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Molecular Dissection of the Sec62/63p Complex, a Member of Protein Translocation Machinery of the Endoplasmic Reticulum Membrane

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Abstract

The heterotetrameric Sec62/63p complex associates with the heterotrimeric Sec61p complex to assemble into the heptameric Sec complex. This complex is necessary and sufficient for posttranslational protein translocation across the membrane of the endoplasmic reticulum. The transmembrane proteins Sec62p and Sec63p are two essential members of this complex.

In my PhD project, I showed that Sec63p is phosphorylated at its C-terminal domain by protein kinase CK2 in vivo and in vitro. Both threonine 652 and threonine 654 of Sec63p are phosphorylated and the tight assembly of Sec62/63p complex is dependent on the phosphorylation of both residues in vivo and in vitro. Exchanging either threonine 652 or threonine 654 against the nonphosphorylatable alanine in Sec63p impairs the binding to Sec62p and causes protein translocation to occur less efficiently across the membrane of the endoplasmic reticulum.

Sec63p is constitutively phosphorylated and the fraction of phosphorylated Sec63p does not change upon certain stress conditions. A phosphorylation mediated regulation of protein translocation was therefore not observed.

Furthermore, I mapped the complete binding site of Sec63p for the N-terminal domain of Sec62p to the last 24 C-terminal residues.

In summary, my work shows for the first time that phosphorylation of Sec63p is required to tightly recruit the putative signal sequence receptor Sec62p to the Sec complex. This tight association is required to efficiently initiate posttranslational protein translocation across the membrane of the endoplasmic reticulum.

Molekulare Kartierung des Sec62/63p Komplexes, ein Mitglied der Proteintranslokationsmaschinerie in der Membran des endoplasmatischen Retikulums

Zusammenfassung

Der heterotetramere Sec62/Sec63p Komplex assoziiert mit dem heterotrimeren Sec61p Komplex zu dem heptameren Sec Komplex. Dieser heptamere Sec Komplex ist sowohl notwendig als auch ausreichend für die posttranslationale Translokation von Proteinen über die Membran des endoplasmischen Retikulums. Die beiden Transmembranproteine Sec62p und Sec63p sind essentielle Komponenten dieses Komplexes.

In meiner Arbeit konnte ich zeigen, dass Sec63p an seiner C-terminalen Domäne von der Proteinkinase CK2 sowohl in vivo als auch in vitro phosphoryliert wird. Die Phosphorylierung der Threonine in Position 652 und 654 ist eine Voraussetzung für die enge Bindung zwischen Sec63p und Sec62p. Dies bestätigen sowohl biochemische Bindungstests als auch Messungen in der lebenden Zelle. Wird eines der beiden Threonine gegen ein nicht phosphorylierbares Alanin ausgetauscht, so wird eine Schwächung der Sec62/63p Bindung und damit einhergehend eine weniger effiziente Proteintranslokation über die Membran des endoplasmischen Retikulums beobachtet.

Sec63p wird von CK2 konstitutiv phosphoryliert und der Grad dieser Phosphorylierung scheint sich unter verschiedenen zellulären Bedingungen nicht zu verändern. Eine Regulation der Proteintranslokation konnte daher nicht nachgewiesen werden.

Durch weitere genetische und biochemische Experimente konnte ich die vollständige Bindungstelle von Sec63p für die N-terminale Domäne von Sec62p auf die letzten 24 Aminosäuren eingrenzen.

Meine Arbeit weist so zum erstenmal den Einfluss einer Phosphorylierung auf die Eigenschaften des heptameren Sec Komplexes nach. Erst wenn Sec63p phosphoryliert wird kann es den putativen Signalsequenzrezeptor Sec62p eng genug rekrutieren, um die Translokation von Proteinen über die Membran des endoplasmischen Retikulums auch effizient zu initiieren.

Table of Contents

Abbreviations

APS	ammonium persulfate
BSA	bovine serum albumin
CPY	carboxypeptidase Y
CIP	calf intestinal phosphatase
CK2	casein kinase 2
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ER	endoplasmic reticulum
E.coli	*Escherichia coli*
EDTA	ethylendiamine-N,N-tetracetate
Endo H	endoglycosidase H
5-FOA	5-fluoroorotic acid
GEF	Guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
Ha	hemagglutinin
HEPES	N-hydroxyethylpiperazine-N'-2-ethansulphoxide
HRP	horseradish peroxidase
Hsp	heat shock protein
Ig	immunoglobulin
IP	immunoprecipitation
IPTG	Isopropyl-beta-D-thiogalactopyranoside
MT	mutant
MW	molecular weight
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethyl sulfonyl fluoride

RNC	ribosome nascent chain
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRP	signal sequence recognition particle
SR	SRP receptor
TCA	trichloroacetic acid
TEMED	N,N,N´,N´-tetramethylethylenediamine
TRIS	tris-(hydoxymethyl)-aminomethane
UPR	unfolded protein response
Ub	ubiquitin
UV	ultraviolet
WT	wild type
YPD	yeast peptone dextrose
YPG	yeast peptone galactose

1. Introduction

1.1 Protein sorting

A eukaryotic cell consists of several membrane enclosed compartments. Each compartment, or organelle, contains its own characteristic set of proteins that confer upon their host compartment its characteristic structural and functional properties. The resident proteins catalyze the reactions that occur in the host organelle or selectively transport molecules into and out of its interior, or lumen.

In eukaryotic cells, most of the proteins are synthesized on ribosomes in the cytosol, except for the few that are synthesized by the ribosomes of the mitochondria or by the plastids of plant cells. To reach their destination in one of the membrane enclosed compartments, the proteins contain short stretches in their amino acid sequences that direct their transport from the cytosol across the membrane of the corresponding organelle. These stretches of sequences are called signal sequences and are bound by one or more signal sequence specific receptors in the cytosol of the cell. These receptors then guide the signal sequence containing protein to a channel where the translocation across the membrane occurs (Blobel et al., 1979). Translocation requires energy which is obtained by ATP or GTP hydrolysis at either the cytosolic or lumenal site of the membrane.

1.2 The endoplasmic reticulum (ER)

All proteins that are secreted from the cell or that localize in the endosome, the vacuole, the Golgi apparatus or in the plasma membrane first have to enter the endoplasmic reticulum (ER) (Katz et al., 1977; Palade, 1975). As a consequence, in addition to lipid production and regulation of the intracellular Ca^{2+} concentration, the ER plays a central role in protein folding, maturation and sorting of most proteins of the secretory pathway.

Beside promoting the folding and sorting of the secretory proteins, the ER is also a major quality control compartment where all proteins are checked for proper folding before they leave the ER. Proteins that do not fold correctly are translocated back from the ER into the cytosol, where they are deglycosylated, ubiquitylated, and degraded by the proteasomes (Bonifacino and Weissman, 1998; Johnson and Haigh, 2000). Once misfolded proteins accumulate excessively in the ER, the unfolded protein response is initiated, leading to the expression of appropriate proteins that help the ER to cope with this stress (Chapman et al., 1998; Hampton, 2000; Liu and Kaufman, 2003; McMillan et al., 1994; Pahl, 1999; Sidrauski et al., 1998). This unique signaling pathway recognizes unfolded proteins in the lumen of the ER and transmits this signal via the unconventional mRNA splicing of a transcription factor to the nucleus of the cell (Shamu, 1997; Shamu, 1998; Sidrauski et al., 1998).

1.3 Protein translocation across the ER membrane

The first step in correctly sorting proteins of the secretory pathway is their targeting to and translocation across the membrane of the ER. Protein translocation into the ER can occur while the protein is still being synthesized by the ribosome (cotranslational protein translocation) or after its translation has been completed (posttranslational protein translocation) (Rapoport et al., 1996). In mammalian cells most proteins are translocated cotranslationally. In contrast in the yeast *Saccharomyces cerevisiae*, both the cotranslational and the posttranslational targeting pathways are extensively used (Ng et al., 1996).

1.3.1 The nature of the signal sequences

Common to both eukaryotic targeting pathways is a signal sequence at the N-terminus of the protein. This signal sequence is necessary and sufficient to target the protein to the ER.

Signal sequences vary in their amino acid composition and length, but they all contain a positively charged N-terminus of one to five residues, a central hydrophobic core of six to fifteen amino acids and a C-terminal polar region of three to seven amino acids (von Heijne, 1985). Studies with signal sequence mutants revealed that the hydrophobic core region is the most essential part required for protein targeting and translocation (von Heijne, 1985). The polar region contains a recognition site for signal peptidases which remove the signal sequence from the protein, once its targeting function has been completed (von Heijne, 1990). The cleavage occurs either during translocation or soon after completion of translocation. The yeast peptidase is a membrane bound heterotetrameric protein complex whose enzymatic activity is provided by the subunit Sec11p (Bohni et al., 1988; YaDeau et al., 1991) and Spc3p (Fang et al., 1997; Meyer and Hartmann, 1997). The active site of the peptidase is surrounded by an extended hydrophobic patch. This patch is suggested to be near the membrane, where it meets the cleavage site of the translocating substrates (Paetzel et al., 1998).

In yeast *Saccharomyces cerevisiae,* signal sequences can choose between the cotranslational or posttranslational mode of targeting to the ER membrane (Hann and Walter, 1991). It was found that the degree of the hydrophobicity of the signal sequence determines by which pathway the signal sequence bearing protein will be translocated across the membrane (Ng et al., 1996). The proteins with very hydrophobic signal sequences, including transmembrane proteins, use predominantly the cotranslational route (Ng et al., 1996), whereas the proteins containing less hydrophobic sequences translocate predominantly posttranslationally across the ER membrane (Johnsson and Varshavsky, 1994b; Ng et al., 1996).

1.3.2 Cotranslational protein translocation

During cotranslational translocation, targeting to the membrane of the endoplasmic reticulum begins as soon as the signal sequence of the nascent polypeptide chain emerges from the ribosome (Walter and Johnson, 1994). A cytosolic factor, the signal

recognition particle (SRP) immediately binds to the accessible signal sequence (Walter and Blobel, 1981). Upon binding the further synthesis of the nascent chain is stopped or at least slowed down. SRP consists of one copy of 7S RNA and six polypeptides (Walter and Blobel, 1982). The 54 kDa subunit of the SRP (SRP54) interacts with the signal sequence (Krieg et al., 1986; Kurzchalia et al., 1986). SRP54 comprises a GTPase domain (Bernstein et al., 1989), and a methionine-rich M-domain that has been shown to contain the signal sequence binding site (High and Dobberstein, 1992; Zopf et al., 1990). It was proposed that the highly flexible side chains of the methionines line the walls of a hydrophobic pocket, forming a "methionine-bristle" that would allow the recognition of a wide variety of signal sequences (Bernstein et al., 1989). A structural analysis of a bacterial homologue of SRP54 confirmed the existence of a hydrophobic groove in the M-domain (Keenan et al., 1998).

Ribosomes carrying a nascent polypeptide together with a SRP bound to its signal sequence are then targeted to the endoplasmic reticulum by two affinities. The ribosome interacts with its membrane receptor, the Sec61p complex (Jungnickel and Rapoport, 1995), and SRP interacts with the SRP receptor (SR) (Gilmore et al., 1982; Meyer et al., 1982). After binding of SRP to SR, the signal sequence is released from SRP54 (Rapiejko and Gilmore, 1997). It is then free to contact the translocation site at the ER membrane (translocon). Protein translocation through the membrane is initiated and protein synthesis is resumed.

The SRP receptor has two subunits, SRα and SRβ. Like SRP54, they both contain GTPase domains (Connolly and Gilmore, 1989; Miller et al., 1995). It is therefore assumed that the whole targeting process is regulated by three G proteins, SRP54, SRα and SRβ and the hydrolysis of GTP to GDP and Pi. The nucleotide-free SRP54 binds to the signal sequence directly. The complex is thought to remain in a state of low affinity for GTP. The interaction of SRP with the ribosome might increase the affinity of SRP for GTP (Bacher et al., 1996). A further increase occurs upon targeting of the ribosome-nascent chain-SRP (RNC-SRP) to the ER membrane and

interaction of SRP with its receptor (SR) (Miller et al., 1993; Rapiejko and Gilmore, 1997). SR and SRP interact with each other in the GTP bound state. SRP-GTP remains associated with SR and would dissociate only after GTP hydrolyzes into GDP (Connolly et al., 1991). It has been shown that SRP54 and SRα act as GTPase-activating proteins for each other while they are in their GTP-bound form (Lu et al., 2001). After GTP hydrolysis SRP54 dissociates from the membrane, releases GDP

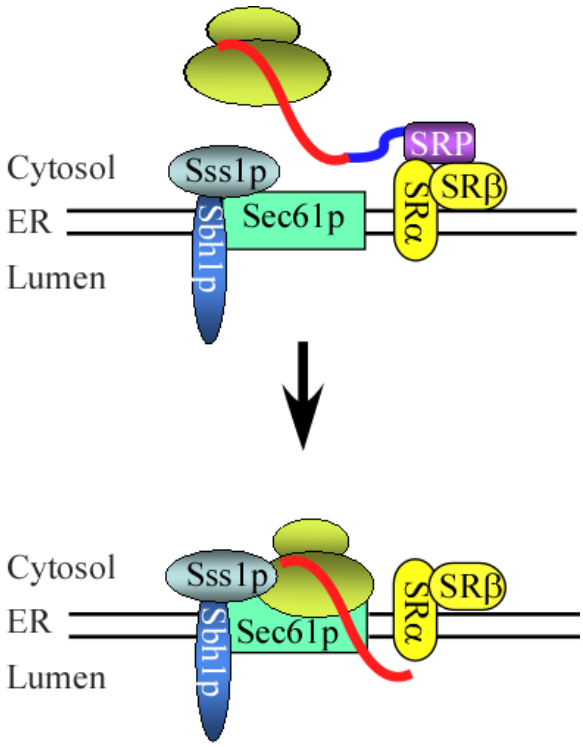

Figure 1.1 A model for cotranslational protein translocation: As soon as it emerges from the ribosome, the N-terminal signal sequence of the nascent polypeptide is recognized by the signal recognition particle (SRP). The complex of SRP, nascent polypeptide and ribosome binds to the ER membrane through the SRP receptor. The signal sequence is then transferred to the trimeric Sec61p complex. Protein synthesis is resumed and the nascent chain is translocated across the membrane.

and remains in a nucleotide-free form until the next round of targeting (Connolly et al., 1991; Rapiejko and Gilmore, 1997). In this process, a guanine nucleotide exchange factor (GEF) is not required for G domain function (Powers and Walter, 1995).

Structural and biochemical data suggest that the dimerization of the eukaryotic SRP receptor is also regulated by a GTP/GDP switch cycle (Schwartz and Blobel, 2003). Here only the GTP bound form of SRβ can form the stable dimer with SRα. Surprisingly, SRβ recruits SRP-RNCs to the protein-conducting channel even in the absence of its N-terminal membrane anchor (Ogg et al., 1998). This suggests that SRβ associates with additional components of ER membrane, probably its potential guanine nucleotide exchange factor (GEF) Sbh1p (Helmers et al., 2003; Wittke et al., 2002). It was observed that in the absence of a functional Sec61 complex, the signal sequence could not be released from the SRP54 protein (Song et al., 2000). The authors therefore proposed that both the GTP hydrolysis of SRP and its receptor are coordinated by the Sec61 complex.

1.3.3 Posttranslational protein translocation

During posttranslational translocation the protein is already synthesized before it contacts the membrane of the ER. Once synthesized, the full-length substrate first interacts with several different cytosolic proteins that keep the protein from aggregating and misfolding and therefore competent for translocation. At least two distinct protein complexes were identified that contain among other proteins either 70 kDa heat shock protein (Hsp70) or the chaperon containing TCP1 (TRiC/CCT) (Deshaies et al., 1988; Plath and Rapoport, 2000). The targeting to the ER membrane is apparently SRP independent, but still requires the signal sequence. However the hydrophobicity does not suffice to make a stable contact with the SRP of yeast. Instead, the signal sequence interacts specifically with the proteins Sec62p and Sec61p at the membrane of the ER (Dünnwald et al., 1999; Plath et al., 1998; Plath et al., 2004). Cross-linking studies indicate that the signal sequence interacts in an ATP-independent reaction with Sec61p by intercalation into transmembrane domains 2 and 7 (Plath et al., 1998). While bound to Sec61p, the signal sequence forms a helix that is contacted at the same time by Sec62p and Sec71p (Plath et al., 1998; Plath et al., 2004). The translocation substrate is then stripped of all cytosolic proteins, allowing it

to subsequently be transported through the membrane channel without the interference of its cytosolic binding partners (Plath and Rapoport, 2000).

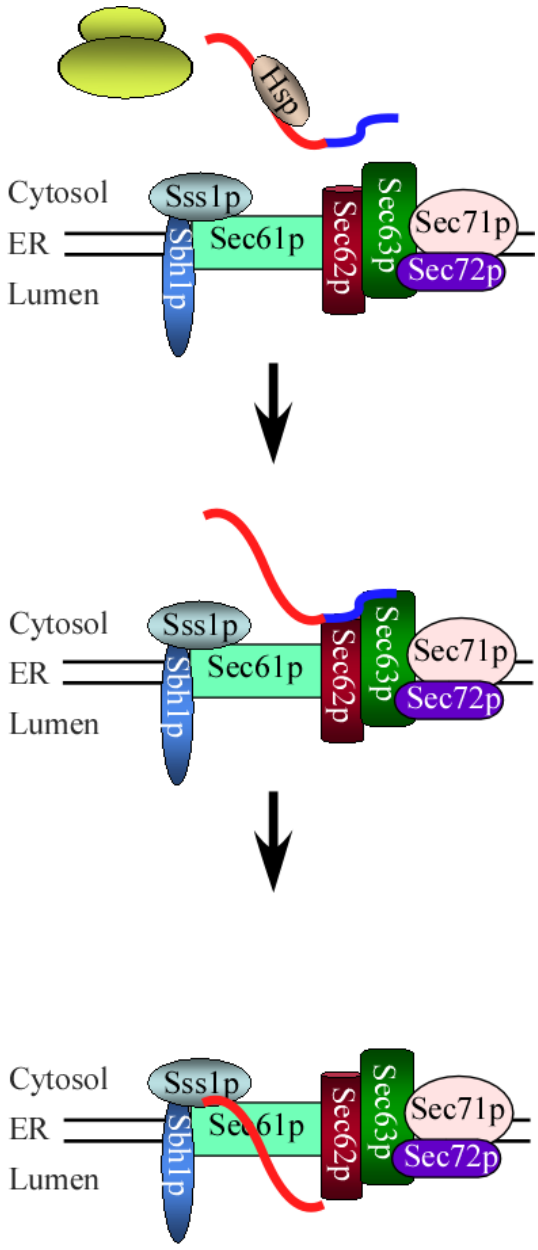

Figure 1.2 A model for posttranslational protein translocation: The completely synthesized protein binds to chaperons to remain in a translocation competent state. The signal sequence is recognized by the heptameric Sec complex at the ER membrane and the polypeptide is translocated into the ER lumen via the Sec61p channel.

1.3.4 The translocation channel

After the targeting to the ER membrane is completed, the nascent chain is translocated across the membrane via an aqueous pore (Gilmore and Blobel, 1985). The central component of the channel is identical for both targeting pathways. It is a multispanning integral membrane protein named Sec61p. Sec61p was first identified by genetic studies in yeast (Deshaies and Schekman, 1987; Stirling et al., 1992; Wilkinson et al., 1996). Sec61p is required for the translocation of both secretory and membrane proteins and is proposed to form the actual translocation channel across the ER membrane. To form a functional channel, Sec61p interacts with Sss1p (Esnault et al., 1993; Wilkinson et al., 1997) and Sbh1p (Panzner et al., 1995) to assemble into the trimetric Sec61p complex. These three components Sec61p, Sbh1p and SSS1p are also found in higher eukaryotes including mammals and are termed the alpha, beta and gama subunit of the Sec complex. Yeast cells have homologues of Sec61p and Sbh1p, named Ssh1p and Sbh2p, respectively (Finke et al., 1996; Toikkanen et al., 1996). These proteins together with Sss1p form an alternative trimeric complex that functions solely in the cotranslational translocation of protein across the membrane of the ER (Finke et al., 1996; Wittke et al., 2002).

The crystal structure of the trimeric Sec61p complex from *Methanococcus jannaschii* has recently been solved (Van den Berg et al., 2004). The solved structure shows the channel of the Sec61p complex in a closed conformation. The twelve transmembrane segments of the three subunits closely align into one single complex. The transmembrane segments 2, 7, 10 of the α subunit form a narrow channel that is proposed to be the pore through which the translocation substrate moves across the membrane. The transmembrane domain 2a of Sec61p, which is called the plug blocks the entry of the channel on the lumenal site of Sec61p. The authors propose that upon signal sequence binding the transmembrane segments 2 and 7 move slightly relative to each other to remove the plug during protein translocation.

Since the ER membrane encloses a special folding milieu, it is important to maintain it impermeable to even small molecules. The Sec61p translocation channel must open to translocate proteins and close when the work is done. The crystal structure of the Sec61p complex confirms the existence of a very narrow channel that is closed in the absence of a translocation substrate (Van den Berg et al., 2004). However Hamman et al. demonstrated with quenching agents of different sizes that the actual size of the channel could expand up to 60 Å (Hamman et al., 1997). By which mechanism the channel as pictured by its X-ray structure can accommodate an opening of 60 Å is still mysterious. Independent of its actual size, Crowley et al. first illustrated the existence of a tight seal by fluorescent quenching studies. They demonstrated that even small molecules from the cytoplasmic side of the membrane could not reach the translocating polypeptide that was labeled with fluorescent probes (Crowley et al., 1993). Since their studies demonstrated that the channel is impermeable even to ions, the opening and closing of the channel must be tightly regulated.

The cytoplasmic surface of Sec61p is the binding site for the ribosome and has been proposed to interact with the signal recognition particle receptor (Morrow and Brodsky, 2001). This model was partially confirmed by a three dimensional image reconstruction of a ribosome bound to the yeast Sec61 complex. The pictures revealed an alignment of the translocation channel with the site on the large ribosomal unit which is believed to be the exit site for the nascent polypeptide (Beckmann et al., 1997). As the protein is elongated, it has only one way out, and protein synthesis directly drives translocation. Electron microscopy studies also verified that the Sec61p complex forms a channel (Hanein et al., 1996). Purified complexes from mammalian and yeast cells formed ring-like structures in detergent with a pore diameter of about 20 Å. Each pore consisted of three or four trimeric Sec61p complexes. Similar ring structures were also seen in reconstituted and native membranes. The addition of ribosomes was seen to increase the number of ring structures in reconstituted membranes (Hanein et al., 1996). Yet, when the purified mammalian Sec61p complex was reconstituted into proteoliposomes, no rings were seen. However, the ring-like structures reappeared when the membranes were incubated with ribosomes. This was

confirmed with proteoliposomes containing the yeast Sec61p complex (Hanein et al., 1996). Again, ring-like structures were seen after addition of ribosomes to the vesicles. Interestingly, the same structures appeared after addition of the Sec62/63p complex. Sec63/63p is part of a tetrameric complex of membrane proteins that interacts with the trimeric Sec61p complex to facilitate posttranslational translocation of proteins (Deshaies et al., 1991). All these data suggest that oligomerization of the Sec61p complex may be induced by its interacting partner(s) in either cotranslational or posttranslational protein transport. This interpretation is in conflict with the X-ray structure of the Sec61 complex. This conflict is probably only resolved once the structure of the signal sequence bound Sec61p complex will have been analyzed by X-ray crystallography.

