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A Sec62p-related component of the secretory protein translocon from *Drosophila* displays developmentally complex behavior

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The isolation and characterization of a *Drosophila melanogaster* gene (*Dtrp1*) that encodes a protein displaying the properties of both a structural and functional homolog of the yeast endoplasmic reticulum membrane-bound translocation protein Sec62p is reported. We show that *Dtrp1* can not only rescue the lethality associated with a *SEC62* gene knockout in yeast, but also complement the *sec62*-associated defective transport of a precursor polypeptide from the cytoplasm into the lumen of the endoplasmic reticulum. Expression of the *Dtrp1* gene throughout *Drosophila* development is characterized by peaks in mid-embryogenesis and mid-pupation, followed by a sustained period of mRNA accumulation in adults. The examination of male reproductive tissues showed a very high level of preferential expression of a 1.6 kb message, while a 2.2 kb message was confined almost exclusively to the non-reproductive tissues. Within the reproductive tract itself the 1.6 kb message was expressed in testes, ejaculatory duct and particularly strongly in the paragonial glands. Since these latter organs are specialized secretory tissues we suggest that the 1.6 kb message may encode a protein isoform that performs a unique, tissue-specific role in the protein translocation pathway. Such observations may indicate a hitherto unexpected diversity in components of the protein translocation pathway in respect to stage, tissue and, potentially, substrate specificity.

Key words: *Drosophila/Dtrp1* gene/protein translocation/ *SEC62* analog/secretory pathway gene

Introduction

Translocation into the lumen of the endoplasmic reticulum (ER) is an early step for proteins entering the secretory pathway. Secretory protein precursors are selected in the cytoplasm by the presence of a cleavable, ER-specific signal sequence in the N-terminus of such polypeptides. The initial stage of the translocation process consists of the targeting of polypeptides to the ER membrane. To date, two distinct signal sequence-dependent targeting pathways have been described.

The first is a signal recognition particle (SRP), GTP-dependent, co-translational process wherein the SRP (Walter and Blobel, 1981) recognizes the signal sequence of a growing polypeptide, and a complex consisting of the SRP, nascent protein and the attached ribosome is directed to the ER membrane via an interaction between the SRP and the membrane-bound SRP receptor (Gilmore *et al.*, 1982; Meyer *et al.*, 1982). The SRP receptor then mediates the GTP-dependent displacement of the SRP (Connolly and Gilmore, 1989). While this pathway exists in all eukaryotic organisms that have been analyzed, it is considered to be the principal mechanism of ER membrane targeting in mammalian systems. The second targeting pathway is a post-translational process, in which full-length cytoplasmic polypeptides are transported to the ER membrane surface by cellular chaperones such as the hsc70 proteins in an ATP-dependent step (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Weich *et al.*, 1990). In mammals this process may act as a salvage pathway for proteins that have somehow missed the SRP-dependent co-translational pathway (reviewed in Rapoport, 1992). However, chain length of the precursor protein may also be an important factor in the choice of pathway, with smaller proteins targeted preferentially by the post-translational pathway (Klappa *et al.*, 1993). Post-translational targeting is clearly important in lower eukaryotes such as *Saccharomyces cerevisiae*, where its existence has been demonstrated both *in vitro* and *in vivo* (Toyn *et al.*, 1988; Rothblatt *et al.*, 1989).

Details of the actual mechanism of protein translocation across the ER membrane are not yet clarified for either targeting pathway nor, indeed, whether distinct or common components are involved. It has long been thought that polypeptide transfer takes place through a hydrophilic, proteinaceous 'pore' that is transiently assembled through the interaction of integral membrane proteins (Blobel and Dobberstein, 1975). In support of this view, recent analyses have begun to identify some of the protein components of an ER membrane-bound 'translocon'. Among the initial translocon components to be identified were the Sec61, Sec62 and Sec63 proteins of *S.cerevisiae*, the genes encoding which (*SEC61*, *SEC62* and *SEC63*) were uncovered in a screen designed to identify mutant yeast strains unable to transport secretory protein precursors into the ER (Deshaies *et al.*, 1988). All three *SEC* genes were found to be essential for yeast cell viability (Rothblatt *et al.*, 1989). Biochemical studies have begun to probe the nature of the contacts that these three proteins display with each other, with other proteins of the yeast ER membrane and lumen, and with the translocating polypeptide chain itself (Deshaies *et al.*, 1991; Musch *et al.*, 1992; Sanders *et al.*, 1992; Brodsky and Schekman, 1993).

