62_{\Delta C125}$-Dha overlay assay of extracts obtained at different times after the shift to the restrictive temperature revealed that the amount of phosphorylated Sec63p increased over time in the wild-type strain, whereas only traces of phosphorylated Sec63p were detected after 2 or 3 hours in the *cka2*ts strain (Fig. 6D, upper panel; compare lanes e, j). As Sec63p expression levels increased in both strains during the incubation at 37.5°C, the experiment provides further evidence that full-length Sec63p is phosphorylated at its C-terminal domain by CK2 in vivo (Fig. 6D, lower panel). Note that the endogenously expressed Sec63-ProA is only very slowly dephosphorylated during the course of the experiment (Fig. 6D; lanes i, j). The kinetics of dephosphorylation suggested that a defect in protein translocation might become apparent only after extended times of CK2-inactivation. To measure the translocation of CPY during the depletion of the CK2 activity, the *ck2ts* cells were pulsed with [^{35}S]-methionine at different times after the shift to the restrictive temperature (Fig. 7A). Although the synthesis of CPY declined in the ck2ts strain during the incubation at 37.5°C, the small amount of phosphorylated Sec63p that persisted even 8 hours after the shift seemed to suffice to translocate the newly made CPY across the membrane (Fig. 7A).

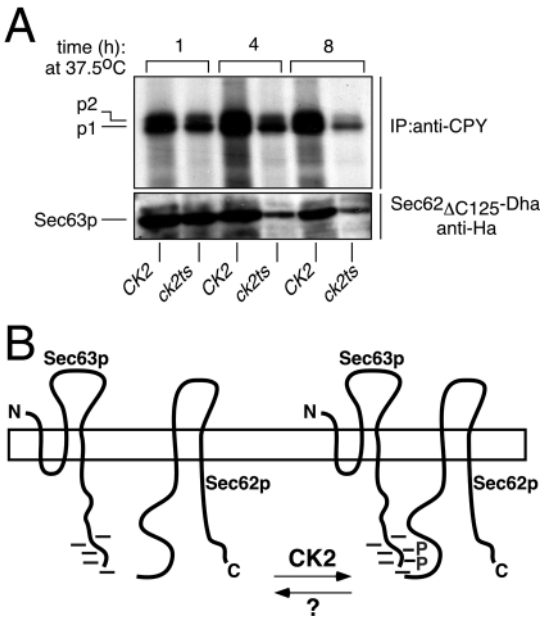

Fig. 7. Phosphorylated Sec63p is only slowly turned over. (A) Wild-type or *cka2ts* cells were shifted to the restrictive temperature, pulsed at the indicated times with [^{35}S]-methionine and subjected to CPY immunoprecipitation (upper panel). Extracts of the same cells were also probed for the presence of the phosphorylated Sec63p by the Sec$62_{\Delta C125}$-Dha overlay assay (lower panel) (B) Summary. Newly made Sec63p is rapidly phosphorylated by protein kinase CK2. The phosphorylated Sec63p binds strongly to Sec62p and becomes protected against dephosphorylation. Certain conditions might induce a phosphatase activity that dephosphorylates Sec63p and thereby shifts the ratio from the bound to the free form.

Discussion

Our molecular analysis of the interaction between Sec63p and Sec62p revealed an unexpected role for CK2 in the formation of the tetrameric Sec62/63 complex and established a new link

between the activity of CK2 and post-translational protein translocation in yeast.