1.3.5 The heptameric Sec complex

The trimetric Sec61p complex assembles with four additional subunits into the heptameric Sec complex. This complex constitutes the functional unit for posttranslational protein translocation (Deshaies et al., 1991; Panzner et al., 1995). The four additional subunits are organized into a subcomplex that is named the tetrameric Sec62/63p complex. Besides the two essential membrane proteins Sec62p and Sec63p, the complex contains the nonessential membrane proteins Sec71p and Sec72p (Deshaies et al., 1991; Lyman and Schekman, 1997).

The nonlethal sec71-1 and sec72-1 mutations were identified by a genetic screen that monitored membrane protein integration into the endoplasmic reticulum membrane of the yeast *Saccharomyces cerevisiae*. The mutations inhibited integration of various chimeric membrane proteins and translocation of a subset of water soluble proteins (Fang and Green, 1994). *SEC71* encodes the 31.5 kDa glycoprotein with one domain of sufficient length and hydrophobicity to span a lipid bilayer (Feldheim et al., 1993). *SEC72* encodes the 23 kDa subunit of the Sec62/63p complex with no transmembrane domain (Feldheim and Schekman, 1994). Sec72p and Sec71p might contribute to

protein translocation by the selective recognition of a subset of signal sequences (Feldheim and Schekman, 1994).

Sec62p is proposed to be involved in signal sequence recognition during the targeting of the nascent chain to the translocation channel (Dünnwald et al., 1999; Plath et al., 2004; Wittke et al., 2000). Sec62p is a 32 KDa protein which consists of two large cytosolic domains at its N- and C-terminus. The two domains are connected via two transmembrane elements (Deshaies and Schekman, 1989). The N-terminal domain of Sec62p directly interacts with an acidic stretch located at C-terminal end of Sec63p (Willer et al., 2003; Wittke et al., 2000). The C-terminal domain of Sec62p harbors an additional binding site to the Sec complex (Wittke et al., 2000). The corresponding binding partner for this site is not yet identified.

Sec63, an ER integral membrane protein that shares homology with the *Escherichia coli* DnaJ protein, has three transmembrane domains (Feldheim et al., 1992). Its J-domain is located between the transmembrane regions two and three in the lumen of the ER. Its large cytosolic 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 substrate from sliding back to the cytosol (Matlack et al., 1999; Sanders et al., 1992). One role of Sec63p in protein translocation is therefore to recruit the active form of Kar2p to the translocating polypeptide and to keep it there in a high local concentration. By this mechanism the interplay of both proteins provides the otherwise random movement of the nascent chain with the required directionality. In contrast to the other members of the tetrameric Sec62/63p complex, Sec63p together with Kar2p fulfills additional functions during cotranslational translocation (Young et al., 2001) and retrotranslocation (Plemper et al., 1997). Whether Sec63p performs these roles in the context of the tetrameric Sec62/63p complex or in combination with other partner proteins is unknown.

1.4 Protein modification in the ER and retrotranslocation

Protein translocation is not an isolated activity. Since the nascent chain translocates as an unfolded polypeptide across the membrane, it has to fold in the lumen of ER. During translocation the nascent chain is glycosylated by a complex of enzymes that is closely located at the lumenal exit site of the translocation channel (Scheper et al., 2003). The glycosylation of the nascent chain helps the protein to fold properly and at the same time serves as a signal whether folding of the protein is achieved or not. Unfolded and unglycosylated proteins are bound by heat shock proteins and free cysteines are oxidized and reshuffled by protein disulfide isomerases (Frand et al., 2000; Stevens and Argon, 1999; Tanner and Lehle, 1987). If this folding machinery fails to fold a nascent chain into its proper structure, the polypeptide is escorted to the Sec61p complex where it is translocated back to the cytosol. Here the polypeptide is deglycosylated, ubiquitinated and subsequently degraded by the proteasome (Pilon et al., 1997; Wiertz et al., 1996).

1.5 Aims of my project

Both cotranslational and posttranslational targeting pathways share the same trimeric Sec61p complex as the channel for translocation. While it is known that the trimeric Sec61p complex cannot initiate posttranslational translocation without the help of the tetrameric Sec62/Sec63p complex, it is assumed that vice vera the heptameric Sec complex can not initiate cotranslational translocation (Panzner et al., 1995). To be able to adjust the two pathways to a changing composition of signal sequences in the cell, I postulated at the beginning of my work that the assembly or disassembly of the heptameric Sec complex might be regulated. Conditions that induce the disassembly of the heptameric Sec complex would free the trimeric Sec61p complex to receive more cotranslational translocation substrates, while conditions that strengthen the heptameric Sec complex would increase the capacity to translocate proteins that use the posttranslational translocation pathway.

To learn more about the assembly of the Sec complex, I decided to further map the interactions between the members of the Sec complex and focused especially on the interaction between Sec62p and Sec63p.

2. Materials and Methods

2.1 Materials

2.1.1 Instruments

Name	**Source**
Agarose gel electrophoresis chamber	Peqlab, Erlangen
Bacteria/yeast incubator	Heraeus, Stuttgart
Bacteria/yeast shaker	Infors,Bottmingen, Switzerland
Developing machine	Kodak, New Haven,USA
Cold centrifuge 5810R	Eppendorf, Hamburg
PCR thermocycler PTC-200	MJ Research, Waltham, USA
pH meter	Eppendorf, Hamburg
Photometer	Eppendorf, Hamburg
Proteinblot chamber TE77	Hoefer, San Francisco, USA
Proteinelectrophoresis chamber	Hoefer, San Francisco, USA
Thermomixer	Eppendorf, Hamburg
Table centrifuge Biofuge fresco	Heraeus, Stuttgart
Table centrifuge Biofuge pico	Heraeus, Stuttgart
Ultracentrifuge L7-65	Beckman, Stuttgart
Vortex Genie2	Bender und Hobein, Karlsruhe

2.1.2 Consumable materials

Name	**Source**
Eppendorf tube	Eppendorf, Hamburg
Film	Amersham, Freiburg
Glass beads (425-600 um)	Sigma, Steinheim
Nitrocellulose membrane	Biorad, München

PCR tube	Roth, Karlsruhe
Whatman paper	Bender und Hobein, Karlsruhe

2.1.3 General Chemicals

Name	**Source**
Acrylamide	Roth, Karlsruhe
Agarose	Peqlab, Erlangen
Ampicillin	Roth, Karlsruhe
APS	Roth, Karlsruhe
Bromophenolblue	Sigma, Steinheim
BSA	Promega, Mannheim
Calcium acetate	Roth, Karlsruhe
Calcium chloride	Roth, Karlsruhe
Chloroform	Roth, Karlsruhe
DMSO	Fluka, Buchs, Swiss
DTT	Roth, Karlsruhe
EDTA	Roth, Karlsruhe
Ethanol	Roth, Karlsruhe
Ethidium bromide	Roth, Karlsruhe
Galactose	Roth, Karlsruhe
Geniticin	Invitrogen, Carlsbad, USA
Glucose	Roth, Karlsruhe
Glycin	Roth, Karlsruhe
Glycerol	Roth, Karlsruhe
HEPES	Roth, Karlsruhe
Isoamylalcohol	Roth, Karlsruhe
Isopropanol	Roth, Karlsruhe
Methanol	Roth, Karlsruhe

Natrium chloride	Roth, Karlsruhe
Natrium hydroxide	Roth, Karlsruhe
PEG	Roth, Karlsruhe
Phenol	Roth, Karlsruhe
Ponceau S	Sigma, Steinheim
SDS	Roth, Karlsruhe
TEMED	Roth, Karlsruhe
Tris	Roth, Karlsruhe
Triton X-100	Sigma, Steinheim
Tween	Roth, Karlsruhe

2.1.4 Chemicals for media

Name	**Source**
Adenine	Sigma, Steinheim
Arginine	Roth, Karlsruhe
Bacto-Agar	Nordwald, Hamburg
Bacto-Yeast-Extract	Roth, Karlsruhe
Bacto-Pepton	Roth, Karlsruhe
Histidine	Ducheta, Haarlem, Netherlands
Isoleucine	Ducheta, Haarlem, Netherlands
Leucine	Ducheta, Haarlem, Netherlands
Lysine	Merck, Darmstadt
Methionine	Ducheta, Haarlem, Netherlands
Phenylalanine	Ducheta, Haarlem, Netherlands
SD (Difco Yeast Nitrogen Base)	Becton, Dickinson, Sparks, USA
Threonine	Sigma, Steinheim
Tryptophan	Ducheta, Haarlem, Netherlands

Uracil	Sigma, Steinheim

2.1.5 Other chemicals

Name	**Source**
1Kb DNA ladder	Biozym, Oldendorf
dNTP	New England Biolabs, Frankfurt
ECL Western Blotting Analysis System	Amersham, Freiburg
IgG-Sepharose 6 Fast Flow	Amersham, Freiburg
Milk powder	Saliter, Obergünzburg
Molecular Marker for SDS-PAGE	Amersham, Freiburg
Phosphatase Inhibitor 1 and 2	Sigma, Steinheim
Protease Inhibitor Cocktail	Roche, Mannheim
PMSF	Serva, Heidelberg
Protein-G-Plus-Agarose	Oncogene, Cambridge
Anti-Ha Antibody Agarose	Hiss Diagnostics, Freiburg

2.1.6 Kits

Name	**Source**
Easy pure DNA Purification Kit	Biozym, Oldendorf
High Pure PCR-Product Purification Kit	Roche, Mannheim

2.1.7 Media for bacterial culture

2.1.7.1 LB liquid medium

0.5% (w/v) Bacto-Yeast-Extract

1% (w/v) Bacto-Peptone

1% (w/v) NaCl

The medium was autoclaved and 0.01% (w/v) ampicillin was added to the medium after it was cooled down to 40° C.

2.1.7.2 LB agar plates

The same recipe as for the liquid medium was used except for adding 2% (w/v) Bacto-Agar before autoclaving. For the ampicillin plates, 0.01% (w/v) ampicillin was added.

2.1.8 Media for yeast cultures

2.1.8.1 Liquid media

YPD or YPG	**Selective medium (SD or SG)**
1% (w/v) Bacto-Yeast-Extract	0.67% (w/v) SD
2% (w/v) Bacto-Peptone	2% (w/v) Glucose or 2% (w/v) Galactose
2% (w/v) Glucose or 2% (w/v) Galactose	0.002% (w/v) Arginine
0.004% (w/v) Adenine in 0.1 M NaOH	0.006% (w/v) Isoleucine
	0.004% (w/v) Lycine
	0.001% (w/v) Methionine
	0.006% (w/v) Phenylalanine
	0.001% (w/v) Threonine
	0.002% (w/v) Adenine in 0.1 M NaOH
	According to the selective need:
	0.002% (w/v) Histidine
	0.006% (w/v) Leucine
	0.004% (w/v) Tryptophan
	0.005% (w/v) Uracil in 0.1 M NaOH

2.1.8.2 Agar plates

The same recipes as for the liquid medium were applied except for adding 2% (w/v) Bacto-Agar. For the geniticin plates, 0.02% (w/v) geniticin was added.

2.1.9 General buffers and solutions

6× DNA loading buffer	**2× SDS loading buffer**
30% (v/v) Glycerol 0.01% (w/v) Bromophenolblue 0.001% (w/v) RNase	120 mM Tris-HCl pH 6.8 4% (w/v) SDS 20% (v/v) Glycerol 100 mM DTT (freshly added) 0.01% (w/v) Bromophenolblue
Protein extraction buffer	**Lysis buffer**
50 mM Hepes pH 7.5 150 mM NaCl 1 mM EDTA pH 8.0 With or without 0.2% (v/v) Triton X-100	2% (v/v) Triton X-100 1% (w/v) SDS 100 mM NaCl 10 mM Tris-HCl pH 8.0 1 mM EDTA pH 8.0
SDS-PAGE laemmli buffer	**Transfer buffer**
25 mM Tris 250 mM Glycin 0.1% (w/v) SDS	24 mM Tris 190 mM Glycin 20% (v/v) Methanol 0.05% (w/v) SDS
TBST	**Minipreparation solution I**
20 mM Tris-HCl 150 mM NaCl 0.2% (v/v) Tween20, adjust to pH 7.6	25 mM Tris-HCl pH 8.0 10 mM EDTA pH 8.0 50 mM Glucose
Minipreparation solution II	**Minipreparation solution III**
200 mM NaOH	3 M Kalium acetate

1% (w/v) SDS	
TE buffer	**TAE buffer**
10 mM Tris-HCl pH 8.0 1 mM EDTA pH 8.0	40 mM Tris-Acetate 1 mM EDTA pH 8.0

2.1.10 Plasmids

Name	**Description**	**Reference**
pRS313	*CEN6 ARSH4 LacZ Amp^r HIS3*	Sikorski and Hieter, 1989
pRS314	*CEN6 ARSH4 LacZ Amp^r TRP1*	Sikorski and Hieter, 1989
pRS315	*CEN6 ARSH4 LacZ Amp^r LEU2*	Sikorski and Hieter, 1989
pRS316	*CEN6 ARSH4 LacZ Amp^r URA3*	Sikorski and Hieter, 1989
pRS304	*LacZ Amp^r TRP1*	Sikorski and Hieter, 1989
pRS305	*LacZ Amp^r LEU2*	Sikorski and Hieter, 1989
pYM8	*TEV-ProA kanMX6*	Knop et al., 1999
pFA6-P_{GAL1}	*Amp^r P_{GAL1} kanMX6*	Dr. Markus Albertsen
pRS314*Flag-FPR1-SEC63$_{C14}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P_{CUP1} Flag-FPR1-Sec63$_{C14}$*	Lab Dr. Nils Johnsson
pRS314*Flag-FPR1-SEC63$_{C14mutants}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P_{CUP1} Flag-FPR1-Sec63$_{C14mutants}$*	This work
pRS315*SEC62$_{\Delta C125}$-Dha*	*CEN6 ARSH4 LacZ Amp^r LEU2 P_{CUP1} Sec62$_{\Delta C125}$-Dha*	Lab Dr. Nils Johnsson
pRS314*Flag-FPR1-*	*CEN6 ARSH4 LacZ Amp^r TRP1*	Lab Dr. Nils Johnsson

$SEC63_{C47}$	*P_{CUP1} Flag-FPR1-$Sec63_{C47}$*	
pRS314*Flag-FPR1-$SEC63_{C47mutants}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P_{CUP1} Flag-FPR1-$Sec63_{C47mutants}$*	This work
pRS314*Flag-$SEC63_{\Delta N244}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P_{CUP1} Flag-$Sec63_{\Delta N244}$*	Lab Dr. Nils Johnsson
pRS314*Flag-$SEC63_{\Delta N244\Delta C47}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P_{CUP1} Flag-$SEC63_{\Delta N244\Delta C47}$*	Lab Dr. Nils Johnsson
pRS314*Flag-$SEC63_{\Delta N244\Delta C14}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P_{CUP1} Flag-$SEC63_{\Delta N244\Delta C14}$*	Lab Dr. Nils Johnsson
pRS314*N_{ui}-$SEC62_{\Delta C125}$-Dha*	*CEN6 ARSH4 LacZ Amp^r TRP1 P_{CUP1} N_{ui}- $SEC62_{\Delta C125}$-Dha*	Lab Dr. Nils Johnsson
pRS314*N_{ug}- SEC62*	*CEN6 ARSH4 LacZ Amp^r TRP1 P_{CUP1} N_{ug}- SEC62*	Lab Dr. Nils Johnsson
pRS313*SEC63-C_{ub}-RUra3*	*CEN6 ARSH4 LacZ Amp^r HIS3 P Met SEC63-C_{ub}-RUra3*	Lab Dr. Nils Johnsson
pRS313*$SEC63_{mutants}$-C_{ub}-RUra3*	*CEN6 ARSH4 LacZ Amp^r HIS3 P Met $SEC63_{mutants}$-C_{ub}-RUra3*	This work
pRS314 *N_{ug}- Ubc6*	*CEN6 ARSH4 LacZ Amp^r TRP1 P_{CUP1} N_{ug}-Ubc6*	Lab Dr. Nils Johnsson
pRS313*SEC62-GFP*	*CEN6 ARSH4 LacZ Amp^r HIS3 P_{CUP1} SEC62-GFP*	Lab Dr. Nils Johnsson
pRS313 *CPY*	*CEN6 ARSH4 LacZ Amp^r HIS3 P_{CUP1} CPY-C_{ub}-URA3*	Lab Dr. Nils Johnsson
pRS313 *Mfα1*	*CEN6 ARSH4 LacZ Amp^r HIS3 P_{CUP1} Mfα1-C_{ub}-URA3*	Lab Dr. Nils Johnsson
pRS313 *SUC2*	*CEN6 ARSH4 LacZ Amp^r HIS3 P_{CUP1} SUC2-C_{ub}-URA3*	Lab Dr. Nils Johnsson
pRS313 *KAR2*	*CEN6 ARSH4 LacZ Amp^r HIS3 P_{CUP1} KAR2-C_{ub}-URA3*	Lab Dr. Nils Johnsson

pRS316*Met- SEC63*	*CEN6 ARSH4 LacZ Amp^r URA3 P Met SEC63*	Lab Dr. Nils Johnsson
pRS314*Met-SEC63$_{\Delta C47C17}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P Met SEC63$_{\Delta C47C17}$*	This work
pRS314*Met-SEC63$_{\Delta C47C20}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P Met SEC63$_{\Delta C47C20}$*	This work
pRS314*Met-SEC63$_{\Delta C47C24}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P Met SEC63$_{\Delta C47C24}$*	This work
pRS314*Met-SEC63$_{\Delta C47C28}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P Met SEC63$_{\Delta C47C28}$*	This work
pRS314*SEC62*	*CEN6 ARSH4 LacZ Amp^r TRP1 P$_{CUP}$ SEC62-Dha*	Lab Dr. Nils Johnsson
pRS314*SEC62$_{\Delta C19}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P$_{CUP}$ SEC62$_{\Delta C19}$-Dha*	Lab Dr. Nils Johnsson
pRS314*SEC62$_{\Delta C35}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P$_{CUP}$ SEC62$_{\Delta C35}$-Dha*	Lab Dr. Nils Johnsson
pRS314*SEC62$_{\Delta N144}$*	*CEN6 ARSH4 LacZ Amp^r TRP1 P$_{CUP}$ SEC62$_{\Delta N144}$-Dha*	Lab Dr. Nils Johnsson
pRS316*Flag-SEC62*	*CEN6 ARSH4 LacZ Amp^r URA3 P$_{CUP}$ Flag-SEC62*	Lab Dr. Nils Johnsson
pET15b*SEC62$_{\Delta C}$-Ha*	*CEN6 ARSH4 LacZ Amp^r P SEC62$_{\Delta C}$*-Ha	This work

2.1.11 Primers

2.1.11.1 Standard primers

Name	Sequence
Sec63C14T1	5'-GGC TGC AGG AAT TCA TCG ATG CGG ATA CAG AAG CTG AAG ATG-3'

Sec63C14T2	5’-GGC TGC AGG AAT TCA TCG ATA CGG ATG CAG AAG CTG AAG ATG-3’
Sec63C14D1	5’-GGC TGC AGG AAT TCA TCG ATG ATG ATA CAG AAG CTG AAG ATG-3’
Sec63C14D2	5’-GGC TGC AGG AAT TCA TCG ATA CGG ATG ATG AAG CTG AAG ATG-3’
Sec63C14E1	5’-GGC TGC AGG AAT TCA TCG ATG AGG ATA CAG AAG CTG AAG ATG-3’
Sec63C14E2	5’-GGC TGC AGG AAT TCA TCG ATA CGG ATG AGG AAG CTG AAG ATG-3’
Sec63C14T1T2	5’-GGC TGC AGG AAT TCA TCG ATG CGG ATG CAG AAG CTG AAG ATG-3’
Sec63C14S3	5’-GGC TGC AGG AAT TCA TCG ATA CGG ATA CAG AAG CTG AAG ATGAT GAA GCA CCA GAA TAG-3’
Sec63ProA-Fw	5’-CTG ATA TCG ATA CGG ATA CAG AAG CTG AAG ATG ATG AAT CAC CAG AAC GTA CGC TGC AGG TCG AC-3’
Sec63T2ProA-Fw	5’-CTG ATA TCG ATA CGG ATG CAG AAG CTG AAG ATG ATG AAT CAC CAG AAC GTA CGC TGC AGG TCG AC-3’
Sec63ProA-Re	5’-ATA TAC GTC TAA GAG CTA AAA TGA AAA ACT ATA CTA ATC ACT TAT ATA TCG ATG AAT TCG AGC TCG-3’
Sec63 Gal-Fw	5’-GAC TGG AAC AAT AGT CAG TTA TAA TTG ACC AAA GAA GGC CTT CGC GCA TAG GCC ACT AGT GGA TC-3’
Sec63 Gal-Re	5’-CGG CCA CGT CTC ACT AGC CTC ATC ATA CTC GTA ATT TGT AGG CAT TTT GAG ATC CGG GTT TTT TCT C-3’
CKA1-Fw	5’-GAT GGG ACA CCC GTG GTT TGC CCC AAT AAG GGA ACA AAT TGA AAA ACG TAC GCT GCA GGT CGA C-3’
CKA1-Re	5’-TTG TAC AGA TGG TAA AAA AAA GTA ATC GTT ATA TCG TTT GTC AGT GAT CGA TGA ATT CGA GCT CG-3’
Sec63C17	5’-GAC CAC CTG CAG TGT ATA CTG ATA TCG ATA CGG AT-3’
Sec63C20	5’-GAC CAC CTG CAG TGG CTA GCG ATT ATA CTG ATA TC -3’
Sec63C24	5’-GAC CAC CTG CAG TGG ATG AAA GTG ATG CTA GCG AT -3’
Sec63C28	5’-GAC CAC CTG CAG TGA CCG AAA GTG ATG ATG AAA GT -3’

Sec62pET Fw	5'-CCT CCC CAT ATG TCA GCC GTA GGT CCA GGT AGC-3'
Sec62pET-Ha Re	5'-CCT CCC CTC GAG CTA GCC CGC ATA GTC AGG AAC ATC GTA TGG GTA CGC ATC CAT GTAGGT TCT AGG GTT -3'

2.1.11.2 Mutagenesis primers

Name	Sequence
Sec63C14D1D2-Fw	5'-CAA CTC TGA TAT CGATGA TGA TGA TGA AGC TG -3'
Sec63C14D1D2-Re	5'-CAG CTT CAT CAT CAT CAT CGA TAT CAG AGT TG-3'

2.1.12 Enzymes

All restriction endonucleases and other modifying enzymes were purchased from Invitrogen GmbH (Karlsruhe), Promega (Mannheim), New England Biolabs (Frankfurt), and Roche (Mannheim) unless otherwise stated.