Homologs of yeast Sec61p and SSS1p (a suppressor of *sec61* mutants) are present in both bacterial and mammalian cells (Ito *et al.*, 1983; Görlich *et al.*, 1992a; Hartmann *et al.*, 1994). The existence of such homologs in prokaryotic and both simple and complex eukaryotic systems

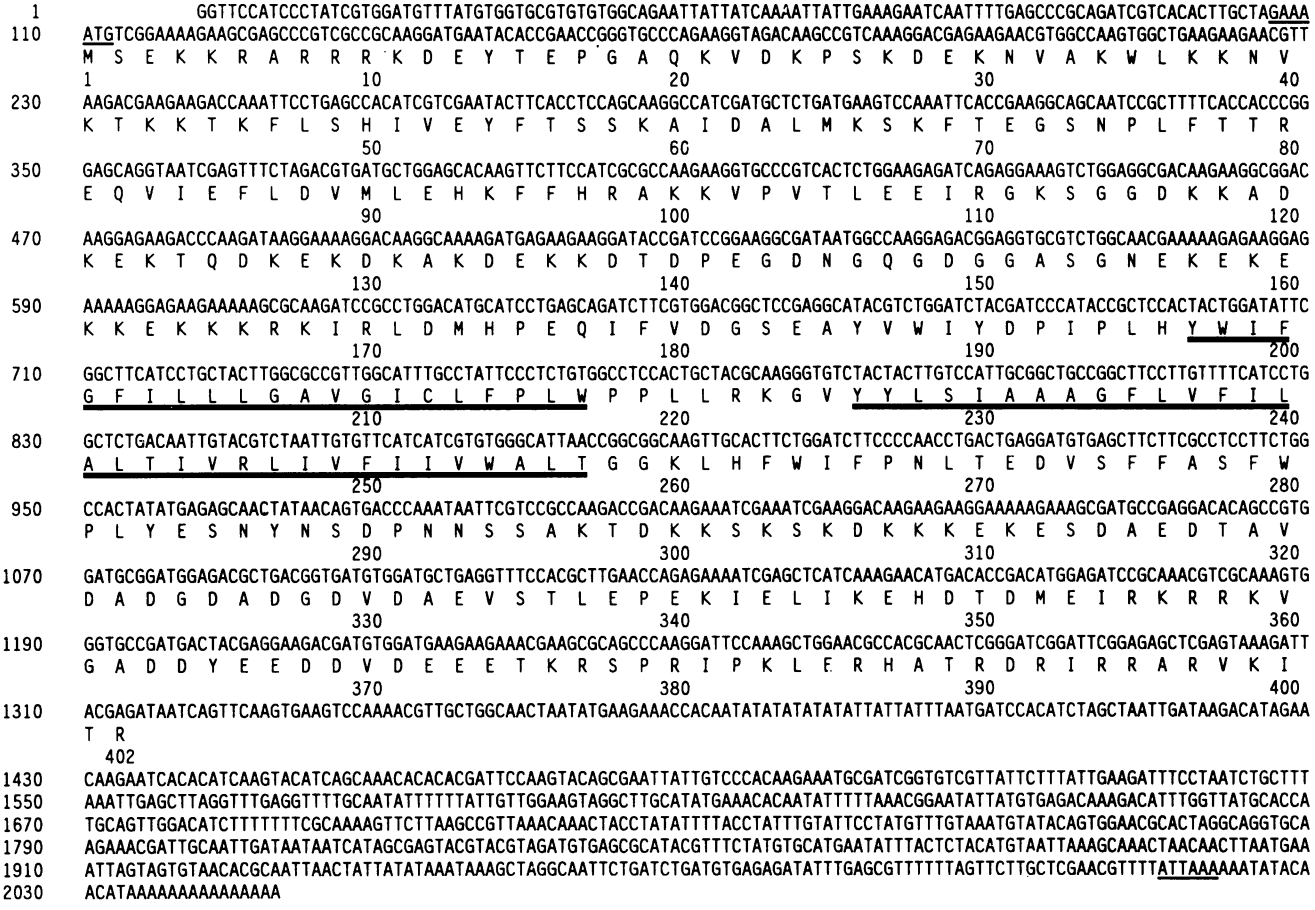

Fig. 1. Nucleotide sequence of the 2.0 kb *Dtrp1* cDNA and the predicted amino acid sequence of the corresponding protein. Numbers in the left-hand column refer to the nucleotide number, with the first nucleotide shown designated as 1. Sequences conforming to a *Drosophila* translational start consensus (see text) and a potential polyadenylation signal are underlined. The deduced amino acid sequence is given in single-letter notation and is numbered directly below the codons. Two potential transmembrane domains, amino acids 197–217 and 226–257, are shown heavily underlined. The 402 amino acid ORF begins with the ATG at nucleotide position 110 and terminates with a stop codon at nucleotide position 1316.

implies a comparable molecular environment for translocating polypeptides and the conservation throughout evolution of a similar transfer mechanism. The failure to detect mammalian proteins with homology to either Sec62p or Sec63p has led to a supposition that these might be components of a distinct, perhaps species-specific, translocon. However, we now report the identification of a gene in *Drosophila melanogaster* that encodes a protein with a domain of sequence homology to Sec62p, and that displays a pronounced Sec62p-like functional activity. Moreover, our studies have uncovered a hitherto unexpectedly complex pattern of developmental regulation for a gene that is involved in the general secretory protein pathway. With the identification of this gene, which we have called *Dtrp1* (for *Drosophila* translocation protein 1), we anticipate the inauguration of *in vivo* studies of protein translocation in a genetically accessible, multicellular organism, something not yet approached in the mammalian systems studied so far.