The interaction between Sec63p and Sec62p is phosphorylation dependent

In vivo [$^{32}PO_4$] labeling and the phosphatase-induced decrease in the apparent molecular weight of a fusion protein harboring the last 14 residues of Sec63p or mutants thereof identified threonines 652 and 654 as phosphorylation sites in Sec63p (Figs 1 and 2). In vitro binding experiments and a newly developed overlay assay further revealed that the phosphorylation of both threonines are required for a strong interaction between the N-terminal domain of Sec62p and the C-terminus of Sec63p (Figs 2 and 3). Coprecipitation of the full-length proteins and the split-ubiquitin technique confirmed the necessity of phosphorylation for the formation of a stable Sec62p-Sec63p complex in vivo (Fig. 4). The threonine/alanine exchanges at position 652 and 654 of Sec63p mimic the effects of dephosphorylating Sec63p or its C-terminal peptides on their in vitro binding to Sec62p. Both alanine replacements also impair protein translocation in living cells. Together, this provides strong evidence that Sec63p phosphorylation is a prerequisite for tightly recruiting Sec62p to the Sec complex (Fig. 5). This proximity then allows Sec62p, which is part of the signal-sequence receptor, to efficiently deliver the signal sequences to Sec61p, the pore-forming subunit of the translocation channel (Matlack et al., 1997; Plath et al., 1998; Dünnwald et al., 1999; Wittke et al., 2000; Plath et al., 2004). The disruption of the phosphorylation-dependent Sec62p-Sec63p interaction impairs but does not prohibit protein translocation (Fig. 5). We therefore suspected a residual interaction between Sec62p and Sec63p that is independent of Sec63p phosphorylation. Indeed, such a residual interaction could be detected by the split-ubiquitin assay for the full-length proteins and by in vitro binding experiments between the N-terminal domain of Sec62p and the last 47 C-terminal residues of Sec63p (Figs 2 and 4) (Wittke et al., 2000). Previous experiments have already identified an additional binding site for the Sec complex at the C-terminus of Sec62p (Wittke et al., 2000). Our hypothesis that this site helps to keep Sec62p loosely attached to the Sec complex in the absence of a functional C-terminal domain of Sec63p was supported by the observed synthetic lethality between the alleles *sec62Δ$_{C35}$-DHA* and *sec63T654A* (Fig. 5).

CK2 phosphorylates Sec63p in vivo and in vitro

The inability of a *ck2ts* strain to phosphorylate Sec63p at the restrictive temperature identified CK2 as the responsible kinase (Fig. 6). The in vitro phosphorylation of the C-terminal peptide of Sec63p by the enriched CK2 confirmed this conclusion (Fig. 6C). Considering the contribution of the phosphorylatable threonines to the translocation of CPY and other signal-sequence-bearing test substrates (Fig. 5), the lack of a detectable translocation defect in the *ck2ts* strain was unexpected (Fig. 7). The slow dephosphorylation of Sec63p during the CK2 heat inactivation might account for this observation as it leaves the cells with sufficient translocation capacity even hours after the inactivation of the kinase has occurred (Fig. 6D, Fig. 7A). We propose that, under the conditions tested, the phosphorylated Sec63p as part of the Sec62/63 complex is protected against rapid dephosphorylation (Fig. 7B). Consequently, those CK2-dependent processes displaying a higher phosphate turnover sense the depletion of CK2 much earlier and stop cell growth before a strong translocation defect manifests (Padmanabha et al., 1990; Hanna et al., 1995; Rethinaswamy et al., 1998).

The slow turnover of the phosphorylated Sec63p makes it less likely but does not necessarily exclude a phosphorylation-dependent regulation of the Sec complex assembly. A signal-dependent dephosphorylation of Sec63p could induce the dissociation of the Sec62/63 complex thereby freeing either of the subunits for alternative tasks (Fig. 7B). We know of two activities of Sec63p that are not shared by Sec62p – cotranslational protein translocation and nuclear fusion during karyogamy (Ng and Walter, 1996; Young et al., 2001). Both processes might require the dissociation of Sec62p from Sec63p, although a direct proof for this is still lacking (Prinz et al., 2000; Helmers et al., 2003; Schwartz and Blobel, 2003).

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References

Beckmann, R., Bubeck, D., Grassucci, R., Penczek, P., Verschoor, A., Blobel, G. and Frank, J. (1997). Alignment of conduits for the nascent polypeptide chain in the ribosome-Sec61 complex. *Science* **278**, 2123-2126.

Bonifacino, J. S. and Dell'Angelica, E. C. (1998). Immunoprecipitation. In *Current Protocols in Cell Biology* (ed. J. S. Bonifacino, M. Dasso, J. B. Harford, J. Lippincott-Schwartz and K. Yamada). New York, NY: John Wiley & Sons.

Brodsky, J. L. and Schekman, R. (1993). A Sec63p-BiP complex from yeast is required for protein translocation in a reconstituted proteoliposome. *J. Cell Biol.* **123**, 1355-1363.

Clifften, P., Sudarsanam, P., Desikan, A., Fulton, L., Fulton, B., Majors, J., Waterston, R., Cohen, B. A. and Johnston, M. (2003). Finding functional features in Saccharomyces genomes by phylogenetic footprinting. *Science* **301**, 71-76.