2.1.13 Antibodies

2.1.13.1 Primary antibodies

Name	Supplier
Anti-Flag, mouse monoclonal	Sigma, Steinheim
Anti-Ha, mouse monoclonal	Covance, Freiburg
Anti-CPY, mouse monoclonal	Molecular Probes, Portland, USA
Anti-Sec63p, rabbit polyclonal	Lab Dr. Enno Hartmann, Lab Dr. Jeff Brodaky
Anti-Sec62p, rabbit polyclonal	Lab Dr. Enno Hartmann

2.1.13.2 Secondary antibodies

Name	Supplier
Goat-anti-Mouse	Biorad, München
Goat-anti-Rabbit	Biorad, München

Rabbit-anti-Goat	DakoCytomation, Hamburg

2.1.14 Yeast strains

Strain	**Relevant Genotype**	**Source/Comment**
JD53	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52*	Dohmen et al., 1995
JD53Gal-63	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 GAL-SEC63-KanMX6*	Lab Dr. Nils Johnsson
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* $sec63_{T654A}$*-PROA-kanMX4*	this work
NJY148	*MATα his3-Δ200 leu2-3,112 lys252-801 trp1-Δ63 ura3-52* $sec63_{T652A}$*::pRS304*	this work
NJY149	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52* $sec63_{T654A}$*::pRS304*	this work
NJY150	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52* $sec63_{T654D}$*::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 cka2-1::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:kanMX4sec63$_{T654A}$::pRS304P$_{CUP1}$SEC62:pRS316*	this work
NYJ126ΔN144	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-63 ura3-52 SEC62::kanMX4 P$_{CUP1}$sec62$_{N144}$:pRS314*	Wittke et al., 2000

2.2 Methods

2.2.1 Plasmid constructions

2.2.1.1 Preparation of chemically competent *E.coli* cells

A single colony of *E.coli* (DH5α) from an LB agar plate was inoculated into 3 ml LB liquid medium and incubated overnight at 37ºC with shaking (220 rpm). 1 ml of this culture was diluted to 100 ml fresh LB medium and allowed to grow to an OD_{600} of 0.3-0.5. Bacteria were collected by centrifugation at 4000 rpm for 10 minutes at 4°C. The pellet was washed with 25 ml of chilled sterile $CaCl_2$ (100 mM) and recovered by centrifugation at 4000 rpm for 10 minutes at 4°C. Then the pellet was resuspended in 5 ml chilled sterile $CaCl_2$ (100 mM), incubated on ice for at least 30 minutes and precipitated by centrifugation at 4000 rpm for 5 minutes at 4°C. Finally, the bacteria were resuspended in 6 ml chilled $CaCl_2$/glycerol (4.8 ml 100 mM $CaCl_2$ plus 1.2 ml 86% (v/v) glycerol). After 5 minutes of incubation on ice, the bacteria were dispensed in 200 µl aliquots to 30 pre-chilled eppendorf tubes and snap-frozen in liquid nitrogen before being stored at -80°C.

2.2.1.2 Chemical transformation

Depending on the application, 1 µg of super coiled plasmid or 1 µl of a ligation mix (usually a 1/10 of the ligation) was added to 100 µl ice-thawed competent cells and left on ice for a period of 30 minutes. Following this, the cells were heat-shocked at

42°C for 90 seconds before rapidly returning to ice for a few minutes. After addition of 1 ml antibiotic free LB medium, the bacteria were incubated for 45 minutes at 37°C with shaking. Finally the bacteria were spread on LB agar plates supplemented with the correct antibiotic and the plates were incubated 18-24 hours at 37°C.

2.2.1.3 Small scale plasmid preparation (minipreparation)

Individual colonies were picked from LB plate and inoculated into 3 ml of antibiotic containing LB medium. After overnight incubation at 37°C with shaking, one ml of bacteria was pelleted by centrifugation at 13,000 rpm for 10 seconds. The pellet was resuspended completely in 100 µl of solution I and incubated on ice for 5 minutes. 200 µl of solution II was then added and mixed gently by inverting the eppendorf tube six times. After 5 minutes of incubation, 150 µl solution III was added, mixed gently and incubated on ice for 5 minutes. The protein-DNA complex was cleared off by centrifugation at 13,000 rpm for 15 minutes, and the supernatant was transferred to another eppendorf tube, mixed well with 2 volume of 100% ethanol, and incubated at room temperature for 5 minutes. The plasmid was finally recovered by centrifugation at 13,000 rpm for 15 minutes, washed with 70% ethanol once, and dissolved in 50 µl of TE or dH_2O containing RNase A (20 µg/ml).

2.2.1.4 Determination of nucleic acid concentration

The concentration of DNA was determined by the spectroscopic measurement of their optical density (OD) at 260 nm and 280 nm. The OD_{260} value of one is equivalent to 50 µg/ml of double stranded DNA. Pure DNA in aqueous solution should have an OD_{260}/OD_{280} ratio of 1.6-1.8.

2.2.1.5 Restriction endonuclease digestion of DNA

Usually 2-3 units of a restriction enzyme for each µg DNA were used. DNA was digested at a concentration of 1 µg/10 µl in a buffer recommended by the supplier. The reaction was carried out between 2 hours to overnight at 37°C (unless otherwise

recommended by the supplier). The quality of the digestion was checked by DNA agarose gel electrophoresis.

2.2.1.6 Nucleic acid analysis by agarose gel electrophoresis

The required amount of agarose (final concentration between 0.8 and 2%) was dissolved in 1× TAE buffer. Ethidium bromide was added at a concentration of 0.3 µg/ml. The molten gel was poured into a horizontal chamber. Combs with the appropriate number and size of the teeth were used to make the loading slots. The gel (when set) was immersed with TAE buffer and run at 35-45 mA (50-100 V) at room temperature for the required time. Samples were loaded onto the gel in loading buffer. DNA was visualized by transillumination with 302 nm ultraviolet radiation.

2.2.1.7 Isolation/purification of DNA from agarose gels

In general, the EasyPure DNA purification Kit was used to isolate the appropriate DNA fragments in the agarose gel (e.g. restriction-digested vectors and PCR products). The DNA band of choice was cut out from the gel, under long wave UV radiation with the aid of a scalpel. 3 volumes of "salt buffer" (all reagents provided in the kit) were added to the DNA containing gel block. The gel piece was melted in the buffer by incubation at 55°C before the binding resin was added. After two subsequent washing steps the resin with bound DNA was air dried and the DNA was finally eluted by the addition of dH_2O.

2.2.1.8 Ligation

In all cases, the insert and vector were loaded on an agarose gel to check the DNA content before ligation. Ligation was performed in a total volume of 20 µl with insert, vector, 1× ligation buffer and 1 µl of T4 ligase and incubated at 15°C overnight. The ratio of insert:vector was about 4:1.

2.2.1.9 Precipitation of nucleic acids

In order to recover nucleic acids from solution, the salt concentration was adjusted to 200 mM with 3 M sodium acetate (pH 5.0) and then 2 volumes of cold ethanol were added. After a short incubation at room temperature, the nucleic acids were precipitated by centrifugation with 13,000 rpm for 15 minutes. The pellet was dried after one wash with 70% ethanol. Finally, the nucleic acids were resuspended in sterile dH_2O or TE buffer.

2.2.1.10 Polymerase Chain Reaction (PCR)

All PCRs were performed in a total volume of 100 µl, in the presence of 10 ng template, 200 µM dNTPs, 0.2 µM of each primer, 1× reaction buffer, 10% DMSO and 1 µl GoTaq DNA polymerase or pfu DNA polymerase. The reactions were carried out in a MJ PCR thermocycler, using specific cycling parameters depending on the application.

2.2.1.11 Site-directed mutagenesis

Mutagenesis was performed according to the instruction manual of QuickChange™Site-Directed Mutagenesis kit. The reaction was set up as indicated below: 5 µl of 10× reaction buffer, 50 ng of DNA template, 2× 125 ng of primers, 1 µl of dNTP mix (provided in the kit), 1 µl of pfu Turbo DNA polymerase (2.5 U/µl) and dH_2O was added to make a final volume of 50 µl. The reaction was subject to the following PCR program: segment 1: 95°C, 30 seconds; segment 2: 95°C, 30 seconds; 55°C, 1 minute; 68°C, 10 to 14 minutes (depending on the vector used); 12 cycles totally. After PCR amplification, 1 µl of the *DpnI* restriction enzyme (10 U/µl, provided in the kit) was directly added to reaction and incubated at 37°C for 1 hour. 10 µl of *DpnI* treated DNA was transferred to 100 µl of *E. Coli* XL1-Blue supercompetent cells. After a heat pulse at 42°C for 45 seconds, the transformation reaction was placed on ice for 2 minutes. 1 ml of LB medium (ampicillin free) was

then added. After 1 hour incubation at 37°C with shaking, all the bacteria were plated on LB plate with 100 µg/ml of ampicillin and incubated overnight at 37°C. Two single colonies were picked for plasmid minipreparations and subsequent sequencing.

2.2.1.12 Construction of fusion proteins

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*65I 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*$_{C14T652A}$, *P*$_{CUP1}$*-F-FPR1-63*$_{C47T654A}$ as well as the other mutations at positions 652, 654, and 661 of Sec63p.

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 previously (Dünnwald et al., 1999; Wittke et al., 2002; Wittke et al., 1999). 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.

Mutants of *SEC63*$_{\Delta C47C17}$, *SEC63*$_{\Delta C47C20}$, *SEC63*$_{\Delta C47C24}$ and *SEC63*$_{\Delta C47C28}$ were created by PCR using *F-FPR1-63*$_{C47}$ as a template and oligos annealing to the DNA coding for the desired residues of Sec63p and an oligo annealing to the 3' untranslated DNA of *SEC63* (Wittke et al., 2000). PCR products were cut with *Pst*I and *Acc*65I and inserted into the pRS314 vector. *P*$_{MET17}$*-SEC63* were cut with *Eag*I and *Pst*I, and, using the common *Pst*I site, inserted together with the P$_{MET17}$-promoter and part of *SEC63* in front of the mutated sequence.

pET15bSEC62$_{\Delta C125}$-Ha was created by PCR using *Flag-SEC62* as template and oligos annealing to the desired sequences of *SEC62*. PCR products were cut with *Nde*I and *Xho*I and inserted into the pET15b vector.

2.2.2 Yeast cell transformation and yeast strain construction

2.2.2.1 Preparation of competent yeast cells

A single colony of yeast cells from YPD-agar or SD-agar plate was inoculated into 5 ml YPD or SD medium and incubated overnight at 30ºC with shaking (220 rpm). The next morning 2 ml preculture was diluted into 100 ml medium (1:50 dilution) and the cells were grown to an OD_{600} of 0.6-0.9. Yeast cells were collected by centrifugation at 3000 rpm for 5 minutes at 4°C. The pellet was washed with 25 ml of chilled distilled H_2O twice. Then the pellet was resuspended in 1 ml 0.1 M lithium acetate. After 5 minutes of incubation on ice, the yeast cells were dispensed in 100 µl aliquots to pre-chilled eppendorf tubes and used immediately for transformation or stored at -80°C.

2.2.2.2 Yeast cell transformation

100 µl aliquot cells were put on ice for several minutes and the supernatant was discarded after a short centrifugation. The cell pellet was resuspended with 240 µl 50% PEG, 36 µl 1M lithium acetate, 10 µl 10 mg/ml salmon sperm DNA (after boiling for 5 minutes) and 50 µl DNA (1 µl of miniprepared DNA in 49 µl distilled water). The mixture was then incubated for 30 minutes at 30°C with shaking and then 20 minutes at 42°C. If the selection was performed by antibiotics, the cells were allowed to grow in YPD for 1 hour 30°C. The cells were then pelleted by a short centrifugation (1 minute at 4000 rpm) and the supernatant was removed before resuspending the cells with 300 µl distilled water. The cells were plated on selective agar plates and incubated at 30°C for 2-3 days.

2.2.2.3 Construction of mutant strains via homologous recombination

To exchange a threonine against an alanine in the sequence of 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. The recombination occured as shown in Figure 2.1 (Wittke et al., 1999). Successful recombination was confirmed by diagnostic PCR and sequencing.

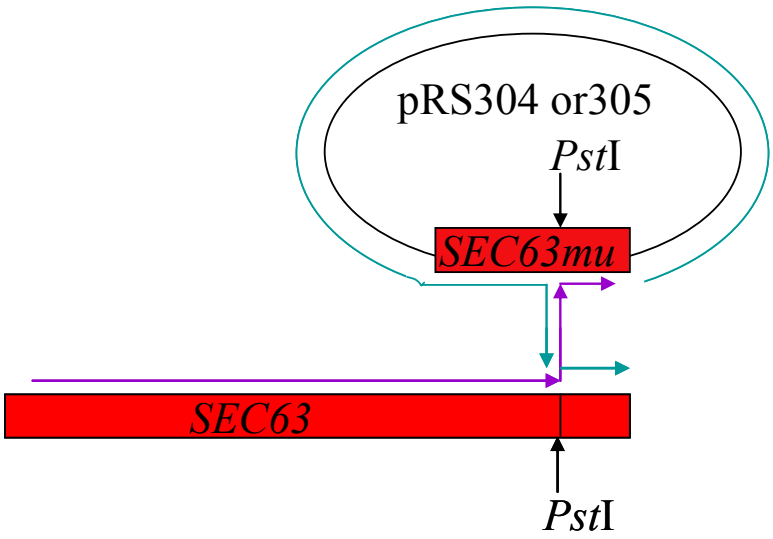

Figure 2.1 Insertion of mutations in the 3'-coding sequences of *SEC63* via homologous recombination. The plasmids pRS304 or 305 carrying *SEC63* spanning the last 448 bp of *SEC63* with the desired mutation were cut with the *Pst*I. The linealized vectors were transformed into JD53 and homologous recombination occured as shown.

Genomic insertions of the sequence coding for protein A (ProA) behind *SEC63* or *CKA1* were performed as shown in Figure 2.2 (Knop et al., 1999). All insertions were tested by diagnostic PCR and by 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).

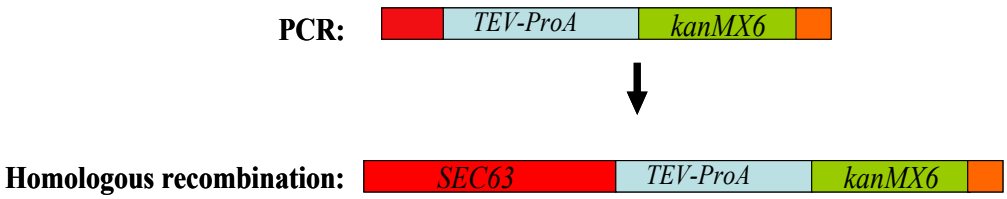

Figure2.2 C-terminal tagging of chromosomal *SEC63* based on a PCR tagging strategy. PCR was performed with the primer Sec63ProA-Fw and Sec63ProA-Re annealing to pYM8 as a template. The PCR product was then transformed into JD53, YDH6 and YDH8 strains and *ProA* was inserted behind *SEC63* via homologous recombination.

2.2.2.4 Preparation of genomic DNA from yeast cells

10 ml cells were cultured for 1-2 days at 30°C. The cell pellet was centrifuged and washed once with distilled water. The pellet was resuspended with 200 µl lysis buffer. 200 µl glass beads and 200 µl of a mixture of phenol, chloroform, isopropanol (25:24:1) were added to the suspension. After vortexing for 5 minutes, 200 µl TE buffer was added and the whole suspension was centrifuged at 13,000 rpm. The upper phase was transferred to a fresh eppendorf tube and 400 µl chloroform was added. After vortexing and centrifugation, the upper phase was transfered to a new eppendorf tube. 1 ml 100% ethanol was used to precipitate the DNA and 70% ethanol to wash the DNA after centrifugation at 13,000 rpm for 15 minutes. The precipitated DNA was finally resuspended in 50 µl TE.

2.2.3 Yeast cell extracts preparation

2.2.3.1 Cell extracts for direct SDS-PAGE

10 ml cells were grown to OD_{600} 1.0. The cells were pelleted and washed twice with distilled water and resuspended in 100 µl 2× SDS sample buffer containing 0.1 M DTT. The samples were boiled for 3 minutes and transferred into liquid nitrogen. After four consecutive cycles of boiling, vortexing and freezing in liquid nitrogen, the samples were then stored at -20°C or applied for SDS-PAGE immediately.

2.2.3.2 Cell extracts for binding assay

100 ml of cell culture were grown to OD_{600} 1.0-1.5 and the cells were pelleted and washed twice with distilled water and then dropped into liquid nitrogen. The cell pellets was stored in -80°C or used immediately. The cell pellets were grinded into powder in liquid nitrogen using a mortar and resuspended with protein extraction buffer containing protease inhibitor cocktail, 1 mM DTT, 0.5% (v/v) phosphatase inhibitor cocktail 1 and 2 and 0.4 mM PMSF. The cell extracts were vortexed 6× 10 seconds on ice and centrifuged for 20 minutes at 13,000 rpm at 4°C. The supernatants were carefully removed from the pellet and then used for the binding assay.

2.2.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

For most applications a polyacrylamide separating gel of 12.5% and a 5% stacking gel were used. Reagents for the separating gel were 6 ml of 30% acrylamide, 3.8 ml of 1.5 M Tris-HCl pH 8.8, 75 µl of 20% SDS, and 5 ml of H_2O. To the mixture 150 µl of 10% ammonium persulphate (APS) was added and the reaction was initiated with 20 µl of TEMED. The gel mix was poured between two glass plates. Upon polymerization, a stacking gel was poured on top and a plastic comb was inserted. Reagents for stacking gel were 1.2 ml of 30% acrylamide, 1 ml of 1 M Tris-HCl pH 6.8, 40 µl of 20% SDS, 5.8 ml of dH_2O, 80 µl of 10% APS and 15 µl of TEMED. The gel was run in laemmli buffer until the desired separation had been reached.

2.2.5 Western blotting

Proteins resolved by SDS-PAGE were transferred to nitrocellulose membrane using a Semi-dry Transfer chamber containing 6 whatman paper immersed in transfer buffer. Transfer was performed at room temperature at a constant current of 210 mA for 1.5 hours.

2.2.6 Probing

The membrane was blocked for 1 hour in blocking buffer (5% dry milk or BSA in TBST) and incubated with primary antibodies of variant dilutions (1:500 to 1:1500, depending on the antibody) in blocking buffer for 1 hour at room temperature or overnight at 4°C. After 4 times of washing with TBST, the membrane was incubated with HRP labeled secondary antibody (1:2000) in blocking buffer at room temperature for 1 hour and washed again with TBST for 4 times. The antibody bound protein was visualized by ECL Western blotting reagents and ECL-Hyperfilm following the manufacture's instructions.

2.2.7 Stripping the Western blot membrane

To probe one membrane with more than one antibody, the membrane was stripped by the following procedure: incubation with a stripping solution (62.5 mM Tris, pH 6.8, 2% SDS, 0.8% DTT) at 50°C for 30 minutes with shaking. The membrane was washed twice with TBST and then blocked in 5% milk as usual.

2.2.8 Split-ubiquitin assay

Cells were first grown at 30°C in liquid selective media containing uracil to an OD_{600} of 1. 4 µl of these cultures, and serial 1:10 dilutions in water were spotted on agar plates selecting for the presence of the fusion constructs and lacking uracil. The assay was performed on media containing 10 mM methionine to reduce the expression of P_{MET17}-*SEC63*-C_{ub}-*RURA3*. All experiments were performed without adding

additional amounts of copper to the medium. The same dilutions were also spotted onto plates containing uracil to check for cell numbers. The plates were incubated at 30°C for 2–5 days.

2.2.9 ^{35}S-pulse and 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 mix (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. The TCA mixtures were incubated on ice for at least 20 minutes. After centrifugation at 13,000 rpm for 10 minutes, the cell pellets were washed once with ice cold acetone, again centrifuged at 13,000 rpm for 10 minutes and allowed to air dry. The pellets were resuspended with 100 µl SDS urea buffer (50 mM Tris-HCl pH7.5, 1 mM EDTA, 1% SDS, 6 M urea) and incubated at room temperature for 15 minutes. Glass beads were added to 90% of the sample volume and vortexed for 1 minute. Samples were then heated at 95°C for 5 minutes and vortexed for 30 seconds. 900 µl detergent containing IP buffer (50 mM Tris-HCl pH7.5, 0.1 mM EDTA, 150 mM NaCl, 0.5% Tween20), the samples were vortexed and put on ice for 20 minutes. After centrifugation at 13,000rpm for 15 minutes, 850 µl of the supernatants were transferred to a fresh eppendorf tube. 2.5 µl anti-CPY monoclonal antibody was added overnight at 4°C. Then 20 µl Protein G agarose beads were added and incubated for 2 hours. After washing the beads twice with detergent containing urea buffer (50 mM Tris-HCL pH7.5, 200 mM NaCl, 0.5% Tween20, 2 M urea) and three times with detergent IP buffer, the beads were boiled in 2× sample buffer and the extracted proteins were separated by SDS-PAGE. For Endo H treatment 30 µl glycoprotein denaturing buffer was added to the beads after washing and the samples were boiled for 10 minutes. 3 µl 10× G5 buffer were added with or without 2 units of Endo H and incubated at 37°C for 1 hour (enzyme and buffer from New England Biolabs, Beverly, USA).

2.2.10 Translocation assay

Cells were first grown at 30°C in liquid selective media containing uracil to an OD_{600} of 1. 4 µl of these cultures were spotted on agar plates selecting for the presence of the fusion constructs and lacking uracil. The same dilutions were also spotted onto plates containing uracil to verify that equal cell numbers were applied. The plates were incubated at 30°C for 2–5 days.

2.2.11 Synthetic lethality assay

Cells were first grown at 30°C in liquid selective media containing uracil to an OD_{600} of 1. 20 µl of these cultures were spotted on 0.1% 5-FOA agar plates selecting for the presence of the fusion constructs. The same amount of cells were also spotted onto plates without 5-FOA. The plates were incubated at 30°C for 2–5 days.

2.2.12 In vivo phosphorylation assay

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) (Qiagene, Heidelberg) 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 as described for the CPY immunoprecipitation.

2.2.13 In vitro phosphorylation assay

100 ml 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 grinded 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 and a protease inhibitor cocktail. The cell suspension was vortexed and centrifuged for 20 minutes at 13,000 rpm at 4°C. The supernatant was incubated with 40 µl IgG-sepharose for 4 hours in the cold room, the beads were washed 5 times with 10 ml of extraction buffer 1 and two times 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 medium containing 100 µM copper at 30°C to an OD_{600} of approximately 1. Cells were extracted as described for binding assay. Proteins were precipitated by incubation with anti-Flag antibody for one hour followed by incubation with 40 µl of Protein G agarose for 2 hours. Beads were washed 4 times with protein extraction buffer and treated with phosphatase as described. After phosphatase treatment the beads were washed 4 times with protein extraction buffer 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 in vitro phosphorylation was performed in a reaction containing 20 µl of Cka1-ProA beads, 20 µM ATP, 1 µCi of [γ-^{32}P] GTP (Amersham), 20 µl of Flag peptide elute 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 2× SDS sample buffer and boiling for 3 minutes.