Results

Sequence of the Drosophila Dtrp1 cDNA

The sequence of a 2 kb cDNA derived from the *Dtrp1* gene is shown in Figure 1. The open reading frame (ORF) begins at position 110 and ends at position 1316, followed by a rather long (~700 bp) 3′ untranslated region. The ORF predicts a protein of 402 amino acids with a molecular weight of ~46 kDa. The sequence immediately preceding the putative initiator methionine contains a 3 out of 4 bp match to the *Drosophila* consensus sequence for translational start sites (Cavener and Ray, 1991). The amino acid content of the predicted protein indicates a highly charged molecule, with most of the charged residues clustering at or near the N- and C-termini of the protein, while hydropathy analysis (Kyte and Doolittle, 1982) reveals two centrally located stretches of hydrophobic amino acids long enough to span a lipid bilayer. These potential transmembrane domains are located at amino acid positions 197–217 and 226–257. The predicted protein lacks the N-terminal stretch of hydrophobic residues characteristic of signal peptides.

The *Dtrp1* cDNA was used as a probe for mapping the location of the gene by *in situ* hybridization to *Drosophila* polytene chromosomes. These studies showed that *Dtrp1* homologous sequences are located on the left arm of chromosome 2 in a single band of hybridization at position 31A (unpublished results). The existence of a single gene at this location has been confirmed by restriction analysis of genomic DNA via Southern blotting.

Table I. *Saccharomyces cerevisiae* strains

Strain	Genotype
RDM 15-9B[a]	*ade2, pep4-3, MATα*
RDM 50-94C[b]	*leu2-3, -112, his4, ura3-52, sec62-1, MATα*
RDM 99[b]	*sec62::HIS3/SEC62, leu2-3,-112/LEU2, his3-11,-15/his3-11,-15, trp1-1/trp1-1, ura3-1/ura3-1, ade2-1/ade2-1, can1-100/can1-100, MATa/MATα*
JRM 151[c]	*sec63-1, pep4-3, ura3-52, leu2-3,-112, MATα*
RDM 15-5B[c]	*sec61-2, pep4-3, ade2, ura3-52, leu2-3,-112, MATα*

[a]Deshaies and Schekman (1987).
[b]Deshaies and Schekman (1989).
[c]Rothblatt *et al.* (1989).