Crowley, K. S., Liao, S., Worrell, V. E., Reinhart, G. D. and Johnson, A. E. (1994). Secretory proteins move through the endoplasmic reticulum membrane via an aqueous, gated pore. *Cell* **78**, 461-471.

Deshaies, R. J. and Schekman, R. (1989). SEC62 encodes a putative membrane protein required for protein translocation into the yeast endoplasmic reticulum. *J. Cell Biol.* **109**, 2653-2664.

Deshaies, R. J., Sanders, S. L., Feldheim, D. A. and Schekman, R. (1991). Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex. *Nature* **349**, 806-808.

Dohmen, R. J., Stappen, R., McGrath, J. P., Forrova, H., Kolarov, J., Goffeau, A. and Varshavsky, A. (1995). An essential gene encoding a homolog of ubiquitin-activating enzyme. *J. Biol. Chem.* **270**, 18099-18109.

Dünnwald, M., Varshavsky, A. and Johnsson, N. (1999). Detection of transient in vivo interactions between substrate and transporter during protein translocation into the endoplasmic reticulum. *Mol. Biol. Cell* **10**, 329-344.

Feldheim, D., Rothblatt, J. and Schekman, R. (1992). Topology and functional domains of Sec63p, an endoplasmic reticulum membrane protein required for secretory protein translocation. *Mol. Cell. Biol.* **12**, 3288-3296.

Glover, C. V., III (1998). On the physiological role of casein kinase II in *Saccharomyces cerevisiae*. *Prog. Nucleic Acid Res. Mol. Biol.* **59**, 95-133.

Görlich, D., Prehn, S., Hartmann, E., Kalies, K. U. and Rapoport, T. A. (1992). A mammalian homolog of SEC61p and SECYp is associated with ribosomes and nascent polypeptides during translocation. *Cell* **71**, 489-503.

Gruss, O. J., Feick, P., Frank, R. and Dobberstein, B. (1999). Phosphorylation of components of the ER translocation site. *Eur. J. Biochem.* **260**, 785-793.