2.2.14 Overlay assay

Protein extracts derived from 10 ml yeast cultures were obtained by resuspending the washed cell pellets in 100 µl 2× SDS sample buffer 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. The membrane was incubated for 2 hours in the cold room 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. 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 extract from these cells for the overlay assay was prepared as described for the binding assay.

2.2.15 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 in the cold room, the beads were washed 4 times with protein extraction buffer and resuspended in 40 µl 2× SDS sample buffer. Extraction of the Sec63-ProA or Sec63$_{T654A}$-ProA expressing cells were done as described for F-Fpr1-63$_{C14}$ expressing cells but with a buffer containing 0.2% Triton X-100. The cell extracts were then incubated with 30 µl of IgG-sepharose beads for 2 hours in the cold room. The beads were spun down and 20 µl of the supernatants were boiled directly with 20 µl of 2× SDS sample buffer. The precipitated beads were washed five times with extraction buffer (plus 0.2% Triton) and boiled in 120 µl 2×SDS sample buffer. 15 µl of the supernatants and 40 µl of the bound fractions were used for western blot analysis. To directly test the interaction between F-Fpr1-63$_{C14}$ and Sec62$_{\Delta C125}$-Dha, yeast cells expressing F-Fpr1-63$_{C14}$ together with Sec62$_{\Delta C125}$-Dha were grown to an OD_{600} of 1-1.5 and collected for extraction as described for the cells expressing F-Fpr1-63$_{C14}$. The extract was then incubated with anti-Ha antibody coupled agarose for 2 hours at 4°C. The beads were washed 4 times with 1 ml of extraction buffer and resuspended in 80 µl of 2×SDS sample buffer for SDS-PAGE.

2.2.16 Phosphatase treatment

20 µl of Protein G agarose presenting the anti-Flag antibody bound protein were treated with 10 units of calf intestinal phosphatase (CIP) or without enzyme for 1 hour at 37°C in 20 µl phosphatase buffer (NEB3). Proteins immobilized on the

nitrocellulose membrane were incubated with 5ml phosphatase buffer containing 30 units of CIP or no enzyme at 37°C for 1 hour.

2.2.17 Protein purification from *E. coli*

BL21(DE3) *E. coli* cells containing a pET-15b based expression vector were cultured in 3 ml LB medium overnight. The next morning the cells were diluted into 250 ml LB medium and grown to an OD_{600} of 0.6. Expression of the protein was induced by adding IPTG to a final concentration of 1 mM. After incubation for 3.5 hours at 220 rpm at 24°C, the culture was centrifuged for 10 minutes at 8000 rpm at 4°C. The cell pellet was washed once with 20 ml PBS and resuspended in 10 ml ice cold lysis buffer (150 mM NaCl, 5 mM imidazole, 50 mM KH_2PO4 pH 8.0) containing protease inhibitor, 0.4 mM PMSF and 1 mg/ml lysozyme. The mixture was incubated on ice for 15 minutes and inverted several times. The lysate was completed by sonication and DNaseI addition to a final concentration of 0.01 mg/ml. After 30 minutes of rotation at 4°C, the mixture was centrifuged for 10 minutes at 9000 rpm, yielding a clear lysate. 350 µl of Ni-NTA (Qiagen), previously washed with lysis buffer three times, was added to the lysate. The mixture was incubated for 20 minutes on ice and inverted several times and then added to a polypropylene column which was allowed to drain. The column was washed with 5× 400 µl DNA elution buffer (10 mM Tris-HCl pH 8.5) and with 2× 5 ml wash buffer (300 mM NaCl, 10 mM imidazole, 50 mM KH_2PO_4, pH 7.5). To elute the protein, elution buffer (300 mM NaCl, 150 mM imidazole, pH 7.5) was added to the column. After 10 minutes of incubation, the flow through of the column was collected. The elution was continued stepwise with 150 µl elution buffer until no further protein was detectable in a Bradford assay. Combined fractions were dialyzed overnight against dialysis buffer (50 mM HEPES, 1 mM DTT, 30% glycerol, pH 7.2) and the dialyzed protein was aliquoted and stored at -80°C. Based on SDS gel electrophoresis and staining with commassie blue, the purity of the protein was estimated to be greater than 90%.

2.2.18 Detection of Sec62-GFP

Cells were grown to OD_{600} 0.6 and 100 µM copper were added to the culture. 2 hours later, cells were directly applied on a glass slide and inspected under the laser scanning microscope LSM510 META (Axiovect 200M Zeiss) using UV light of 488nm, appropriate filter sets and a magnification of 60.

3. Results

3.1 Phosphorylation of the C-terminal domain of Sec63p stimulates its interaction with the N-terminal domain of Sec62p

3.1.1 The interaction between the C-terminal domain of Sec63p and the N-terminal domain of Sec62p is phosphorylation dependent

The interaction between the two membrane proteins Sec62p and Sec63p can be reconstituted by expressing in yeast cells the N-terminal cytosolic domain of Sec62p ($Sec62_{\Delta C125}$) linked to an Ha tagged Dihydrofolate reductase ($Sec62_{\Delta C125}$-Dha, Figure 3.1D) and the cytosolic last 14 residues of Sec63p attached to a Flag epitope labeled FK506 binding protein Fpr1p (F-Fpr1-$Sec63_{C14}$, Figure 3.1B) (Wittke et al., 2000). Interestingly, F-Fpr1-$Sec63_{C14}$ could be separated during SDS-PAGE as two forms of slightly different motilities (Wittke et al., 2000). It was observed that only the slower moving species could be precipitated by the N-terminal domain of Sec62p (Wittke et al., 2000). This difference in the electrophoretic motility of F-Fpr1-$Sec63_{C14}$ could be due to either the partial proteolysis of the fusion protein during the preparation of cell extracts or its post-translational protein modification such as protein phosphorylation. To exclude proteolysis during extract preparation as one explanation I harvested F-Fpr1-$Sec63_{C14}$ expressing cells in two fold SDS sample buffer and immediately boiled the extract. This procedure should minimize proteolysis during the preparation of the extracts. As can be seen in Figure 3.2A, two F-Fpr1-$Sec63_{C14}$ species of different mobilities appear on the blot after treatment with anti-Flag antibody. This result indicates that protein modification rather than protein degradation of F-Fpr1-$Sec63_{C14}$ leads to appearance of the two forms of F-Fpr1-$Sec63_{C14}$ during SDS-PAGE. To test whether F-Fpr1-$Sec63_{C14}$ becomes phosphorylated in yeast cells, I purified the fusion protein by immunoprecipitation with anti-Flag antibody and incubated the precipitate with an unspecific phosphatase (CIP, Calf Intestinal Phosphatase). Phosphatase treatment reduces the apparent molecular weight of both forms of

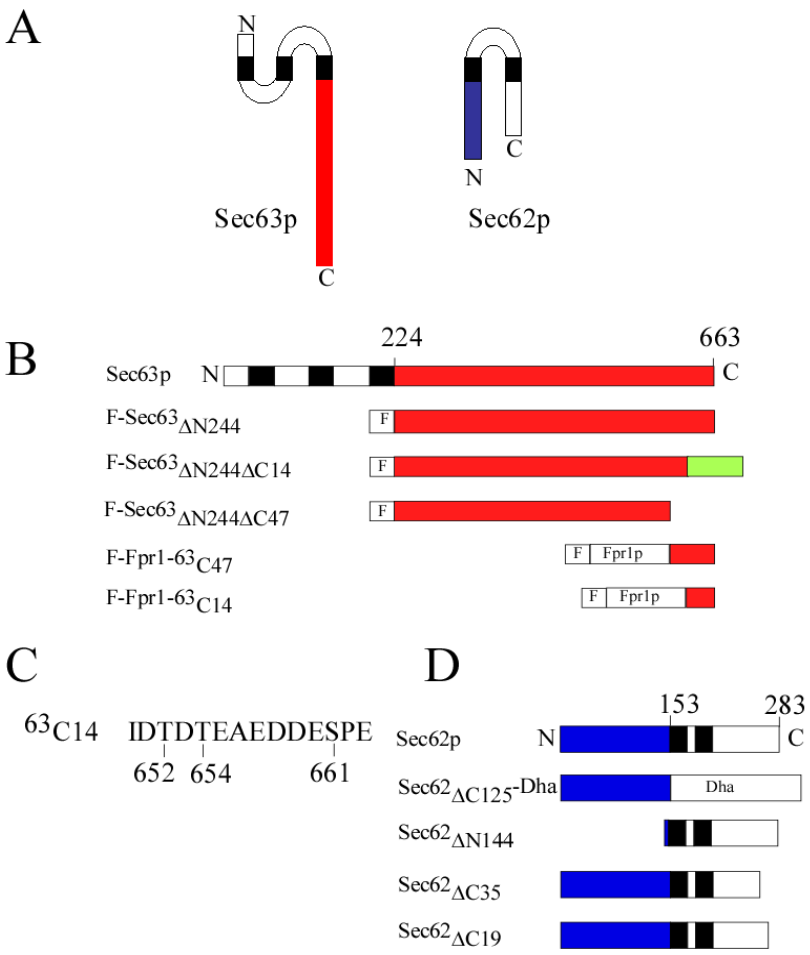

Figure 3.1. Schematic representation of Sec63p, Sec62p and the different constructs used in this work. A. Topological structure of Sec63p and Sec62p. Sec63p contains three transmembrane domains (black bars) and a large cytosolic domain (red bar). Sec62p contains two transmembrane domains (black bars) and a large cytosolic N-terminal domain and C-terminal domain (blue bar and white bar). **B**. Representation of Sec63p and its fusion constructs. **C**. The sequence of the last C-terminal 14 residues of Sec63p is given in the one letter code. **D**. Representation of Sec62p and its fusion constructs.

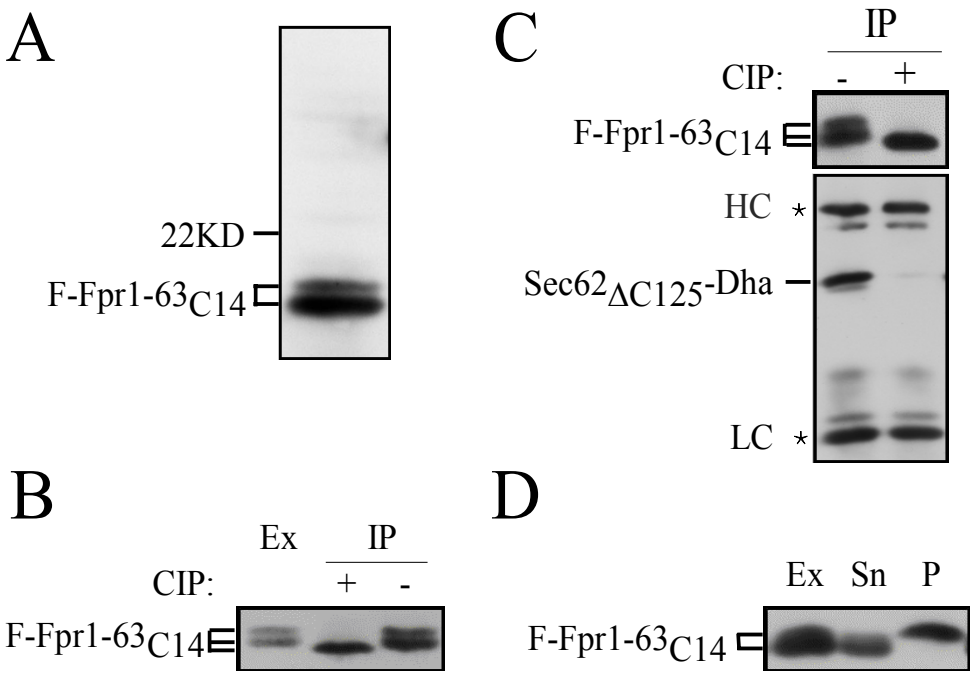

Figure 3.2. The interaction between the last C-terminal 14 residues of Sec63p and the N-terminus of Sec62p is phosphorylation dependent. A. Yeast cells expressing F-Fpr1-63_{C14} were harvested in two fold SDS sample buffer and immediately boiled. The extract was separated by SDS-PAGE transferred on nitrocellulose. The blot was probed with anti-Flag antibody. **B.** Extracts of yeast cells expressing F-Fpr1-63_{C14} were incubated with anti-Flag antibody to precipitate F-Fpr1-$63_{C14.}$ Extracts (Ex) and precipitates (IP) were probed with anti-Flag antibody after mock treatment (-) or treatment of the precipitate with calf intestinal phosphatase (+CIP). **C.** F-Fpr1-63_{C14} enriched on agarose-coupled protein G beads was incubated without or with phosphatase followed by incubation with Sec$62_{\Delta C125}$-Dha containing cell extracts. Precipitates were subjected to 12.5% SDS-PAGE and immunoblot detection with anti-Flag or anti-Ha antibody. The asterisk indicates light chain (LC) or heavy chain (HC) of the antibody. **D.** Extracts of yeast cells expressing F-Fpr1-63_{C14} together with Sec$62_{\Delta C125}$-Dha were incubated with anti-Ha antibody to precipitate Sec$62_{\Delta C125}$-Dha. Extracts (Ex), supernatants (Sn) and precipitates were probed with anti-Flag antibody after SDS-PAGE and transfer onto nitrocellulose to follow the binding of F-Fpr1-Sec63_{C14} to Sec$62_{\Delta C125}$-Dha.

F-Fpr1-Sec63$_{C14}$ during SDS-PAGE, suggesting that the phosphorylation of the fusion protein causes the different motilities of F-Fpr1-Sec63$_{C14}$ during gel electrophoresis (Figure 3.2B). To directly test whether phosphorylation of the C-terminus of Sec63p is needed for the binding to the N-terminal domain of Sec62p, F-Fpr1-Sec63$_{C14}$ was first enriched by immunoprecipitation and then dephosphorylated by incubating the immunoprecipitate with CIP. CIP- treated or untreated F-Fpr1-Sec63$_{C14}$ presenting agarose beads were then incubated with extracts of yeast cells expressing Sec62$_{\Delta C125}$-Dha. The beads were washed and the bound protein was extracted by boiling the agarose in two fold SDS sample buffer. Sec62$_{\Delta C125}$-Dha was detected after SDS-PAGE and transfer onto nitrocellulose by treatment with anti-Ha antibody. The western blot in Figure 3.2C clearly shows that phosphatase treatment of F-Fpr1-Sec63$_{C14}$ indeed abolished its binding to Sec62$_{\Delta C125}$-Dha (Figure 3.2C). To confirm this conclusion I repeated the co-precipitation of F-Fpr1-Sec63$_{C14}$ together with Sec62$_{\Delta C125}$-Dha from yeast cells coexpressing both fusion proteins. As previously observed only the slower migrating form of F-Fpr1-Sec63$_{C14}$ could be precipitated by Sec62$_{\Delta C125}$-Dha whereas the unphosphorylated F-Fpr1-Sec63$_{C14}$ remained in the supernatant (Figure 3.2D). Together both experiments proof that phosphorylation of F-Fpr1-Sec63$_{C14}$ is needed for its tight interaction with the N-terminal domain of Sec62p.

3.1.2 Both threonines in position 652 and 654 of Sec63p are essential for the strong binding to the N-terminal domain of Sec62p

Close inspection of the gel in Figure 3.2B reveals that both bands of F-Fpr1-Sec63$_{C14}$ shift into a single faster moving species after phosphatase treatment (Figure 3.2B). I therefore suspect a minimum of two phosphorylation sites in F-Fpr1-Sec63$_{C14}$. Since only the slowest migrating form is co-precipitated with Sec62$_{\Delta C125}$-Dha (Figure 3.2D), phosphorylation at both sites in F-Fpr1-Sec63$_{C14}$ seems to be required for its binding to Sec62$_{\Delta C125}$-Dha. The last 14 residues of Sec63p contain two threonines and one serine (Figure 3.1C). To map the phosphorylation sites I replaced the threonine residues in F-Fpr1-Sec63$_{C14}$ individually (F-Fpr1-63$_{C14T652A}$; F-Fpr1-63$_{C14T654A}$) or in

combination (F-Fpr1-63$_{C14T652;\ 654A}$) by alanines or exchanged the last three residues of Sec63p including Ser 661 from S-P-E to A-L-P (F-Fpr1-63$_{C14S661A}$). As the first test I analyzed the running behaviors during SDS-PAGE of the mutated F-Fpr1-63$_{C14}$ fusion proteins before and after in vitro phosphatase treatment. Similar to the wild type F-Fpr1-63$_{C14}$, F-Fpr1-63$_{C14S661A}$ displayed two bands during SDS-PAGE that merged into a single faster migrating band after phosphatase treatment (Figure 3.3A, lane 7 and 8). The experiment shows that Ser 661 is either not phosphorylated or that

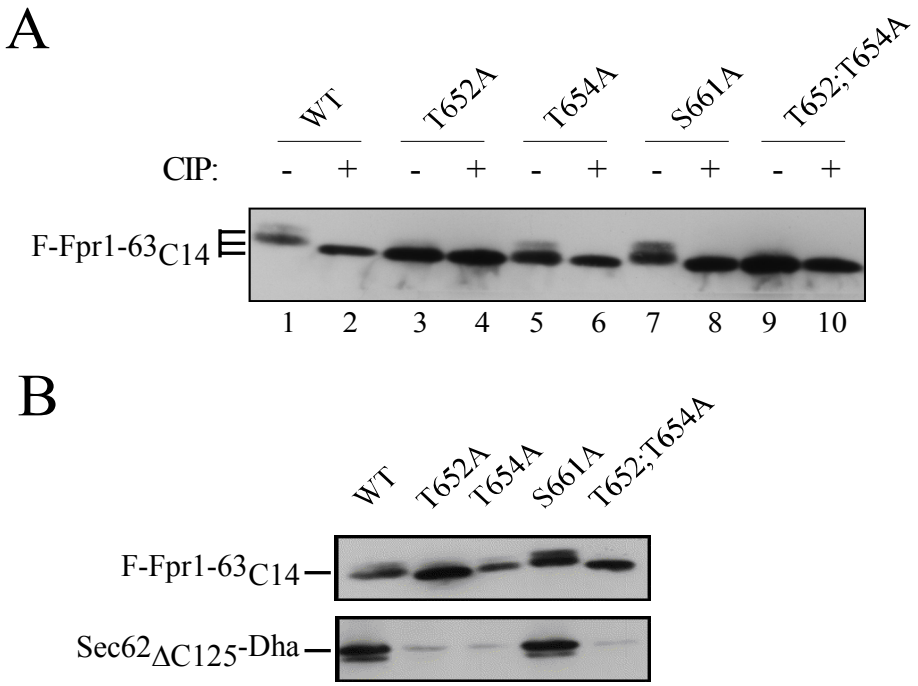

Figure 3.3. The phosphorylatable threonines in position 652 and 654 of Sec63p are required for the binding to the N-terminal domain of Sec62p. A. Immunoprecipitation of F-Fpr1-63$_{C14}$ and its mutants carrying single alanine exchanges in position 652, 654, 661 or two alanine exchanges at position 652 and 654. The precipitate was either mock (CIP: -) or phosphatase (CIP: +) treated and subjected to anti-Flag detection after 12.5% SDS-PAGE. **B.** Extracts of cells expressing F-Fpr1-63$_{C14}$ or its mutants were anti-Flag immunoprecipitated and the precipitates were subsequently incubated with cell extracts containing Sec62$_{\Delta C125}$-Dha. Bound fractions were probed with anti-Flag (upper panel) or anti-Ha antibody (lower panel) after 12.5% SDS-PAGE.

its phosphorylation does no influence the mobility of the mutant protein during SDS-PAGE. A different result was obtained when Thr652 in F-Fpr1-Sec63$_{C14}$ was replaced by alanine. As shown in Figure 3.3A lane 3 and 4, the CIP treated as well as the untreated fusion protein run during SDS-PAGE as one band. The position of this band is similar to the running position of the CIP-treated F-Fpr1-Sec63$_{C14}$ (Figure 3.3A, lane 1 and 2). In contrast, F-Fpr1-63$_{C14T654A}$ displayed a double band during SDS-PAGE. After CIP treatment, the upper band disappeared whereas the running behavior of the lower band remained unchanged (Figure 3.3A, lane 5 and 6). I concluded that F-Fpr1-63$_{C14T652A}$ is not phosphorylated whereas F-Fpr1-63$_{C14T654A}$ is still phosphorylated at a single site. I therefore proposed that the mutant F-Fpr1p fusion protein containing alanines in position 652 and 654 should display the same running behavior as the CIP-treated wild type fusion protein. This was the case since the double mutant F-Fpr1-63$_{C14T652;\ 654A}$ was detected as a single band during SDS-PAGE. The running behavior of this band was identical to the dephosphorylated F-Fpr1-63$_{C14}$ and remained unaltered after phosphatase treatment (Figure 3.3A, lane 9 and 10).

To directly show that F-Fpr1-63$_{C14}$ is phosphorylated in vivo, I labeled yeast cells expressing either F-Fpr1-63$_{C14}$ or F-Fpr1-63$_{C14T652;\ 654A}$ with [^{32}P]O_4. To follow [^{32}P]O_4 incorporation into F-Fpr1-Sec63$_{C14}$ and F-Fpr1-63$_{C14T652;\ 654A}$, the cells were lysed and the fusion proteins were immunoprecipitated with anti-Flag antibody. Two close bands of F-Fpr1-Sec63$_{C14}$ can be detected on the autoradiograph of the gel while no [^{32}P]O_4 labeling was found for F-Fpr1-63$_{C14T652;\ 654A}$ (Figure 3.4, lane 2 and 3). Since both F-Fpr1p fusion proteins could be immuonoprecipitated from the cell extracts with roughly the same efficiency (Figure 3.4, lane 4 and 5), I concluded that F-Fpr1-63$_{C14}$ is phosphorylated in vivo whereas F-Fpr1-63$_{C14T652;\ 654A}$ is not.

The importance of the two phosphorylatable threonines on the binding to the N-terminal domain of Sec62p was further confirmed by the different binding affinities of these F-Fpr1-63$_{C14}$ mutants to Sec62$_{\Delta C125}$-Dha. Again the different F-Fpr1p fusion proteins were immobilized on agarose beads and the beads were incubated with

extracts from yeast cells expressing Sec62$_{\Delta C125}$-Dha. As can be seen from the western blot of the bound fraction, both the single and the double replacements of T652 and T654 by alanine drastically reduced the binding of the corresponding fusion proteins to the N-terminal domain of Sec62p. In contrast, the exchange of serine at position 661 had no detectable effect on this binding (Figure 3.3B).