Sequence similarity between DTRP1 and yeast Sec62p

Although the *Dtrp1* cDNA was isolated during a low stringency library screen using a probe from the v-*ski* oncogene (see Materials and methods), there was no significant sequence similarity between the two genes. However, a search of the protein sequence database with the BLASTP algorithm (Altschul *et al.*, 1990) revealed an intriguing sequence similarity between the yeast translocation protein Sec62p and the deduced product of the *Dtrp1* gene. A sequence comparison of a 110 amino acid region is shown in Figure 2. This region contains the known and putative transmembrane domains of Sec62p and DTRP1, respectively, and extends into the C-terminal domain of each protein. There is 35.4% identity and 67.0% overall amino acid similarity across the entire region.

Besides this region of sequence similarity, the Sec62 and DTRP1 proteins share other general structural characteristics. For example, the membrane-spanning domains of the two proteins, as well as the hinge region, are very similar in size. Each protein contains clusters of highly charged amino acids at the N- and C-termini, and neither protein contains a signal sequence. Detailed study of Sec62p in yeast has shown it to be an integral membrane protein of the ER, spanning the membrane twice and displaying its hydrophilic ends towards the cytosol (Deshaies and Schekman, 1990). Thus, given both the sequence and general structural similarities between the two proteins, it appeared reasonable to investigate the possibility that DTRP1 could have functional properties akin to those of the Sec62 protein.

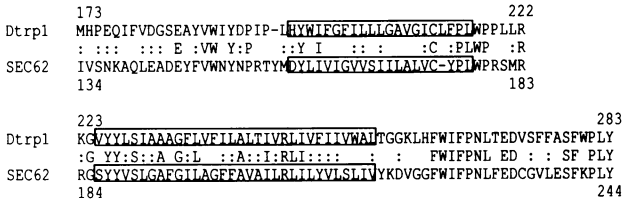

Fig. 2. Regions of amino acid sequence similarity of *Drosophila* DTRP1 to *S.cerevisiae* Sec62p. The amino acid residue number is indicated at both ends of the DTRP1 and Sec62p sequences. The known transmembrane domains of Sec62p and putative transmembrane domains of DTRP1 are boxed. Identical residues are as indicated and conservative changes are delineated by a colon.

Dtrp1 complements a yeast temperature-sensitive (ts) sec62 mutant

We examined the ability of *Dtrp1* to complement a temperature-sensitive mutation in *SEC62* (*sec62*^{ts}) that is carried in the haploid yeast strain RDM 50-94C (Table I; Deshaies and Schekman, 1989). RDM 50-94C is unable to grow at the restrictive temperature of 37°C, while good growth is seen at the permissive temperature of 24°C. Both single- and multi-copy yeast expression vectors containing the *Dtrp1* cDNA (see Materials and methods) were transformed individually into RDM 50-94C cells. The wild-type *SEC62* gene on a single-copy yeast expression vector was included as a positive control. Selected transformants grown initially at 24°C were shifted to the restrictive temperature of 37°C and growth monitored as shown in Figure 3. These curves revealed that neither the parental *sec62*^{ts} strain nor the vector-transformed strains were able to continue growing at the elevated temperature. In contrast, the *sec62*^{ts} strains expressing the *Dtrp1* cDNA and the strain containing the positive control plasmid RSB 482 were all viable at the restrictive temperature (Figure 3). Thus, expression of the *Dtrp1* cDNA was able to rescue the *sec62*^{ts} mutant and confer growth at rates near those of the strains rescued by the yeast *SEC62* gene itself. These results suggest that the *Drosophila* DTRP1 protein is able to functionally replace yeast Sec62p in *sec62* mutants. We investigated the possibility that *Dtrp1* might also be able to complement the functions of *SEC61* and/or *SEC63*, by performing similar experiments with *sec61*^{ts} and *sec63*^{ts} mutant strains (Table I). In neither case was *Dtrp1* expression able to rescue growth at 37°C (unpublished results), from which we infer that DTRP1 specifically complements Sec62p, but not Sec61p or Sec63p function.