Hann, B. and Walter, P. (1991). The signal recognition particle in *S. cerevisiae*. *Cell* **67**, 131-144.
Hanna, D. E., Rethinaswamy, A. and Glover, C. V. (1995). Casein kinase II is required for cell cycle progression during G1 and G2/M in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **270**, 25905-25914.
Helmers, J., Schmidt, D., Glavy, J. S., Blobel, G. and Schwartz, T. (2003). The beta-subunit of the protein-conducting channel of the endoplasmic reticulum functions as the guanine nucleotide exchange factor for the beta-subunit of the signal recognition particle receptor. *J. Biol. Chem.* **278**, 23686-23690.
Johnson, A. E. and van Waes, M. A. (1999). The translocon: a dynamic gateway at the ER membrane. *Annu. Rev. Cell Dev. Biol.* **15**, 799-842.
Johnsson, N. and Varshavsky, A. (1994a). Ubiquitin-assisted dissection of protein transport across membranes. *EMBO J*. **13**, 2686-2698.
Johnsson, N. and Varshavsky, A. (1994b). Split ubiquitin as a sensor of protein interactions in vivo. *Proc. Natl. Acad. Sci. USA* **91**, 10340-10344.
Keenan, R. J., Freymann, D. M., Stroud, R. M. and Walter, P. (2001). The signal recognition particle. *Annu. Rev. Biochem.* **70**, 755-775.
Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K. and Schiebel, E. (1999). Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast* **15**, 963-972.
Lyman, S. K. and Schekman, R. (1997). Binding of secretory precursor polypeptides to a translocon subcomplex is regulated by BiP. *Cell* **88**, 85-96.
Matlack, K. E., Plath, K., Misselwitz, B. and Rapoport, T. A. (1997). Protein transport by purified yeast Sec complex and Kar2p without membranes. *Science* **277**, 938-941.
Matlack, K. E., Misselwitz, B., Plath, K. and Rapoport, T. A. (1999). BiP acts as a molecular ratchet during posttranslational transport of prepro-alpha factor across the ER membrane. *Cell* **97**, 553-564.
Menetret, J. F., Neuhof, A., Morgan, D. G., Plath, K., Radermacher, M., Rapoport, T. A. and Akey, C. W. (2000). The structure of ribosome-channel complexes engaged in protein translocation. *Mol. Cell* **6**, 1219-1232.
Ng, D. T. and Walter, P. (1996). ER membrane protein complex required for nuclear fusion. *J. Cell Biol.* **132**, 499-509.
Ng, D. T., Brown, J. D. and Walter, P. (1996). Signal sequences specify the targeting route to the endoplasmic reticulum membrane. *J. Cell Biol.* **134**, 269-278.
Padmanabha, R., Chen-Wu, J. L., Hanna, D. E. and Glover, C. V. (1990). Isolation, sequencing, and disruption of the yeast CKA2 gene: casein kinase II is essential for viability in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**, 4089-4099.
Panzner, S., Dreier, L., Hartmann, E., Kostka, S. and Rapoport, T. A. (1995). Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. *Cell* **81**, 561-570.
Pilon, M., Schekman, R. and Römisch, K. (1997). Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. *EMBO J.* **16**, 4540-4548.
Pinna, L. A. (2003). The raison d'etre of constitutively active protein kinases: the lesson of CK2. *Acc. Chem. Res.* **36**, 378-384.
Plath, K., Mothes, W., Wilkinson, B. M., Stirling, C. J. and Rapoport, T. A. (1998). Signal sequence recognition in posttranslational protein transport across the yeast ER membrane. *Cell* **94**, 795-807.
Plath, K., Wilkinson, B. M., Stirling, C. J. and Rapoport, T. A. (2004). Interactions between Sec complex and prepro-alpha-factor during posttranslational protein transport into the endoplasmic reticulum. *Mol. Biol. Cell* **15**, 1-10.
Prinz, A., Behrens, C., Rapoport, T. A., Hartmann, E. and Kalies, K. U. (2000). Evolutionarily conserved binding of ribosomes to the translocation channel via the large ribosomal RNA. *EMBO J.* **19**, 1900-1906.
Rethinaswamy, A., Birnbaum, M. J. and Glover, C. V. (1998). Temperature-sensitive mutations of the CKA1 gene reveal a role for casein kinase II in maintenance of cell polarity in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 5869-5877.
Sanders, S. L., Whitfield, K. M., Vogel, J. P., Rose, M. D. and Schekman, R. W. (1992). Sec61p and BiP directly facilitate polypeptide translocation into the ER. *Cell* **69**, 353-365.
Schnell, D. J. and Hebert, D. N. (2003). Protein translocons: multifunctional mediators of protein translocation across membranes. *Cell* **112**, 491-505.
Schwartz, T. and Blobel, G. (2003). Structural basis for the function of the beta subunit of the eukaryotic signal recognition particle receptor. *Cell* **112**, 793-803.
Simon, S. M. and Blobel, G. (1991). A protein-conducting channel in the endoplasmic reticulum. *Cell* **65**, 371-380.
Van den Berg, B., Clemons, W. M., Jr, Collinson, I., Modis, Y., Hartmann, E., Harrison, S. C. and Rapoport, T. A. (2004). X-ray structure of a protein-conducting channel. *Nature* **427**, 36-44.
Willer, M., Jermy, A. J., Young, B. P. and Stirling, C. J. (2003). Identification of novel protein-protein interactions at the cytosolic surface of the Sec63 complex in the yeast ER membrane. *Yeast* **20**, 133-148.
Wirth, A., Jung, M., Bies, C., Frien, M., Tyedmers, J., Zimmermann, R. and Wagner, R. (2003). The Sec61p complex is a dynamic precursor activated channel. *Mol. Cell* **12**, 261-268.
Wittke, S., Lewke, N., Müller, S. and Johnsson, N. (1999). Probing the molecular environment of membrane proteins in vivo. *Mol. Biol. Cell* **10**, 2519-2530.
Wittke, S., Dünnwald, M. and Johnsson, N. (2000). Sec62p, a component of the endoplasmic reticulum protein translocation machinery, contains multiple binding sites for the Sec-complex. *Mol. Biol. Cell* **11**, 3859-3871.
Wittke, S., Dünnwald, M., Albertsen, M. and Johnsson, N. (2002). Recognition of a subset of signal sequences by Ssh1p, a Sec61p-related protein in the membrane of endoplasmic reticulum of yeast *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **13**, 2223-2232.
Young, B. P., Craven, R. A., Reid, P. J., Willer, M. and Stirling, C. J. (2001). Sec63p and Kar2p are required for the translocation of SRP-dependent precursors into the yeast endoplasmic reticulum in vivo. *EMBO J.* **20**, 262-271.