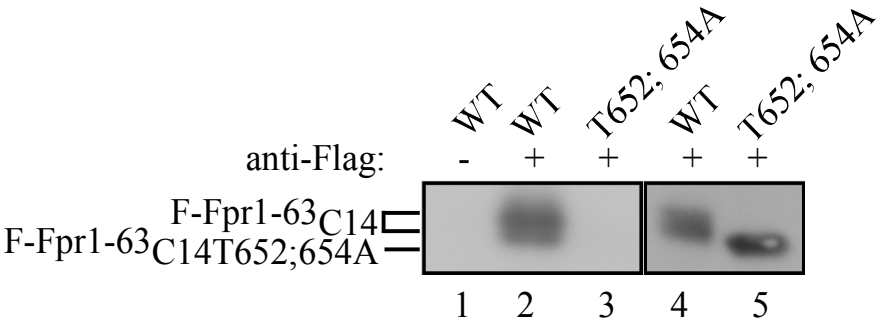

Figure 3.4. In vivo phosphorylation of F-Fpr1-63$_{C14}$. Yeast cells expressing F-Fpr1-63$_{C14}$ (lane 2, 4) or F-Fpr1-63$_{C14T652;\ 654A}$ (lane 3, 5) were labeled with [^{32}P]O_4. Extracts were incubated either with anti-Flag antibody (anti-Flag +) or not (anti-Flag -) followed by the precipitation of the antibody, SDS-PAGE of the bound fraction, and autoradiography of the dried gel (lane 1-3). Anti-Flag western blot of the immunoprecipitates of the unlabeled cells is shown in the right panel (lane 4 and 5).

To test whether the effect of the phosphorylation of the threonines in position 654 and 652 on the binding to the N-terminal domain of Sec62p can be mimicked by the replacements with acidic amino acids, I exchanged the threonine residues individually (F-Fpr1-63$_{C14T652D}$; F-Fpr1-63$_{C14T654D;}$ F-Fpr1-63$_{C14T652E;}$ F-Fpr1-63$_{C14T654E}$) or in combination (F-Fpr1-63$_{C14T652;\ 654D}$) by aspartate or glutamate. The binding of these mutants to the N-terminus of Sec62p was again tested by precipitation experiments as described. As observed for the alanine replacements the threonine exchanges of F-Fpr1-63$_{C14}$ against the acidic residues abolished the binding to the N-terminus of

Sec62p (Figure 3.5 A and B). I concluded that glutamic acid or aspartic acid cannot functionally substitute the phosphorylated threonines of F-Fpr1-63_{C14}.

The N-terminal border of the C-terminal binding site of Sec63p is not yet precisely defined. The region of the negatively charged amino acids extends to the last approximately 50 residues of Sec63p. To test the effect of phosphorylation on the binding of the whole acidic tail of Sec63p to the N-terminal domain of Sec62p, the fusion protein containing the last 47 residues of Sec63p (F-Fpr1-63_{C47}) was enriched by immunoprecipitation from yeast extracts. F-Fpr1-63_{C47} was then dephosphorylated by CIP and CIP- treated or untreated F-Fpr1-$Sec63_{C47}$ presenting agarose beads were then incubated with extracts of yeast cells expressing $Sec62_{\Delta C125}$-Dha. The western blot in Figure 3.6A clearly shows that treatment of F-Fpr1-$Sec63_{C47}$ with CIP significantly reduces its binding to $Sec62_{\Delta C125}$-Dha (Figure 3.6A). To monitor the influences of the phosphorylatable threonines in position 652 and 654 on the binding of F-Fpr1-63_{C47}, I constructed the corresponding mutants and repeated the binding experiments. As can be seen in Figure 3.6B, the binding of the F-Fpr1-63_{C47} mutants to $Sec62_{\Delta C125}$-Dha were again dramatically reduced. Similar to F-Fpr1-63_{C14} the binding of the complete C-terminal acidic domain of Sec63p to $Sec62_{\Delta C125}$ is drastically impaired after phosphatase treatment or through single alanine replacement at positions 652 or 654. The residual affinities that I observed for the mutated or phosphatase treated F-Fpr1-63_{C47} to the N-terminal domain of Sec62p are above the background and therefore significant.

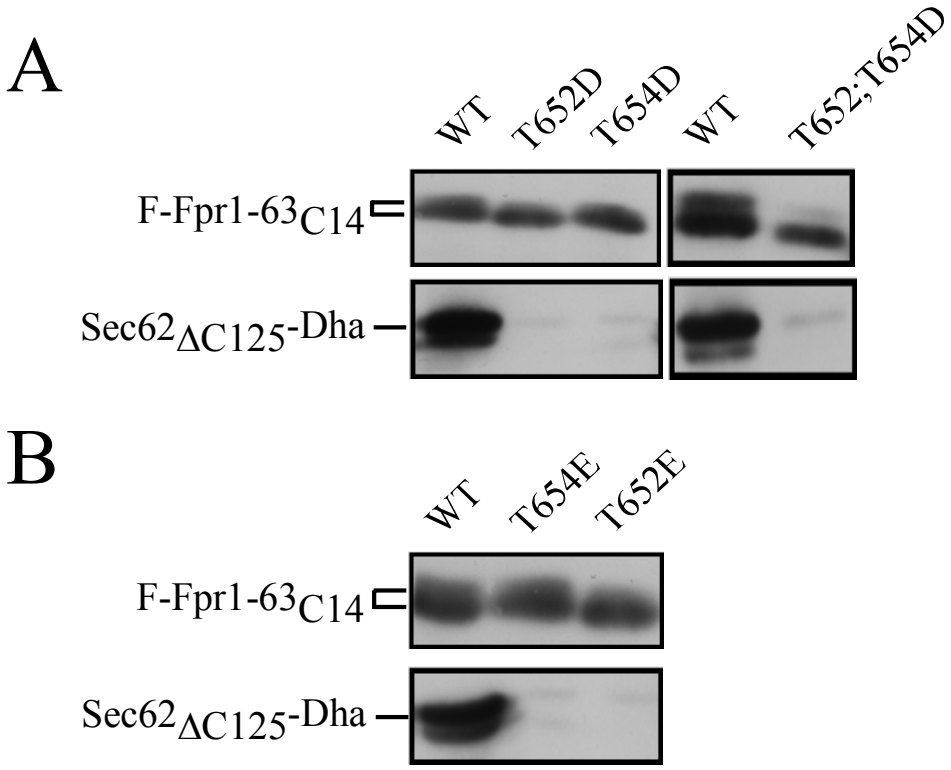

Figure 3.5. Acidic amino acids can not mimic the phosphorylatable threonines in position 652 and 654 of Sec63p. A. Extracts of cells expressing F-Fpr1-63_{C14} or its mutants carrying single aspartate exchanges in position 652, 654 or aspartates at both positions were anti-Flag immunoprecipitated and the precipitates were subsequently incubated with extracts containing $Sec62_{\Delta C125}$-Dha. Bound fractions were probed with anti-Flag (upper panel) or anti-Ha antibody (lower panel) after 12.5% SDS-PAGE. **B.** The same as A except for extracts of cells expressing F-Fpr1-63_{C14} or its mutants carrying single glutamate exchanges in position 652 or 654.

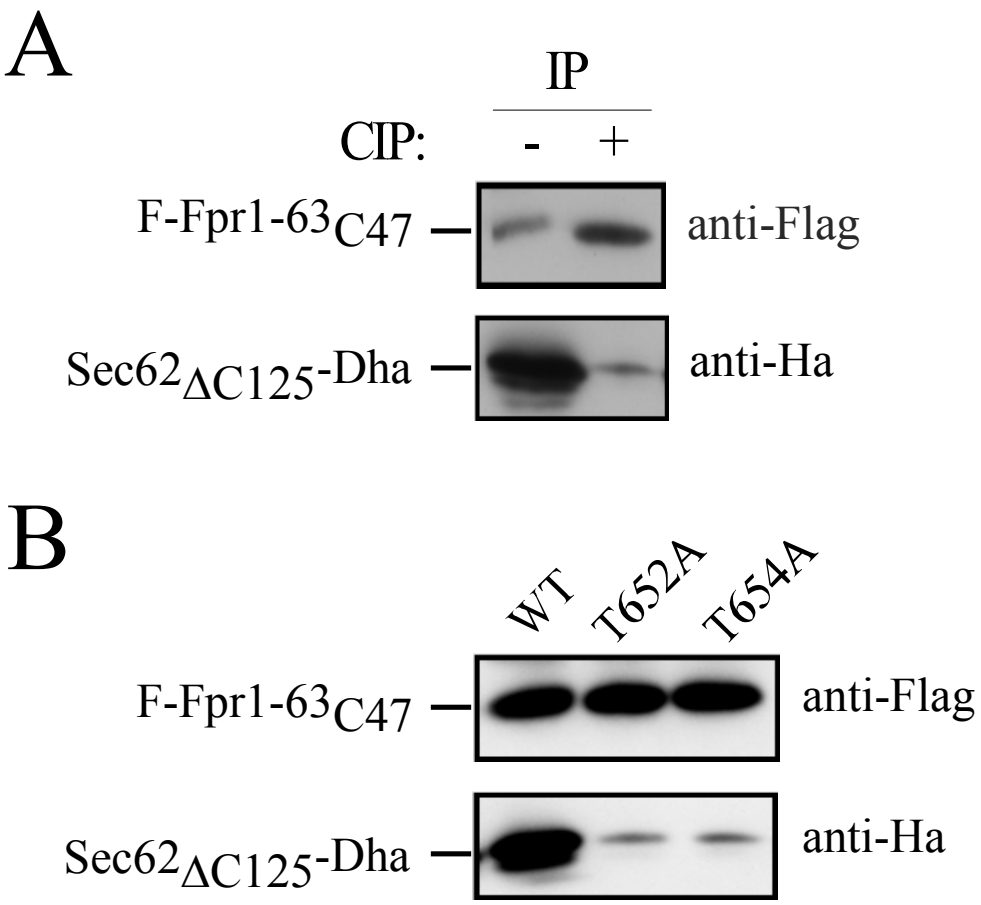

Figure 3.6. The binding of the complete C-terminal acidic domain of Sec63p to N-terminus of Sec62p is phosphorylation dependent. A. Agarose-coupled F-Fpr1-63_{C47} was incubated without or with phosphatase (-, + CIP) followed by incubation with $Sec62_{\Delta C125}$-Dha. Precipitates were subjected to 12.5% SDS-PAGE and immunoblot detection with anti-Flag (upper panel) or anti-Ha antibody (lower panel). **B**. Extracts of cells expressing F-Fpr1-63_{C47} or its mutants carrying alanine exchanges at position 652 or 654 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 antibody (lower panel) after 12.5% SDS-PAGE.

3.1.3 The binding of the full length Sec63p to Sec62p is phosphorylation dependent

3.1.3.1 A newly developed overlay assay is a specific and sensitive method to detect phosphorylated Sec63p

F-Fpr1-63_{C14} is an ectopically expressed artificial fusion protein. The question therefore remained whether the endogenous full length Sec63p is also phosphorylated at its C-terminus. Unfortunately, unlike F-Fpr1-$63_{C14,}$ the full length Sec63p showed no alteration in its running behavior during SDS-PAGE after CIP treatment (data not shown). Either Sec63p is not phosphorylated or its phosphorylation does not influence the mobility of Sec63p during SDS-PAGE. To distinguish between the two different explanations, I therefore tried to devise an alternative assay to measure the phosphorylation of the full length Sec63p. Here the yeast cell extract was first submitted to SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was then incubated with an extract of yeast cells expressing $Sec62_{\Delta C125}$-Dha. The membrane was subsequently incubated with anti-Ha antibody and finally with anti-mouse antibody coupled to horseradish peroxidase. A chemiluminescent signal at the running position of Sec63p should indicate the binding of the N-terminal domain of Sec62p to the C-terminus of Sec63p. Since this binding depends on the phosphorylation of T652 and T654, the detection of $Sec62_{\Delta C125}$-Dha at the running position of the full length Sec63p would reflect the in vivo phosphorylation of the full length Sec63p at both threonines.

To first test the validity of this overlay assay, I transformed into yeast strains either the Flag tagged complete cytosolic domain of Sec63p (F-$Sec63_{\Delta N244}$), a mutant of the cytosolic domain lacking its last 47 residues (F-$Sec63_{\Delta N244\Delta C47}$) or a mutant in which the last 14 residues of Sec63p were replaced with an unrelated and slightly longer sequence (F-$Sec63_{\Delta N244\Delta C14}$). The interactions of these mutants with $Sec62_{\Delta C125}$-Dha were then evaluated with the help of the overlay assay. The assay showed that

Sec62$_{\Delta C125}$-Dha can bind to membrane immobilized F-Sec63$_{\Delta N244}$ but not to F-Sec63$_{\Delta N244\Delta C47}$ or F-Sec63$_{\Delta N244\Delta C14}$ (Figure 3.7, lane 6, 7 and 8). Since both mutants of the cytosolic domain of Sec63p lack the binding site to the N-terminal domain of Sec62p, this outcome confirms the specificity of this assay. Additionally, an interaction signal was seen in all lanes at the running position of the native full length Sec63p (Figure 3.7, right panel). This signal is very specific since it depends on the incubation with extracts containing the Sec62$_{\Delta C125}$-Dha (Figure 3.8A). I therefore conclude that the overlay assay is specific and sensitive enough to detect the full-length Sec63p at its natural expression level. The overlay assay also recapitulates the binding of Sec62$_{\Delta C125}$-Dha to F-Fpr1-63$_{C47}$ (Figure 3.7, lane 9), but surprisingly not to F-Fpr1-63$_{C14}$ (Figure 3.7, lane 10). I will show later that the overlay assay is a more stringent binding assay than the co-precipitation assays and that residues N-terminal to the last 14 residues of Sec63p contribute to the binding strength of the Sec62/63p complex.

3.1.3.2 Sec63p is phosphorylated in vivo

Since I could show that the overlay assay can be used to monitor the binding between the N-terminal domain of Sec62p and the C-terminal tail of Sec63p in the full length protein, I used the assay to ask whether the full length Sec63p is also phosphorylated in vivo. To this end I preincubated the membrane displaying the proteins of the yeast with CIP before incubating with cell extracts expressing Sec62$_{\Delta C125}$-Dha. The result of this overlay showed that the preincubation of the blot with CIP abolished the binding of Sec62$_{\Delta C125}$-Dha to the membrane (Figure 3.8A). This result makes it very likely that the full length Sec63p is phosphorylated at its C-terminus in vivo. To further confirm this result, I engineered alanine exchanges at position 652 or 654 or an aspartate exchange at position 654 into the chromosomal *SEC63* via homologous recombination. Extracts of yeast strains expressing these mutants as well as a deletion mutant lacking the last 14 residues of Sec63p (Sec63$_{\Delta C14}$) were then subjected to the Sec62$_{\Delta C125}$-Dha overlay assay. The absence of an interaction signal in all lanes

containing the mutants of Sec63p clearly documents the significance of the phosphorylatable threonines at position 652 and 654 for this interaction (Figure 3.8B).

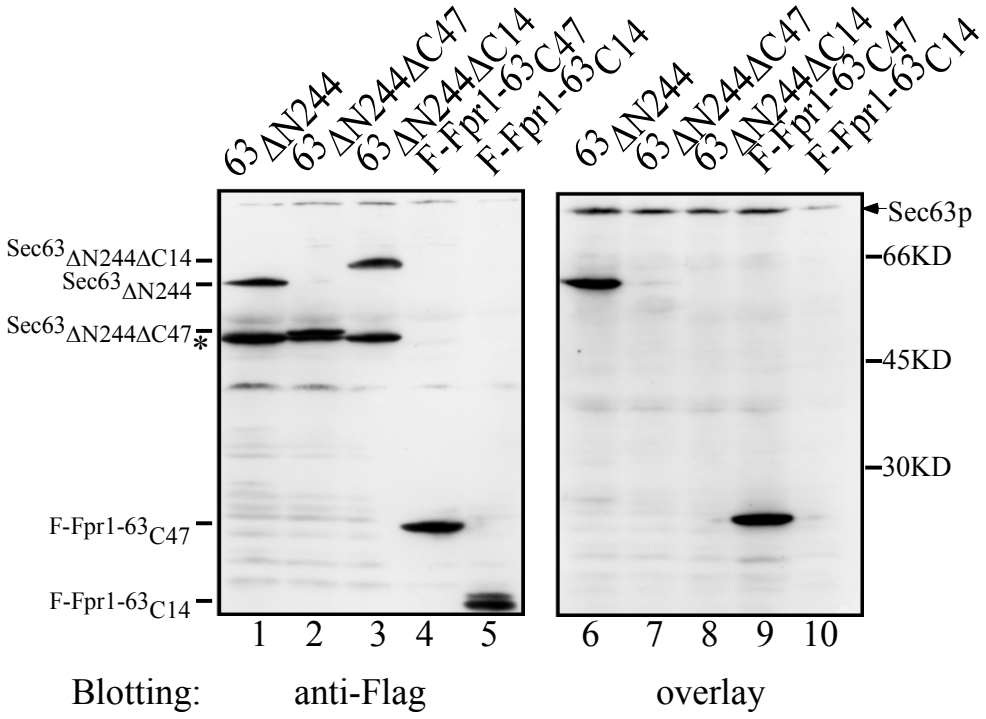

Figure 3.7. Overlay assay. Extracts of cells expressing the Flag tagged cytosolic domain of Sec63p ($Sec63_{\Delta N244}$) or mutants thereof were subjected to SDS-PAGE followed by transfer onto nitrocellulose membrane. The nitrocellulose was probed with anti-Flag antibody (left panel) or incubated with cell extracts expressing $Sec62_{\Delta C125}$-Dha and then probed with anti-Ha antibody (right panel). The bound $Sec62_{\Delta C125}$-Dha was detected by the anti-Ha antibody. The arrow indicates the native Sec63p. The asterisk indicates the degradation products of the ectopically expressed cytosolic domain of Sec63p.

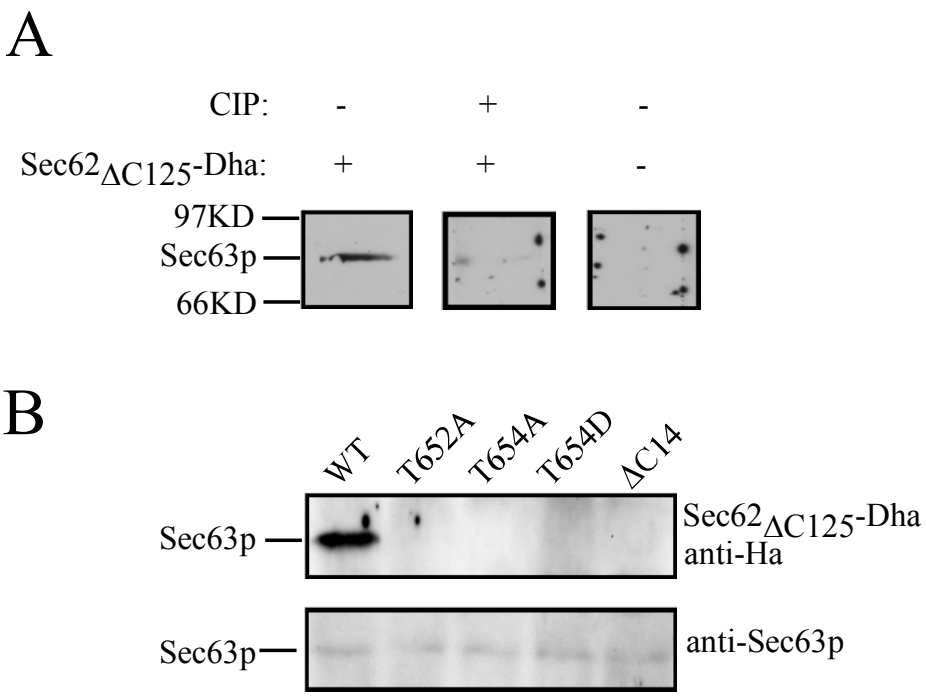

Figure 3.8. Full length Sec63p is phosphorylated at its C-terminal domain in vivo. **A**. Protein extracts from wild type cells were separated by 12.5% SDS-PAGE and transferred onto nitrocellulose. The blot was treated either with or without phosphatase (CIP: +, CIP: -) and subsequently incubated with extracts from yeasts either expressing $Sec62_{\Delta C125}$-Dha or not ($Sec62_{\Delta C125}$-Dha: +, $Sec62_{\Delta C125}$-Dha: -). Bound $Sec62_{\Delta C125}$-Dha was detected with anti-Ha antibody. **B.** Extracts of yeast cells expressing Sec63p or its alleles were subjected to the $Sec62_{\Delta C125}$-Dha overlay assay (upper panel), or detection with anti-Sec63p antibody (lower panel). ΔC14 indicates a mutant of Sec63p lacking the last 14 amino acids.

3.1.3.3 The interaction between the C-terminal domain of Sec63p and the N-terminal domain of Sec62p is direct

All experiments performed so far can not strictly rule out that a third unknown protein might mediate the interaction between the cytosolic domains of Sec62p and Sec63p. However once denatured and separated by SDS-PAGE, all potential binding partners of Sec63p should have been removed. The overlay assay therefore opens the

possibility to ultimately test whether the interaction between the cytosolic domains of Sec62p and Sec63p are direct or mediated by a third protein contained in the yeast extracts. F-Fpr1-63_{C47} or F-Fpr1-$63_{C47T652A}$ were immobilized after SDS-PAGE on the nitrocellulose membrane and the membrane was incubated with a Ha and 6× His tagged N-terminal domain of Sec62p that was expressed in *E.coli*. and purified via Ni-affinity chromatography. The interaction signal that appeared at the position of the immobilized F-Fpr1-63_{C47} but not at the position of its mutant proofs that the interaction between the two cytosolic domains is indeed direct (Figure 3.9).

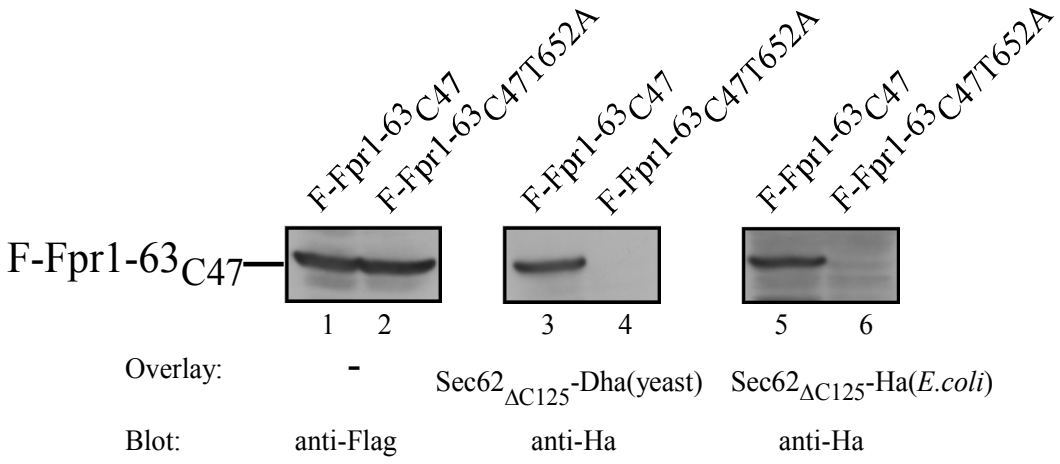

Figure 3.9. The interaction between Sec62$_{\Delta C125}$-Dha and the C-terminal domain of Sec63p is direct. Extracts of yeast cells expressing the Flag tagged last C-terminal 47 residues of Sec63p (lane 1, 3 and 5) or its T652A mutant (lane 2, 4 and 6) were separated by SDS-PAGE followed by transfer onto nitrocellulose. The nitrocellulose was either probed with anti-Flag antibody (lane 1 and 2) or incubated with cell extracts expressing Sec62$_{\Delta C125}$-Dha from yeast cells (lane 3 and 4) or incubated with Sec62$_{\Delta C125}$-Ha purified from *E.coli* (lane 5 and 6). The blot was finally probed with anti-Ha antibody (lane 3-6).