Dtrp1 restores viability to a lethal SEC62 gene knockout in yeast

As a more stringent test of the ability of *Dtrp1* to complement *SEC62* function, we decided to test whether *Dtrp1* could restore viability to yeast cells in a background completely negative for *SEC62* function. RPD 99 is a diploid heterozygous strain in which one copy of the *SEC62* gene has been knocked out by targeted insertion of a *HIS3* gene (Deshaies and Schekman, 1989; Table I). To determine if *Dtrp1* could fully substitute for *SEC62*, RPD 99 was transformed with the single-copy plasmid YCP33GAL7 in which the *Dtrp1* cDNA is under the control of the *GAL1* promoter. Plasmid RSB 482 was again used as a positive control. Transformants were grown up and then sporulated to induce the formation of haploid progeny. Spores were propagated in rich medium and colonies were then screened for the rescue of both

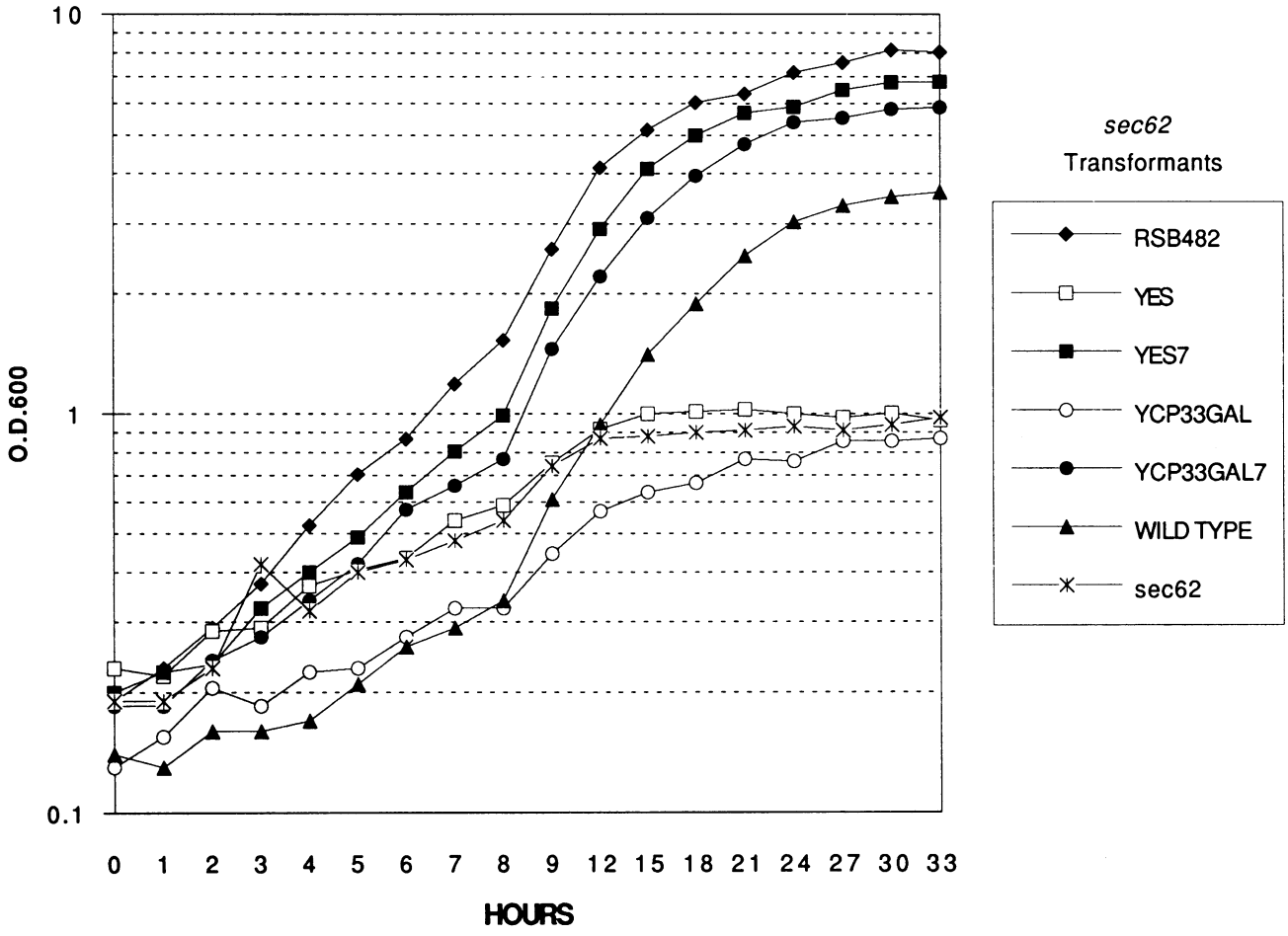

Fig. 3. Growth profiles of *sec62*^{ts} (strain RDM 50-94C) cells alone and transformed with the plasmid constructs shown. (◆) RSB482, single-copy yeast expression vector pSEYc68 containing coding sequence of the wild-type *SEC62* gene; (□) YES, multi-copy yeast expression vector pYES2; (■) YES7, pYES2 containing the *Dtrp1* cDNA; (○) YCP33GAL, single-copy yeast expression vector YCP33GAL; (●) YCP33GAL7, YCP33GAL containing the *Dtrp1* cDNA; (▲) wild-type, wild-type strain RDM 15-9B; (✳) *sec62*, *sec62*^{ts} strain RDM 50-94C. Cultures of the indicated transformed and non-transformed strains growing exponentially at room temperature were diluted into fresh media and shifted to the restrictive growth temperature of 37°C (arbitrarily defined as the zero time point). Growth was monitored by measuring the optical density of the cultures at 600 nm (OD_{600}, vertical axis) over time (h, horizontal axis) until stationary phase was reached.