3.1.3.4 Monitoring the influence of phosphorylation on the interaction between Sec63p and Sec62p in living cells

The overlay assays indicate that the in vitro interaction between the full length Sec63p and the N-terminal domain of Sec62p depends on the phosphorylation of threonine 652 and 654. To confirm the influence of Sec63p phosphorylation on its interaction with Sec62p in living cells, I investigated the interaction between the full length Sec63p and the full length Sec62p in vivo. To be able to selectively precipitate Sec63p from extracts, I replaced in a first step chromosomal *SEC63* with *SEC63-PROA* via homologous recombination. The Sec63-ProA fusion protein still binds to $Sec62_{\Delta C125}$-Dha in the overlay assay (Figure 3.10A) and complements the function of Sec63p in protein translocation (data not shown). When I replaced threonine 654 with alanine in the fusion protein ($Sec63_{T654A}$-ProA), its binding to $Sec62_{\Delta C125}$-Dha was abolished in the overlay assay (Figure 3.10B).

The in vivo interaction between Sec63-ProA and the full length Sec62p was investigated by a co-precipitation experiment. Sec63-ProA or $Sec63_{T654A}$-ProA were precipitated from yeast extracts with IgG-sepharose beads and the bound and unbound fractions were probed with anti-Sec62p antibody. The western blot shows that Sec62p was precipitated only by the wild type Sec63-ProA but not by $Sec63_{T654A}$-ProA (Figure 3.10C). The experiment therefore confirms that the phosphorylatable threonines are also important for the strength of the Sec63p-Sec62p interaction in vivo.

Additional evidence on the influence of the Sec63p phosphorylation on the Sec62/63p complex stability in living cells was obtained by the split-ubiquitin assay (Figure 3.11A) (Johnsson and Varshavsky, 1994a). In this assay Sec63p or its mutants were fused to the C-terminal half of ubiquitin that was attached to RUra3p (Sec63-C_{ub}-RUra3p), whereas Sec62p was fused behind the N-terminal half of ubiquitin (N_{ug}-Sec62p). The RUra3p module monitors the interaction-induced reassociation between N_{ub} and C_{ub} by a simple growth assay. The reassociation of the Ub halves initiates the

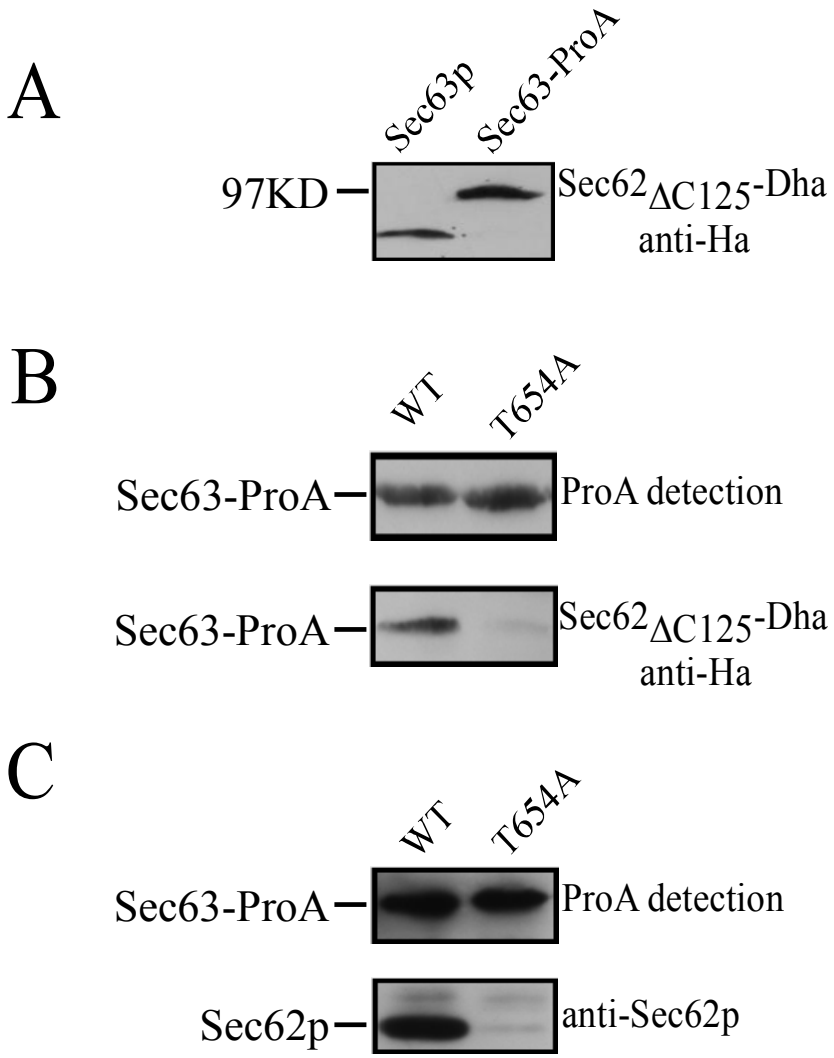

Figure 3.10. Dephosphorylated Sec63p cannot form a stable Sec62/63p complex. A. Overlay assay of cell extracts expressing Sec63p or Sec63-ProA. **B.** Cell extracts containing Sec63-ProA or $Sec63_{T654A}$-ProA were subjected to the overlay assay (lower panel). Similar protein levels were estimated by incubations with rabbit anti-goat/goat anti-rabbit antibodies (upper panel). **C.** Extracts of yeast cells expressing Sec63-ProA or $Sec63_{T654A}$-ProA were precipitated with IgG-sepharose and the precipitates were probed with anti-Sec62p serum (lower panel). Incubation with rabbit anti-goat/goat anti-rabbit antibodies confirmed similar protein levels of Sec63-ProA or $Sec63_{T654A}$-ProA.

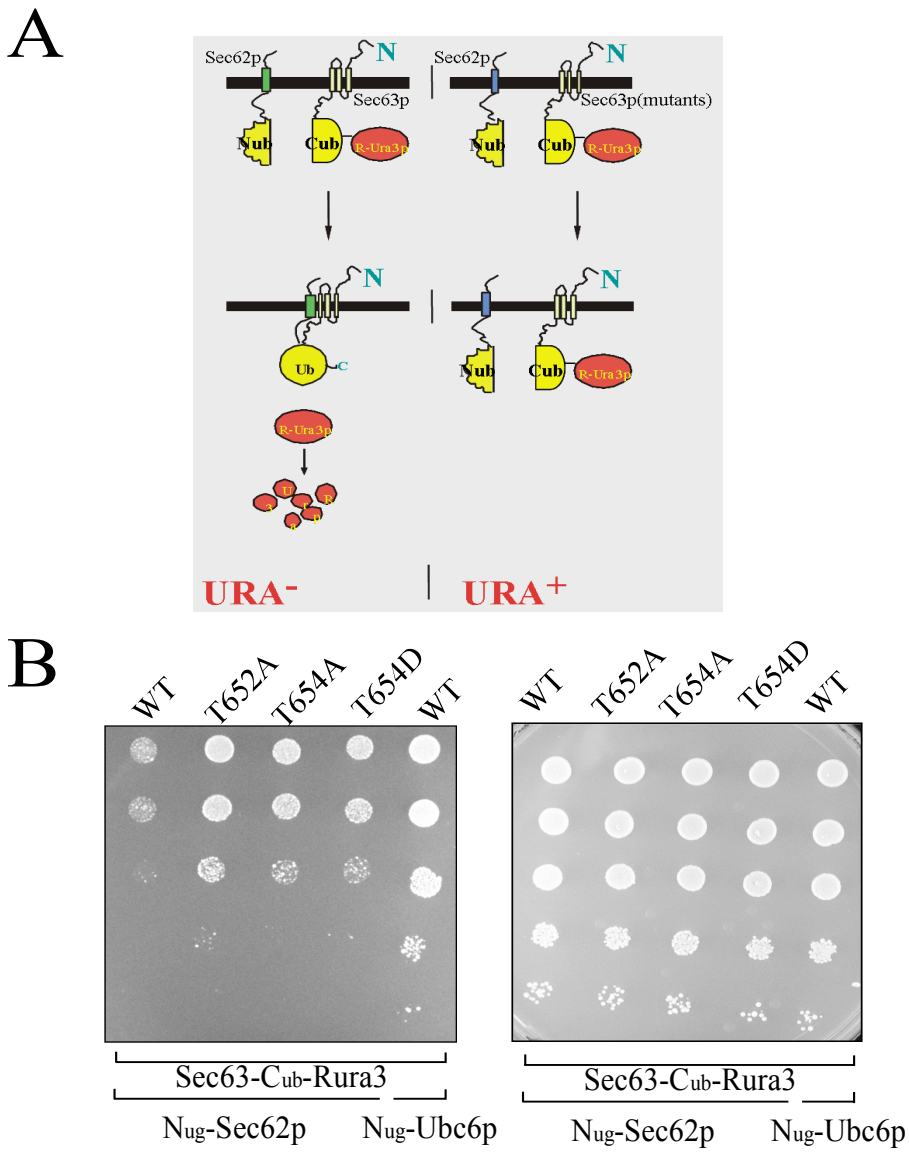

Figure 3.11. Dephosphorylated Sec63p is not proximal to Sec62p in vivo. A. Scheme of the split-ubiquitin assay. **B.**Yeast cells co-expressing Sec63-C_{ub}-RUra3p or its mutants together with N_{ug}-Sec62p or N_{ug}-Ubc6p were grown to an OD_{600} of 1. 4 µl of the cell culture and ten-fold dilutions thereof were spotted on plates lacking uracil (left panel) and tryptophan and histidine to select for the presence of the plasmids. The growth on plates containing uracil confirmed that equal cell numbers were spotted (right panel).

cleavage of the RUra3p from C_{ub}. Due to its exposed N-terminal arginine, the enzymes of the N-end rule pathway of protein degradation rapidly degrade RUra3p. In this assay interactions are therefore detected by the non-growth of the N_{ub}/C_{ub}- co-transformants on SD-ura (Wittke et al., 1999). In this interaction assay the wild type Sec63p binds much stronger to Sec62p than the mutants bearing a alanine residue at position 652 or 654 (T652A or T654A) or an aspartate at position 654 (Figure 3.11B). The small yet reproducible difference in the growth of the co-transformants suggests that the mutants at position 652 had a more severe effect on the binding to Sec62p than the corresponding mutation at position 654. Furthermore residual interactions between Sec62p and the mutants of Sec63p have to be assumed as the cells that co-express the ER membrane protein N_{ug}-Ubc6p together with Sec63-C_{ub}-RUra3p grew slightly better on SD-ura than the cells co-expressing N_{ug}-Sec62p together with the mutants of Sec63-C_{ub}-RUra3p (Figure 3.11B). Ubc6p is a membrane protein of the ER that does not bind to Sec63p and served here as a negative control for the assay (Gilon et al., 2000).

3.2 The disruption of Sec63p phosphorylation causes a protein translocation defect

My in vitro binding experiments showed that alanine exchanges of the threonines at position 652 and 654 mimicked the dephosphorylation of the C-terminal domain of Sec63p in respect to its binding to Sec62p. I therefore proposed that these mutations could also be used to establish the role of phosphorylation on the function of Sec63p in the cell.

A functional Sec62/63p complex is known to be important for the posttranslational translocation of protein across the membrane of the ER (Wittke et al., 2000). To measure the effect of the non-phosphorylatable *sec63* alleles on protein translocation, I followed the maturation of carboxypeptidase Y (CPY) in strains carrying the alleles $sec63_{T652A}$ or $sec63_{T654A}$. CPY is a vacuolar enzyme and a widely used model substrate for the posttranslational translocation pathway (Hann and Walter, 1991).

First CPY is synthesized as preproCPY in the cytosol and then transferred to the lumen of the ER where the signal sequence is removed and the protein is glycosylated to create the P1 and P2 forms of CPY. In the actual experiment wild type cells , cells carrying the $sec63_{T652A}$ or the $sec63_{T654A}$ allele and cells carrying the temperature sensitive allele *sec62-1* (Deshaies and Schekman, 1989) were labeled for 5 minutes with ^{35}S-methionine. The cells were lysed and CPY was immunoprecipitated from the yeast extracts with a monoclonal anti-CPY antibody. After SDS-PAGE and autoradiography the ratio of translocated CPY versus cytosolic preproCPY was estimated and compared between the different yeast strains. As can be seen in Figure 3.12A both threonine exchanges in *SEC63* cause a significant accumulation of preproCPY in the cytosol. The accumulation of cytosolic CPY is however more severe in cells carrying the *sec62-1* allele (Figure 3.12A). The experiment proofs that the exchange of both threonines against alanines causes a partial translocation defect. Again the impact on protein translocation is more severe for the exchange at position 652 of Sec63p. To follow the influence of the *SEC63* alleles on the translocation of other signal sequence-bearing proteins I made use of a sensitive growth assay (Wittke et al., 2002). Here different signal sequences were fused in front of the N-terminus of Ura3p. The fusion proteins were then expressed in wild type cells and cells carrying different *SEC63* alleles. Cells displaying a translocation defect can grow on plates lacking uracil due to the accumulation of the Ura3p activity in the cytosol. The non-growth on SD-ura reflects the efficient import of the translocation substrates into the lumen of the ER and therefore confirms the functionality of the respective allele. According to this assay cells carrying $sec63_{T652A}$, $sec63_{T654A}$, $sec63_{T654D}$ or $sec63_{\Delta C14}$ can only incompletely translocate the fusion proteins containing the signal sequences of the α-factor, invertase, CPY and Kar2p (Figure 3.12B). A similar phenotype was observed for the cells expressing a mutant of Sec62p (Wittke et al., 2000). $Sec62_{\Delta N144}$ lacks the N-terminal binding site for the C-terminus of Sec63p. This mutant was therefore expected to cause a translocation defect that is similar to the alleles of Sec63p that display a mutation in the complementary binding site (Figure 3.12B).

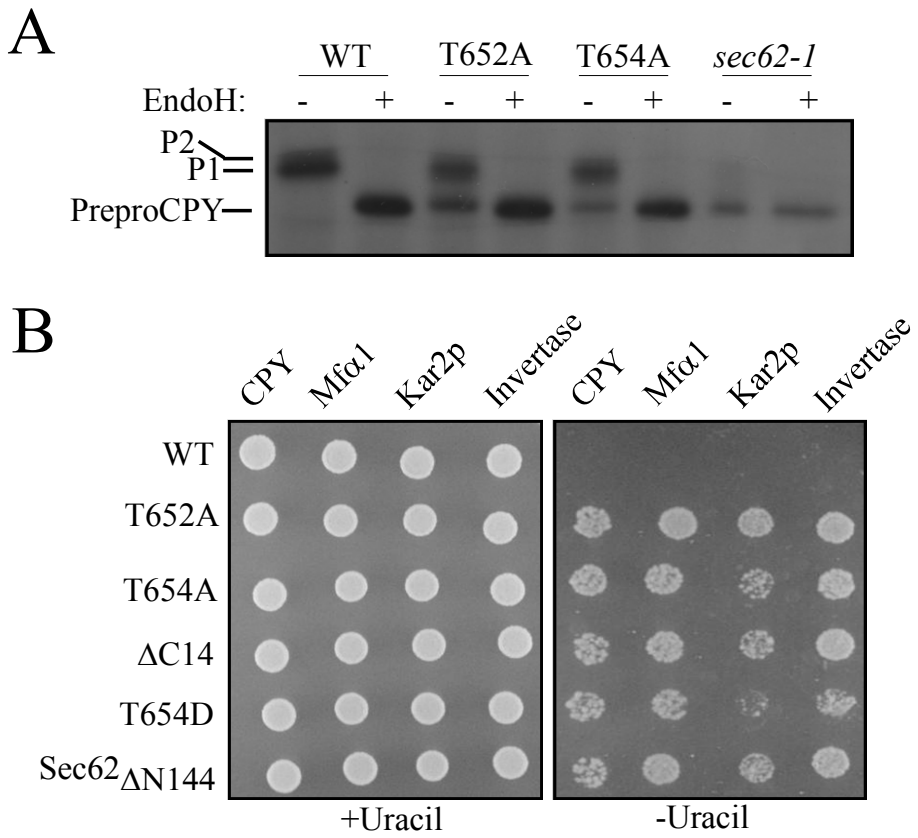

Figure 3.12. Non-phosphorylatable Sec63p causes a protein translocation defect. **A.** Pulse analysis of CPY translocation. Wild type cells, cells carrying the alanine replacements in position 652 or 654 of Sec63p, or *sec62-1* ts cells were labeled with ^{35}S-methionine for 5 minutes and subjected to a CPY immunoprecipitation. P1 and P2 indicate the positions of the translocated and preproCPY the position of the non-translocated fraction of CPY on the autoradiogram of the gel. The shift in the apparent molecular weight after EndoH treatment (EndoH: +) confirmed the localization of P1 and P2 in the early 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 containing or lacking uracil and histidine to select for the presence of the plasmid. Growth of cells on SD-ura plates indicates a translocation defect for the signal sequence of the indicated yeast proteins (CPY, Mfα1, Kar2p and invertase).

3.3 Loss of interaction does not lead to Sec62p mislocalization

One of the direct consequences of a less stable interaction between Sec62p and the other members of the Sec complex might be a mislocalization of Sec62p. To test this assumption I analyzed the localization of a Sec62-GFP fusion protein in wild type cells and cells carrying the alleles *sec63*$_{T652A}$ or *sec63*$_{T654A}$. The fluorescence microscopy revealed a very similar distribution of Sec62p in the *sec63*$_{T652A}$, *sec63*$_{T654A}$ or the wild type cells (Figure 3.13). In all cells the observed localization of Sec62p is similar to other ER resident proteins. I concluded that the disturbance of the interaction between Sec63p and Sec62p did not lead to a dramatic relocalization of Sec62p.

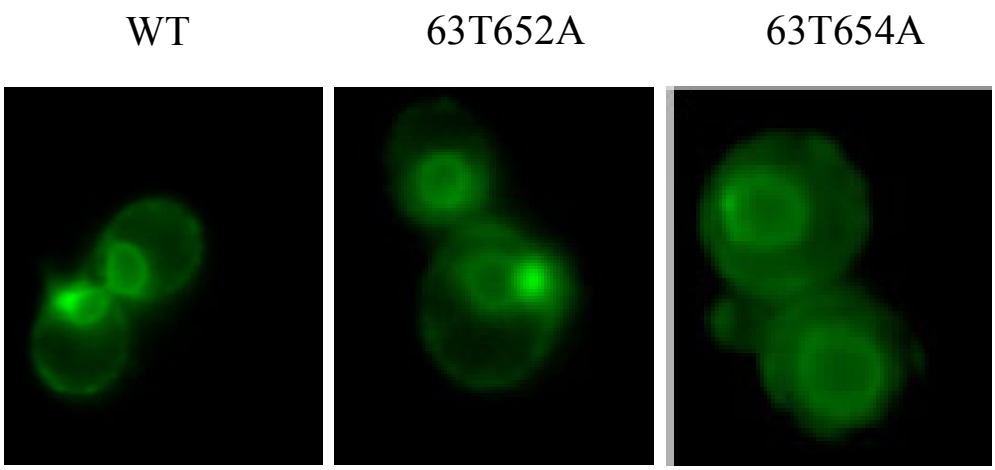

Figure 3.13. Dephosphorylated Sec63p does not change the localization pattern of Sec62p. The cells expressing Sec62-GFP containing *SEC63* or its allele *sec63*$_{T652}$ or *sec63*$_{T654A}$ were cultured to an OD_{600} of 1.0. Cells were directly observed under the fluorescent microscope using light with a wavelength of 488nm.

3.4 The C-terminus of Sec62p contributes a second binding site to the Sec complex

Cells expressing alleles of *SEC63* that lack a functional binding site to the N-terminus of Sec62p or cells expressing alleles that lack the corresponding binding site on Sec62p are still viable. In addition, the translocation defect of cells carrying $sec63_{T652A}$ or $sec63_{T654A}$ is only relatively mild (Figure 3.12A). As Sec62p contains an additional binding site for the Sec complex in its C-terminal domain, I assumed that the phosphorylatable threonines at position 652 and 654 become only essential for the formation of the Sec complex once this alternative binding site had been removed. To prove this assumption, I transformed the empty *TRP1* plasmid or *TRP1* plasmids containing the wild type *SEC62* or different alleles of *SEC62* ($sec62_{\Delta N144}$, $sec62_{\Delta C35}$

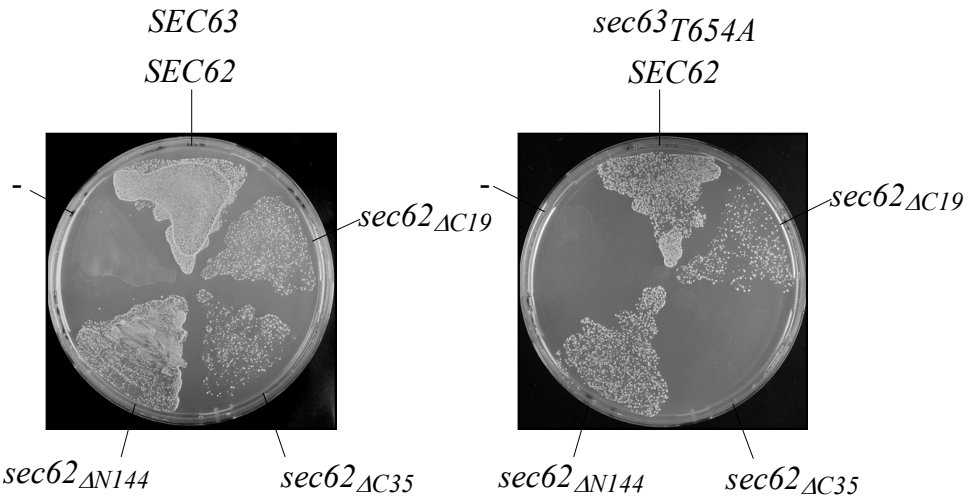

Figure 3.14. Synthetic lethality between the $sec62_{\Delta C35}$ allele and $sec63_{T654A}$ allele. Yeast cell containing *SEC63* or $sec63_{T654A}$ and expressing Sec62p from a *URA3* plasmid were transformed with the different *TRP1* plasmids containing the indicated alleles of *SEC62* carrying the Dha module at their C-termini. Cells were plated on 5-FOA containing media. The non growth of cells carrying $sec63_{T654A}$ and $sec62_{\Delta C35}$-*Dha* indicates a synthetic lethality between $sec63_{T654A}$ and the $sec62_{\Delta C35}$-*Dha* allele.

and *sec62*$_{\Delta C19}$) into yeast cells containing *SEC63* or *sec63*$_{T654A}$ and expressing Sec62p only from a centromeric *URA3* plasmid. The transformed cells were then plated onto medium containing 5-FOA (5-Fluoroorotic acid). 5-FOA can be converted by Ura3p into the toxic substance 5-Fluorouridine monophosphate. To survive on this medium, the transformants have to be able to lose the *URA3* plasmid encoding the native *SEC62*. This however is only possible when the *sec62* allele on the *TRP1* plasmid encodes a functional copy. The non-growth of the different yeast strains on the 5-FOA plates will therefore indicate whether a certain *SEC62* allele is functional in the *sec63*$_{T654A}$ or the wild type background. As can be seen in Figure 3.14, all *SEC62* alleles are functional in the genetic background of the otherwise wild type cells. However cells carrying a mutation in the binding site of Sec63p for Sec62p and containing the *sec62*$_{\Delta C35}$ allele can not survive on 5-FOA medium. The synthetic lethality between the two alleles indicates that the C-terminus of Sec62p harbors an additional binding site for the Sec complex. This binding site becomes essential for the survival of the cells once the interaction between the N-terminus of Sec62p and the C-terminus of Sec63p is destroyed or at least significantly impaired.

3.5 Casein kinase 2 (CK2) phosphorylates Sec63p in vivo and in vitro

3.5.1 The C-terminal domain of Sec63p can be phosphorylated by CK2 in vitro

Casein kinase 2 (CK2) is a highly conserved serine/threonine protein kinase that is found in all eukaryotic organisms. The typical CK2 recognition site has been defined as a serine or threonine residue that is preceded by two and followed by two to five acidic residues (Meggio et al., 1994). The sequence environments of T652 and T654 are both in accord with the consensus target phosphorylation site of the protein kinase CK2 (Figure 3.1C). *S. cerevisiae* possesses two CK2 isoforms that are encoded by an essential gene pair, *CKA1* and *CKA2*. To figure out whether CK2 directly phosphorylates Sec63p, I first set up a simple in vitro system. To isolate CK2 from

yeast, the sequence coding for protein A (ProA) was integrated in frame to the coding sequence of *CKA1*. The resulting fusion protein was then purified from yeast extracts via IgG-sepharose beads. As the substrate for the phosphorylation reaction, I purified the fusion protein F-Fpr1-Sec63$_{C14}$ from yeast extracts by an immunoprecipitation with anti-Flag antibody. The enriched F-Fpr1-Sec63$_{C14}$ was treated on the beads with CIP to remove the phosphates and finally eluted from the anti-Flag antibody by incubating the agarose beads with a solution containing the Flag peptide. The same procedure was repeated with the F-Fpr1-63$_{C14T652;\ 654A}$ that served as a control substrate for the kinase reaction. Dephosphorylated F-Fpr1-63$_{C14}$ and F-Fpr1-63$_{C14T652;\ 654A}$ were incubated with immobilized Cka1p in the presence of [γ-^{32}P] GTP.

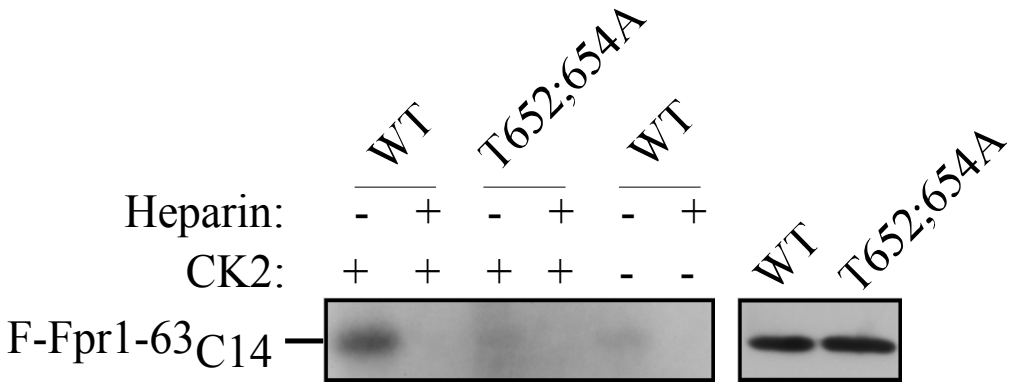

Figure 3.15. The C-terminal tail of Sec63p can be directly phosphorylated by Casein kinase 2 (CK2) in vitro. F-Fpr1-63$_{C14}$ and F-Fpr1-63$_{C14T652;654A}$ were eluted from anti-Flag antibody precipitates with an excess of the Flag peptide. Autoradiogram (left panel) or anti-Flag western blot (right panel) of CIP-treated F-Fpr1-63$_{C14}$ or F-Fpr1-63$_{C14T652;\ 654A}$ incubated in the presence of [γ-^{32}P] GTP and Cka1-ProA attached to IgG-sepharose (CK2: +) or in the presence of mock treated IgG-beads (CK2: -). The CK2 inhibitor heparin was added to the reaction in a concentration of 10 μg/ml.

After the termination of the reaction, F-Fpr1-63_{C14} and F-Fpr1-$63_{C14T652;\ 654A}$ were resolved by SDS-PAGE and the incorporation of [γ-^{32}P] into F-Fpr1-63_{C14} was determined by autoradiography. Significant amounts of [γ-^{32}P] were indeed incorporated into dephosphorylated F-Fpr1-63_{C14} but not into the mutant F-Fpr1-$63_{C14T652;\ 654A}$. This incorporation was completely blocked by heparin, an inhibitor of CK2 activity (Figure 3.15). Together these results demonstrate that CK2 can directly phosphorylate the C-terminal domain of Sec63p in vitro.

3.5.2 The C-terminal Sec63p can be phosphorylated by CK2 in vivo

To test whether CK2 is also responsible for the phosphorylation of Sec63p in vivo, I used the yeast strain YDH8. This strain contains a temperature sensitive *cka2* allele as its sole CK2 encoding copy. As a consequence, CK2 becomes inactivated when the temperature is shifted from 25°C to 37.5°C. Since the phosphorylation of F-Fpr1-63_{C14} can be easily detected by the phosphorylation-induced shift of its molecular weight during SDS-PAGE, I first introduced F-Fpr1-63_{C14} into YDH8 cells and the isogenic strain YDH6 that harbors the native *CKA2* locus. The cells were grown at 25°C and then transferred to 37.5°C. Copper was immediately added to the culture medium to 100 µM after the temperature shift to induce the expression of the fusion protein. Aliquots of the culture were taken after 0, 0.5, 1, 2, and 3 hours at 37.5°C. The phosphorylation status of F-Fpr1-63_{C14} was monitored by SDS-PAGE and subsequent western blotting with anti-Flag monoclonal antibody. The appearance of the indicative double band of F-Fpr1-63_{C14} in the wild type but not in the *cka2ts* strain clearly shows that the in vivo phosphorylation of the last 14 residues of Sec63p depends on the presence of CK2 (Figure 3.16A). Treatment of F-Fpr1-63_{C14} with CIP confirmed that no phosphorylation had occurred in F-Fpr1-63_{C14} from the *cka2ts* strain while F-Fpr1-63_{C14} from the wild type strain was very likely phosphorylated at both threonine residues (Figure 3.16B). The experiments therefore indicate that CK2 phosphorylates the C-terminal domain of Sec63p in vivo.

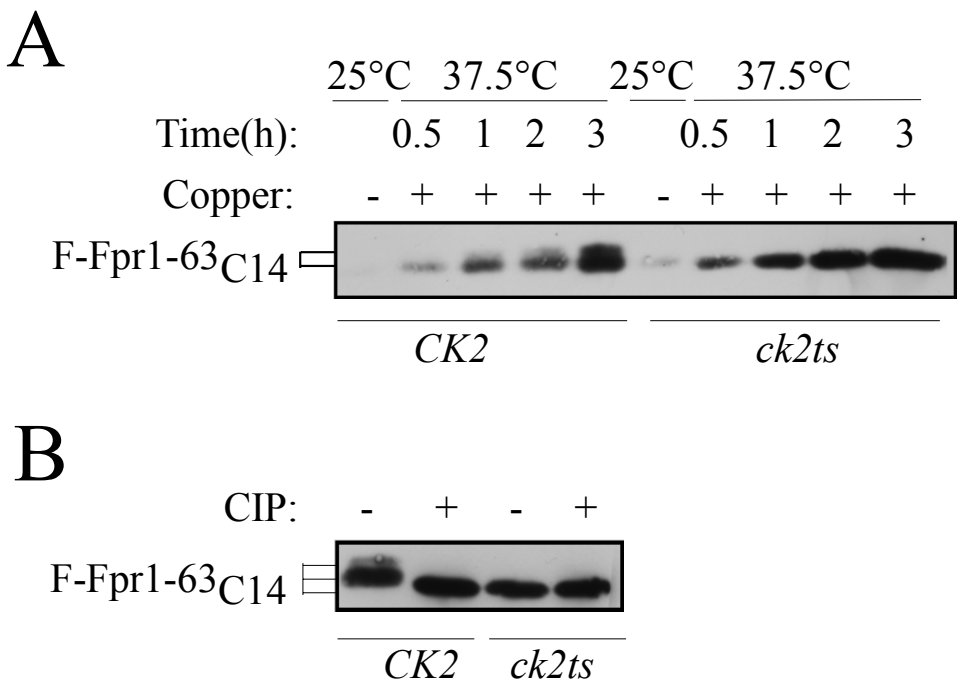

Figure 3.16. The C-terminal tail of Sec63p can be phosphorylated by CK2 in vivo. **A.** Yeast cells carrying the indicated alleles of *CKA2* were shifted from 25°C to 37.5°C and the expression of F-Fpr1-63_{C14} was induced. Cell extracts were prepared at the indicated times and probed with anti-Flag 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 (CIP: -) or treated with an unspecific phosphatase (CIP: +).

3.5.3 Full length Sec63p can be phosphorylated by CK2 in vivo

To test whether the full length Sec63p is also phosphorylated by CK2 in vivo, I used the overlay assay to monitor the phosphorylation of the full length Sec63p in the YDH8 strain after the shift to the restrictive temperature. However, I failed to observe a rapid dephosphorylation of endogenous Sec63p in the YDH8 strain after the shift to the non-permissive temperature (Figure 3.17). This observation raises the question

whether full length Sec63p is indeed a substrate for CK2 in vivo or whether Sec63p is phosphorylated by CK2 but turned over only slowly. To distinguish between these two alternative interpretations I expressed Sec63p ectopically by the inducible P_{MET17}-promoter to selectively follow the modification of the newly made Sec63p in cells without CK2 activity. To separate the induced Sec63p from the naturally expressed Sec63p I replaced the chromosomal *SEC63* with *SEC63-PROA* by homologous recombination. Cells were cultured at 25°C with high concentrations of methionine to reduce the expression of the ectopic Sec63p, transferred to medium without methionine to increase the expression of Sec63 and immediately shifted to 37.5°C to inactivate CK2. Extracts of the yeast cells were prepared at different times after the temperature shift and the proteins were resolved by SDS-PAGE. The proteins were then transferred onto nitrocellulose and the membrane was probed by the $Sec62_{\Delta C125}$-Dha overlay assay. The assay revealed that the amount of phosphorylated Sec63p

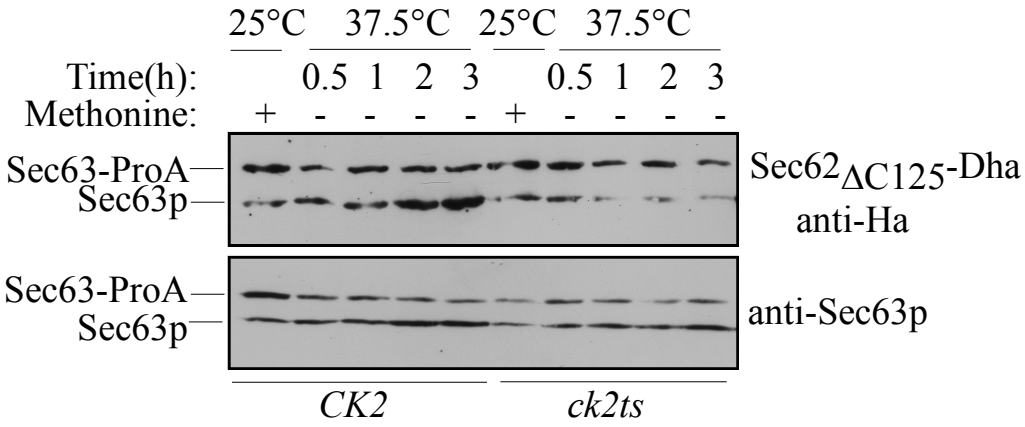

Figure 3.17. The full length Sec63p is phosphorylated by CK2 in vivo. A *CK2* and a *ck2ts* strain containing *SEC63-ProA* and expressing Sec63p ectopically from the P_{MET17}-promotor were grown at 25°C in medium containing 10 mM methionine and shifted to 37.5°C and methionine free medium. Extracts were prepared at different times after the shift and were probed with the $Sec62_{\Delta C125}$-Dha overlay assay (upper panel) or with anti-Sec63p antibody (lower panel).

increases over time in the wild type strain whereas only traces of phosphorylated Sec63p are detected after two or three hours after the temperature shift in the *cka2ts* strain (Figure 3.17, upper panel). Since Sec63p expression levels increased similarly in both strains during the incubation at 37.5°C (Figure 3.17, lower panel), this result provides evidence that full length Sec63p is phosphorylated at its C-terminal domain by CK2 in vivo.

3.5.4 Phosphorylated Sec63p is slowly turned over

The same experiment shows that the endogenously expressed Sec63-ProA is still recognized by the overlay assay even after three hours after the temperature shift had occurred (Figure 3.17, upper panel). I therefore conclude that Sec63p is phosphorylated by CK2 but that the turnover of the protein linked phosphate is rather slow.

As Sec63p is phosphorylated by CK2, depletion of CK2 activity should lead to a measurable defect in posttranslational protein translocation. However the kinetics of dephosphorylation suggested that a defect in protein translocation might become apparent only after extended times of CK2 inactivation. To measure protein translocation 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 and subjected to a CPY immunoprecipitation. Although the synthesis of CPY declined in the *ck2ts* strain during the incubation at 37.5°C, the newly made CPY was still properly translocated across the membrane even after a prolonged incubation at the restrictive temperature (Figure 3.18). The ongoing translocation of preproCPY can be explained by the presence of phosphorylated Sec63p even 8 hours after the shift. The observed reduction in the amount of phosphorylated Sec63p results predominantly from a general decrease of the protein levels in the *ck2ts* cells and not a selective dephosphorylation of Sec63p.

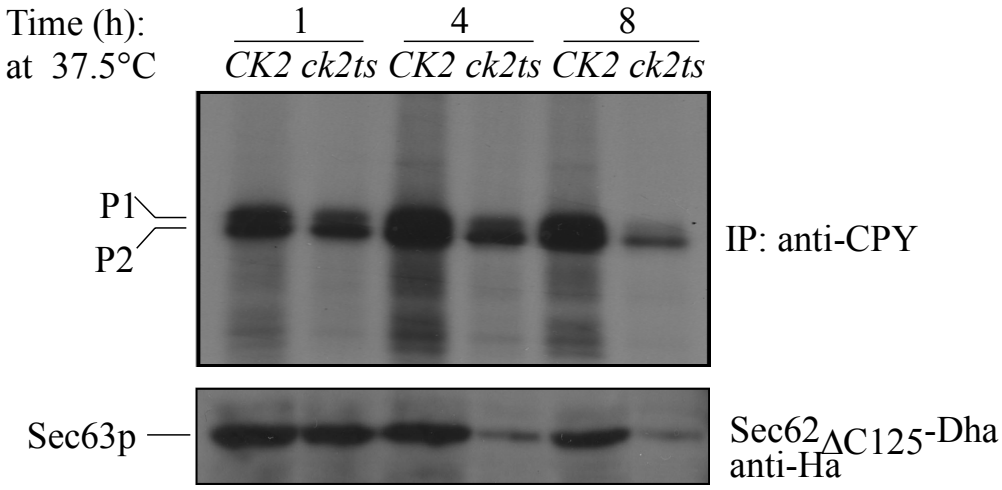

Figure 3.18. Phosphorylated Sec63p is slowly turned over. Wild type or *cka2ts* cells were shifted to the restrictive temperature, pulsed after the indicated times with ^{35}S-methionine and subjected to CPY immunoprecipitation (upper panel). Extracts of the same cells were also probed for the presence of the phosphorylated Sec63p with the Sec62$_{\Delta C125}$-Dha overlay assay (lower panel).

3.6 The phosphorylation status of Sec63p is not altered under some stress conditions

The experiments in Figure 3.17 and 3.18 imply that phosphorylated Sec63p turns over very slowly under favorable growth conditions. Are there certain conditions under which the phosphorylation of Sec63p is regulated? To find this out, I monitored the status of Sec63p phosphorylation under different stress conditions. First I treated cells expressing Sec63-ProA with DTT or tunicamycin. Both reagents increase the amount of the unfolded proteins in the ER and induce a signaling pathway called the unfolded protein response (UPR) (Koizumi et al., 1999; Trotter and Grant, 2002). Under these conditions it is thought that a higher proportion of proteins are translocated back from

the ER lumen to the cytosol. I speculated that under these conditions the molecular composition of the translocation channels might be altered. At different times after addition of DTT or tunicamycin, the cell extracts were prepared and the proteins were

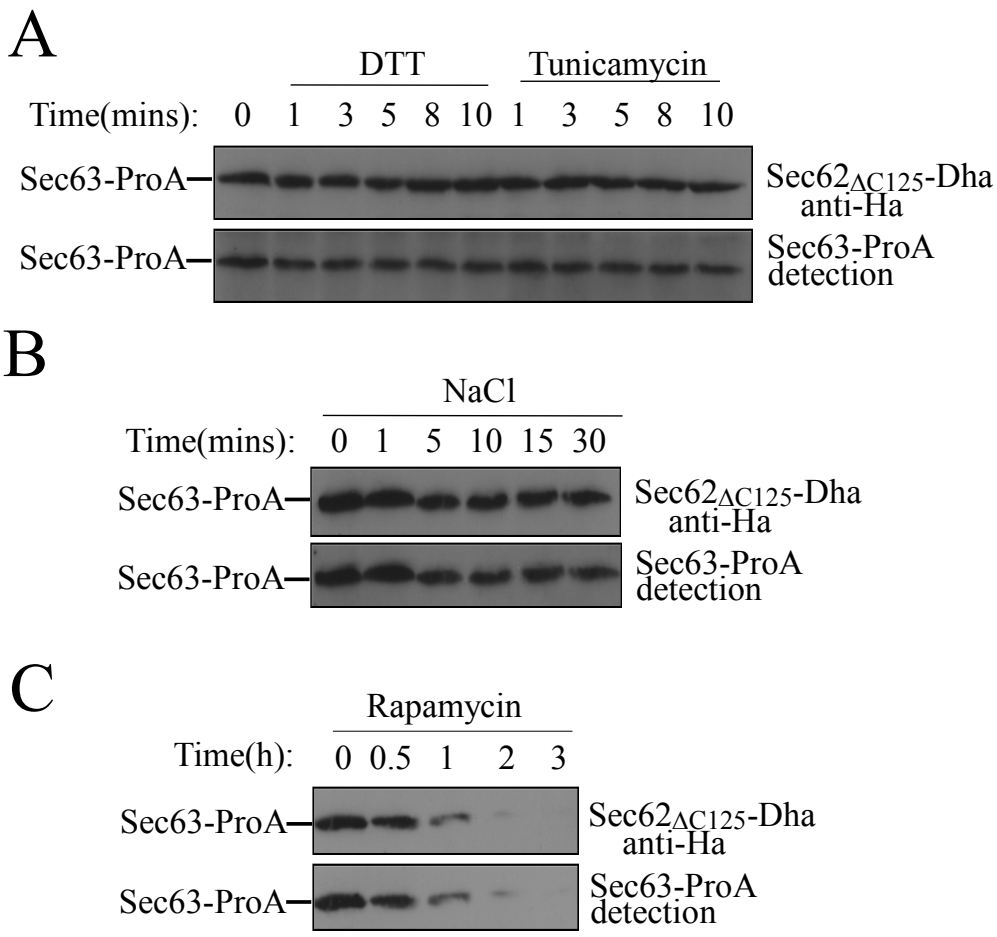

Figure 3.19. Sec63p is constitutively phosphorylated under some stress conditions. A. Extracts of cells expressing SEC63-ProA were prepared after different times of 5 mM DTT or 10 µg/ml tunicamycin treatment and subjected to SDS-PAGE. Extracts were probed with the Sec62$_{\Delta C125}$-Dha overlay assay (upper panel). The same blot was stripped and then probed with goat anti-rabbit/rabbit anti-goat antibodies for the ProA detection (lower panel). **B.** The same as A except for treating the cells with 1 M NaCl. **C.** The same as A except for treating cells with 0.2 µg/ml rapamycin.

separated by SDS-PAGE. The overlay assay was used to detect the phosphorylated Sec63p and ProA detection was used to analyze the amount of Sec63-ProA. As can be deduced from the unaltered strength of the signal of the overlay assay, Sec63p is not rapidly dephosphorylated upon treating cells with DTT or tunicamycin. Similar results were obtained when the Sec63-ProA expressing cells were subjected to treatment with high salt, rapamycin, heat or mating hormone respectively (Figure 3.19A, B, and C or data not shown). Rapamycin inhibits the TOR signaling pathway and affects the initiation and elongation of translation, ribosome biosynthesis and amino acid import (Raught et al., 2001). The decrease that I observed for the amount of phosphorylated Sec63p after rapamycin treatment can be explained by a similar decrease in the amount of total Sec63p.