histidine and uracil auxotrophy on selective plates (*sec62*^{ts} transformants were included as controls for the effects of auxotrophy). In all cases rescue from uracil auxotrophy indicates a colony bearing a plasmid construct, while rescue from histidine auxotrophy additionally identifies those haploid colonies with a *SEC62*-negative chromosomal genotype. Since any *HIS*$^{+}$ haploid colonies cannot, by definition, contain the essential *SEC62* gene, their ability to survive in the absence of histidine must be conferred by plasmid-borne yeast *SEC62* in the case of RSB 482 or, most importantly, by plasmid-borne *Drosophila Dtrp1* in the case of the YCP33GAL7 construct. As shown in Figure 4, individual *sec62*$^{-}$ haploid isolates containing either the control yeast construct RSB 482 or *Drosophila Dtrp1* were able to grow on both Ura^{-} and Ura^{-}/His^{-} media (as expected, the *sec62*^{ts} transformants were inviable in the absence of histidine because of their chromosomal *his4* genotype). Thus, the ability of YCP33GAL7-bearing colonies to grow on doubly selective media decisively established that *Drosophila Dtrp1* was able to replace the cell viability function of *SEC62* in yeast.

α-Factor precursor is processed in sec62 mutants expressing Dtrp1

The ability of the *Dtrp1* gene to substitute for *SEC62* has been demonstrated clearly in the above viability studies. We next decided to examine whether *Dtrp1* was able to mediate translocation of proteins across the ER membrane in a manner analogous to *SEC62*. In yeast cells, prepro-α-factor (ppαF) is translocated from the cytosol into the ER lumen, where it is both glycosylated and proteolytically converted into the core-glycosylated precursor form of α-factor (gpαF). The Sec62 protein contacts ppαF early in the translocation process (Musch *et al.*, 1992), and unprocessed α-factor accumulates in the cytoplasm of *sec62* cells (Rothblatt *et al.*, 1989). The ability of *Dtrp1*-rescued *sec62*^{ts} and *SEC62*-negative cells to convert ppαF to gpαF was examined in the Western blot shown in Figure 5. Wild-type cells contained only the 26 kDa gpαF species. However, when tunicamycin was added to wild-type cells, there was a large accumulation of an unglycosylated, pαF precursor. In *sec62*^{ts} cells grown at 24°C either alone or transformed with the non-recombinant expression