3.7 Fine mapping of the binding site of Sec63p for the N-terminal domain of Sec62p

I could show that only F-Fpr1-63$_{C47}$ but not F-Fpr1-63$_{C14}$ can be detected by the overlay assay (figure 3.7 lane 9 and 10). I assumed that the overlay assay is more stringent than the precipitation assay and that the last 47 residues of Sec63p contain additional sites of contact to Sec62p. If this is true, residues upstream to the last 14 C-terminal residues should increase the affinity of the C-terminal domain of Sec63p to the Sec62$_{\Delta C125}$-Dha. To systematically test this, I constructed plasmids expressing different Sec63p mutants under the control of the inducible P$_{MET17}$-promoter. In these mutants the last 47 residues of Sec63p were replaced by either the last 17 (Sec63$_{\Delta C47C17}$), 20 (Sec63$_{\Delta C47C20}$), 24 (Sec63$_{\Delta C47C24}$), or 28 (Sec63$_{\Delta C47C28}$) residues of Sec63p. I transformed the fusion constructs into a cell strain containing *SEC63-PROA* and prepared the cell extracts for the overlay assay and anti-Sec63p antibody western blotting. The overlay assay revealed a weak binding of Sec62$_{\Delta C125}$-Dha to Sec63$_{\Delta C47C17}$. The binding increases when the C-terminal peptide is extended to 20, 24 or 28 residues. The binding strength of Sec63$_{\Delta C47C20}$ towards the N-terminus of Sec62p is between that of Sec63$_{\Delta C47C17}$ and Sec63$_{\Delta C47C24}$ (Figure 3.20A). To test whether Sec62p binding correlates with the function of Sec63p, I expressed these

mutants in a cell strain in which the expression of the chromosomal Sec63p was placed under the control of the P_{Gal1}-promoter. I investigated the growth of these cells

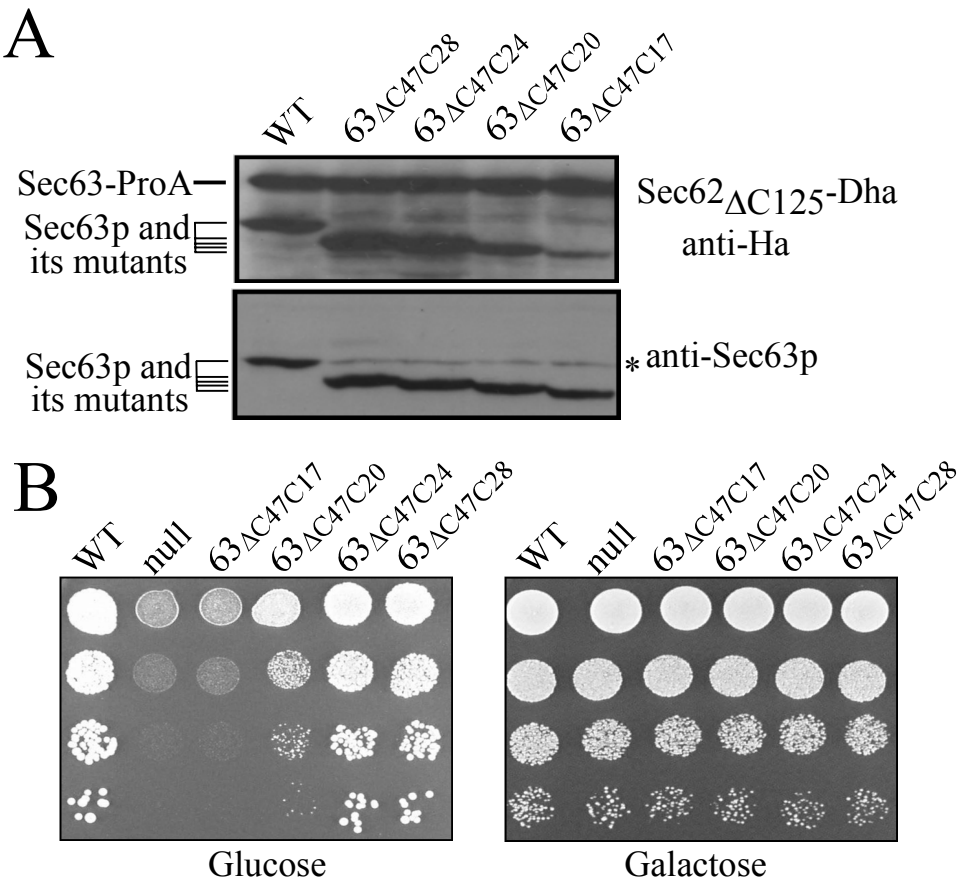

Figure 3.20. Sec63p residues upstream of the last 14 residues of Sec63p are part of the Sec62p binding site. A. The plasmids expressing wild type Sec63p and its mutants were introduced into the yeast strain containing *SEC63-PROA*. Cell extracts were separated by SDS-PAGE and the nitrocellulose was probed with $Sec62_{\Delta C125}$-Dha or with anti-Sec63p antibody. The asterisk indicates a faint cross reacting band that runs at the position of Sec63p. **B** The same plasmids were introduced into a yeast strain containing *Gal-SEC63*. The cells were grown in the galactose medium until an OD_{600} of 1.0. 4 µl of the cell cultures and ten-fold dilutions thereof were spotted on plates containing either glucose (left panel) or galactose (right panel).

on plates containing either glucose or galactose as a carbon source. Under these conditions the expression of the chromosomal Sec63p is either repressed (glucose) and the growth of the cells depends on the functionality of the Sec63p mutants or highly induced (galactose). The growth assay shows that on glucose plates the cells transformed with $Sec63_{\Delta C47C17}$ do not grow, whereas cells expressing $Sec63_{\Delta C47C20}$ grow poorly and cells expressing either $Sec63_{\Delta C47C24}$ or $Sec63_{\Delta C47C28}$ grow as well as the cells expressing the wild type Sec63p (Figure 3.20B). All strains grow equally well on the plate containing galactose (Figure 3.20B). The results of the growth assay therefore reflect the results of the overlay assay quite well. The strength of the binding of the Sec63p mutants to $Sec62_{\Delta C125}$-Dha correlates with their ability to support the growth of yeast strain lacking *SEC63*. Although the last 14 residues of Sec63p are essential for binding to the N-terminal domain of Sec62p, the residues directly proximal to the last 14 residues contribute to its strength and functionality. I further used the growth based translocation assay to investigate how the different

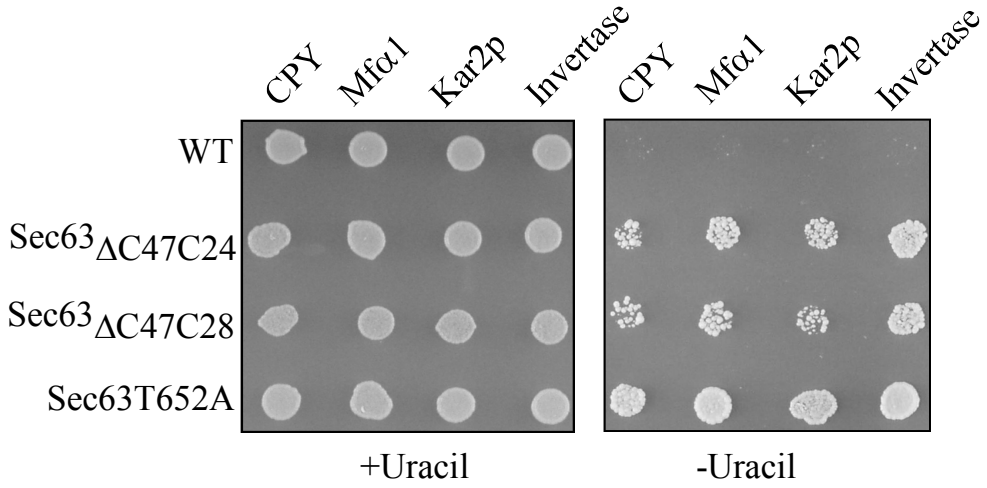

Figure 3.21. Functional analysis of Sec63p deletion mutants with steady state assay. Yeast cells carrying the indicated alleles of *SEC63* and expressing signal sequence bearing C_{ub}-Ura3p constructs were spotted on plates containing or lacking uracil. Growth of cells on SD-ura plates indicates a translocation defect for the signal sequence of the indicated yeast proteins (CPY, Mfα1, Kar2p and invertase).

Sec63p mutants affect the translocation of the signal sequence-bearing Ura3p fusion proteins. To this end the different truncation mutations were integrated via homologous recombination into the *SEC63* locus of the wild type stain JD53. Figure 3.21 shows that defects in protein translocation can be detected for all mutant strains. Interestingly, the translocation defect in the strain carrying $sec63_{\Delta C47C24}$ or $sec63_{\Delta C47C28}$ is less severe than in the strain harboring the $sec63_{T652A}$ allele.

4. Discussion

4.1 The tight assembly of the tetrameric Sec62/63p complex depends on the phosphorylation of Sec63p at its C-terminal domain

The trimetric Sec61p complex is required for both cotranslational and posttranslational protein translocation across the membrane of the endoplasmic reticulum in yeast cells. To transport proteins posttranslationally, the trimeric Sec61p complex has to associate with the tetrameric Sec62/63p complex to constitute a functional heptameric Sec complex. In contrast, Sec61p complex has probably to disassemble from the tetrameric Sec62/63p complex for its role in cotranslational protein translocation (Panzner et al., 1995). A recent quantification of the cellular amounts of all yeast proteins established an 1.5 fold excess of Sec61p over the Sec62/63p complex (Ghaemmaghami et al., 2003). This ratio might be optimal for certain growth conditions. However other cellular conditions might require a rapid redistribution between the free and the Sec62/63p bound Sec61p to adjust the translocation machinery to a changed composition of signal sequences and membrane proteins in the cell. Usually such changes in the composition of a protein complex are induced by posttranslational modifications, phosphorylation being the most common among them. It was already shown that the phosphorylation of some members of the mammalian translocation machinery might be important to promote the efficiency of cotranslational protein translocation (Gruss et al., 1999). In my thesis work, I established for the first time a link between the phosphorylation of one member of the heptameric Sec complex and the stability and functionality of this complex.

In vivo [^{32}P]O_4 labeling experiments 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 the phosphorylation sites in Sec63p (Figures 3.3A and 3.4). 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-terminal domain of Sec63p (Figures 3.3B and 3.8A, B). Coprecipitation of the full length proteins and the split-ubiquitin technique confirmed the necessity of the phosphorylation for the formation of a stable Sec62/63p complex in vivo (Figures 3.10C and 3.11).

Exchanging the threonines 652 and 654 of Sec63p against alanines mimics the negative effect of their dephosphorylation on the in vitro binding to Sec62p. The same alanine replacements in the chromosomally encoded Sec63p impair protein translocation in living cells. Employing two different experimental approaches I could show that the translocation of four different signal sequence-bearing fusion proteins are significantly disturbed. A very similar translocation defect could also be detected in cells expressing a Sec62p mutant that lacks its N-terminal domain (Figure 3.13B). As this N-terminal domain harbors the binding site for the C-terminal sequence of Sec63p, both results independently confirm that Sec63p phosphorylation is a prerequisite for tightly recruiting Sec62p to the Sec complex via its N-terminal domain (Figure 3.3B and 3.13). 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 (Dünnwald et al., 1999; Matlack et al., 1997; Plath et al., 1998; Wittke et al., 2000).

I conclude that the phosphorylation of both threonines in the C-terminus of Sec63p are essential for its strong interaction with N-terminal domain of Sec62p. The phosphorylation of a threonine introduces negative charges at this position. The effect of a phosphorylation can sometimes be mimicked by acidic amino acids (Gryz and Meakin, 2000; Hu et al., 1999). If this is also true for the Sec63p phosphorylation, I could have easily created a mutant of Sec63p whose interaction with Sec62p is constitutive and can not be regulated by phosphorylation. To construct such a Sec63p mutant, I introduced aspartic acid or glutamic acid at position 652 or 654 of Sec63p. The results from the in vitro and in vivo binding experiments showed that the corresponding alleles of Sec63p do not strongly interact with Sec62p any longer

(Figures 3.5 and 3.8B). Probably the N-terminal domain of Sec62p forms a phosphothreonine binding pocket which recognizes more than just the negative charge of the phosphate. Examples of such phosphoamino acid binding pockets can be found in proteins like the human vaccine H1 related phosphatase (Schumacher et al., 2002).

4.2 CK2 phosphorylates Sec63p in vivo and in vitro

Casein Kinase 2 (CK2) is highly conserved Ser/Thr protein kinase that is ubiquitously distributed among eukaryotic organisms. The enzyme was one of the first protein kinases discovered and has been studied for over 40 years in cell culture and tissue extracts (Pinna, 1994; Wojda, 2000). However both the mechanism of regulation of CK2 and its physiological role remained poorly understood. Clues regarding the function of CK2 in vivo have been obtained via the identification of its physiological substrates and molecular genetic studies in genetically tractable organisms (Pinna, 1994). Over hundreds of well-characterized substrates of CK2 are now known (Meggio et al., 1994; Meggio and Pinna, 2003). The enzyme phosphorylates a broad spectrum of targets, including both nuclear and cytoplasmic and both enzymatic and structural proteins. The multiplicities of substrates which play important roles in transcription, translation, signal transduction, cell cycle regulation, etc (Meggio et al., 1994). Therefore CK2 functions to globally regulate cellular metabolism (Litchfield, 2003). Consistent with this viewpoint, molecular genetic studies have shown that CK2 is essential for viability in *Saccharomyces cerevisiae* (Padmanabha et al., 1990).

In *Saccharomyces cerevisiae*, CK2 has been purified to homogeneity and characterized both structurally and functionally (Bidwai et al., 1994). The purified enzyme is a tetramer composed of two distinct catalytic subunits, α and α', and two distinct regulatory subunits, β and β' subunits. The *CKA1*, *CKA2*, *CKB1* and *CKB2* genes encoding the α, α', β and β' subunits respectively have been sequenced (Bidwai et al., 1992; Bidwai et al., 1994; Bidwai et al., 1995; Pilon et al., 1997). Deletion of *CKA1* or *CKA2* alone has no obvious phenotype, but simultaneous deletion of both

genes is lethal, demonstrating that the two catalytic subunits are interchangeable (Birnbaum and Glover, 1991). Deletion of either or both regulatory subunits has no detectable phenotype under optimal growth conditions but results in an increased sensitivity to high concentration of Na^+ and Li^+ (Bidwai et al., 1995).

The inability of a *ck2ts* strain to phosphorylate Sec63p at the restrictive temperature identified CK2 as the responsible kinase of Sec63p (Figure 3.17). The in vitro phosphorylation of the C-terminal peptide of Sec63p by the enriched CK2 confirmed this conclusion and demonstrated that the phosphorylation of Sec63p by CK2 is most probably direct and not mediated by a further kinase(s) (Figures 3.15). Considering the contribution of the phosphorylatable threonines to the translocation of CPY and other signal sequence-bearing test substrates (Figure 3.12), the lack of a detectable translocation defect in the *ck2ts* strain was unexpected (Figure 3.18). However, 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 inactivation of the kinase has occurred (Figures 3.18). It therefore seems that, under the conditions tested, the phosphorylated Sec63p as part of the Sec62/63p complex is protected against rapid dephosphorylation and that Sec63p is constitutively phosphorylated by CK2 under normal growth conditions.

4.3 Sec63p is constitutively phosphorylated

After defining the interaction between the C-terminal domain of Sec63p and the N-terminal domain of Sec62p as phosphorylation dependent, I wondered whether the function of this complex is also regulated by phosphorylation. Until now there are two described activities of Sec63p that are not shared by Sec62p: cotranslational protein translocation and nuclear fusion during karyogamy (Young et al., 2001; Ng and Walter, 1996). Both processes might require the dissociation of Sec62p from Sec63p although a direct proof for this is still lacking. Only indirect evidence suggests that the heptameric Sec complex can not initiate SRP dependent protein translocation and that conversely the trimeric Sec61 complex fails to efficiently recognize posttranslational

translocation substrates (Helmers et al., 2003; Prinz et al., 2000a; Prinz et al., 2000b; Schwartz and Blobel, 2003). Manipulating the stability of the heptameric Sec complex via Sec63p phosphorylation might therefore be one mechanism to reversibly readjust the balance between cotranslational and posttranslational translocation.

Since I have shown that dephosphorylation of Sec63p is very slow, I tested different experimental conditions that might specifically increase the rate of Sec63p dephosphorylation. As I mentioned in the introduction, the nascent chain translocates as an unfolded polypeptide into the lumen of the ER, where it becomes glycosylated, oxidized and folded. Improper folded proteins are retrotranslocated via the Sec61p complex back into the cytosol. There the proteins are deglycosylated, ubiquitylated, and subsequently degraded by the proteasome (Frand et al., 2000; Pilon et al., 1997; Stevens and Argon, 1999; Tanner and Lehle, 1987; Wiertz et al., 1996). Since this retrotranslocation is also achieved by the Sec61p channel, I speculated that the regulated disassembly of the tetrameric Sec62/63p complex might be used to rapidly increase the amount of trimeric Sec61p complex for the process of retrotranslocation. However, in the cells which were treated with DTT or tunicamycin to induce the accumulation of misfolded protein in the ER, the fraction of phosphorylated Sec63p remained constant (Figure 3.19A). Furthermore, I tested the status of Sec63p phosphorylation under other stress conditions. I treated the cells with NaCl, rapamycin or with mating hormone. Upon high salt treatment a signaling pathway is induced to cope with the osmotic stress (Kuo et al., 1997; Varela et al., 1992). Rapamycin treatment suppresses the TOR (target of rapamycin) kinase pathway which acts within an intracellular regulatory network to adjust cell growth according to nutrient availability (Powers et al., 2004). This network affects all aspects of gene expression, including transcription and translation. Mating hormone treatment induces a specific signaling cascade to prepare the cells for cell fusion and to stop their growth (Konopka and Fields, 1992). Under all these different conditions tested, Sec63p remained phoshorylated to the same extent as observed for the unstressed cells (Figure 3.19B, C).

A further evidence against a highly regulated phosphorylation of Sec63p was obtained by the artificial overexpression of Sec63p. When the synthesis of Sec63p was placed under the control of the P_{Gal1} promoter and the cells were incubated in medium with galactose, I always observed a strong increase in the amount of phosphorylated Sec63p that corresponded closely to the increase in the total amount of Sec63p (data not shown). It therefore seems that CK2 can immediately phosphorylate newly synthesized Sec63p that is not bound to the other components of the Sec complex.

All these results imply that Sec63p is constitutively phosphorylated and that a rapid dephosphorylation of Sec63p does not occur under the conditions tested. However one has to realize that the overlay assay that I used to detect the phosphorylated Sec63p is probably not sensitive enough to reveal subtle changes in the amount of phosphorylation. Furthermore I cannot exclude a phosphorylation dependent regulation of the Sec complex assembly under conditions that were not tested in this study. A special yet unknown signal might still induce the dephosphorylation of Sec63p and the subsequent dissociation of the Sec62/63p complex, finally freeing either of the subunits for alternative tasks. Such a readjustment might be required for yeast cells to adapt to physiological situations in which a specific spectrum of proteins has to be translocated across the ER membrane.

4.4 Additional factors contribute to strength of the Sec62p-Sec63p interaction

Although the coprecipitation experiments pointed to an essential role of the last 14 residues of Sec63p to the binding to the N-terminal domain of Sec62p, the overlay assay revealed that additional residues proximal to these 14 amino acids contribute to its strength (Figure 3.20A). When the C-terminal 47 residues of Sec63p were replaced by the last 17 amino acids, a weak but specific binding of the N-terminal domain of Sec62p was detected by the overlay assay. The binding of $Sec63_{\Delta C47C17}$ to $Sec62_{\Delta C125}$-Dha was therefore stronger than the binding of $Sec63_{T652A}$ or F-Fpr1-63_{C14}. Surprisingly whereas cells expressing $Sec63_{T652A}$ were still viable, cells expressing

Sec63$_{\Delta C47C17}$ were not. To resolve this contradiction, I tested further *SEC63* alleles containing 3, 7 or 11 more N-terminal residues than Sec63$_{\Delta C47C17}$. Indeed the binding strength increased until a length of 24 residues was achieved. The results make it very likely that the binding site to the N-terminal domain of Sec62p is confined to the C-terminal 24 residues of Sec63p (Figure 3.20). I expected that binding to the N-terminal domain of Sec62p is the only function of these 24 residues. If true, the amino acid exchanges that impair this binding should also abolish the functionality of this allele. Surprisingly, cells expressing Sec63$_{\Delta C47C24T652A}$ still survive whereas cells expressing Sec63$_{\Delta C47C20T652A}$ do not (data not shown). According to the results of my overlay assay both mutants do not bind to the N-terminal domain of Sec62p any longer. I therefore assumed that the last 24 residues contain an extra activity that is not provided by the last 20 residues of Sec63p. A clue about this possible activity was provided by combining different alleles of *SEC63* and *SEC62* in one genome. As the disruption of the phosphorylation dependent Sec62p-Sec63p interaction impairs but does not prohibit protein translocation (Figure 3.12), I 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 (Figures 3.11 and 3.6B). Previous experiments have already identified an additional binding site for the Sec complex at the C-terminus of Sec62p (Wittke et al., 2000). The 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 *sec62$_{\Delta C35}$-Dha* and *sec63$_{T654A}$* (Figure 3.14). The C-terminus of Sec62p is positively charged, whereas the last 47 residues of Sec63p are highly negatively charged. As Sec63$_{\Delta C47C24T652A}$ is partially functional whereas Sec63$_{\Delta C47C20T652A}$ is not, I further proposed that the last 24 residues of Sec63p contain in addition to the binding site for Sec62$_{\Delta C125}$ a further weak binding activity for the C-terminal domain of Sec62p. This proposal was supported by the survival of cells that contained *sec63$_{\Delta C47C24}$* together with *sec62$_{\Delta C35}$* or *sec62$_{\Delta N144}$* (data not shown). This model is still hypothetical since I failed to verify the interaction between the two C-

terminal domains of Sec63p and Sec62p biochemically. A weak affinity between the two domains might be the cause for this failure. Different experimental strategies are therefore needed to either support or discard this aspect of my model for the interaction between Sec62p and Sec63p.

4.5 Relevance to human cell biology

The major components of the heptameric Sec complex, Sec61p, Sec62p and Sec63p have also been biochemically identified in human cell culture. All three proteins are abundantly expressed in most tissue examined (Meyer et al., 2000; Tyedmers et al., 2000). In contrast to the proteins of yeast, the majority of the human Sec proteins are not isolated as one complex. This finding is consistent with the observation that protein translocation in higher eukaryotes is predominantly cotranslational (Rapoport et al., 1996). Like Sec63p from yeast, the human Sec63p contains a negatively charged C-terminal tail domain (Meyer et al., 2000). Inserted into this sequence is a serine that might serve as a CK2 phosphorylation site. I propose that this sequence is not, or only to a small extent phosphorylated in human cells. As a consequence the Sec62p-Sec63p interaction is weak and Sec61p is mainly involved in cotranslational protein translocation. Evidence for a functional role of the human Sec63p and Sec62p in protein translocation is still missing. However mutations in Sec63p were found to be associated with the autosomal dominant polycystic liver disease (PCLD) (Davila et al., 2004). This disorder is characterized by the progressive development of liquid filled biliary epithelial cysts in the liver. These cysts are not functional any longer and will lead to a steady impairment of liver function. One of the PCLD associated *SEC63* alleles contains a C-terminal truncation where the complete C-terminal domain together with some additional residues are deleted. This observation allows me to propose that the intact binding of between the cytosolic domains of Sec63p and Sec62p is also important for protein maturation and sorting in human cells.

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