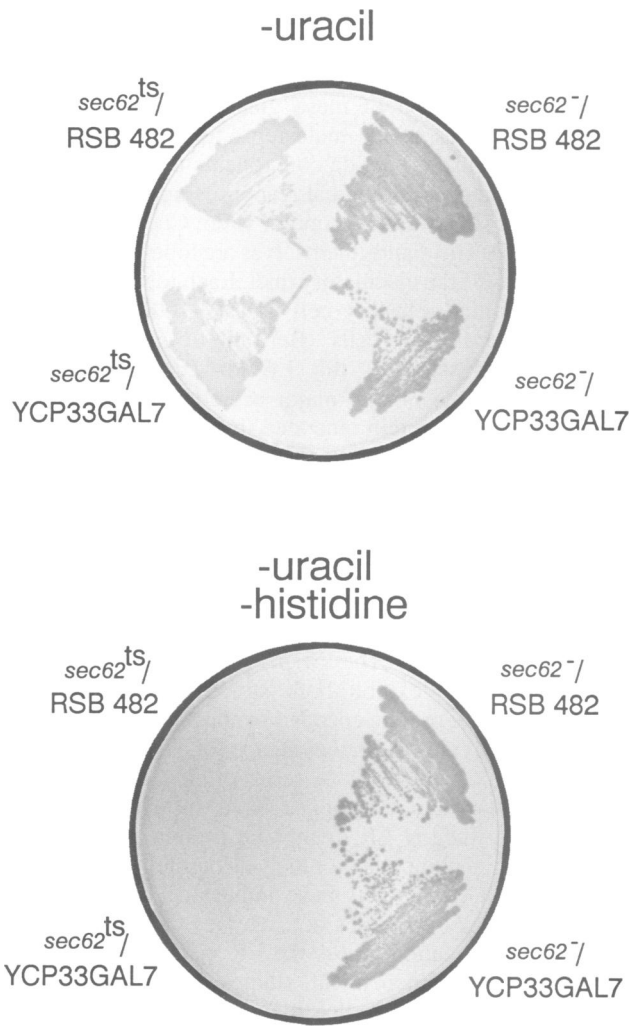

Fig. 4. Growth on selective media of *sec62*^{ts} mutant and haploid *sec62::HIS3* gene-disrupted strains (*sec62*$^{-}$) transformed with either yeast genomic *SEC62* or *Drosophila Dtrp1* cDNA sequences. In the case of the *sec62*$^{-}$ strains, haploid cells were obtained by sporulation of diploid strain RPD 99 that had been transformed by the single-copy *Dtrp1* cDNA expression construct (YCP33GAL7), or by the wild-type *SEC62* plasmid (RSB 482). The selective medium used is indicated above each plate.

vector YCP33GAL, there was a very large accumulation of ppαF (which runs faster than pαF in SDS gels), indicating an inability to efficiently transport ppαF into the ER lumen for core glycosylation (Figure 5). In contrast, in *sec62*^{ts} cells rescued by expression of *Dtrp1* and grown at the restrictive temperature of 37°C, the 26 kDa gpαF species was evident and almost none of the ppαF remained. Similarly, *Dtrp1*-rescued *SEC62*-negative cells contain only the gpαF species (Figure 5), a result similar to those obtained for both the wild-type and *SEC62*-negative cells rescued by the positive control plasmid RSB 482. Therefore, the translocation defect that occurs in *sec62* mutant cells was cured by the expression of *Dtrp1*. These data strongly suggest that the *Dtrp1* gene product functions mechanistically in a fundamentally similar manner to the yeast Sec62 protein.

Developmental expression of Dtrp1 in Drosophila

In conjunction with the above functional studies of the *Dtrp1* gene product in yeast, a molecular analysis of *Dtrp1*

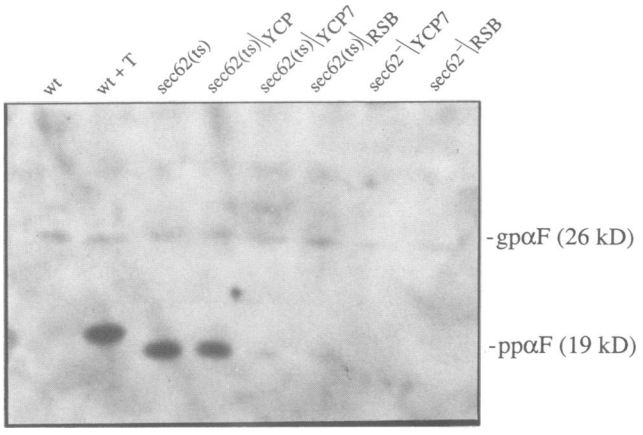

Fig. 5. Western blot showing the conversion of ppαF to gpαF in *sec62*^{ts} mutant and *sec62*$^{-}$ cells expressing the *Dtrp1* cDNA. Lanes from left to right are as follows: wt, wild-type strain RDM 15-9B; wt + T, RDM 15-9B plus addition of tunicamycin during the last 60 min of growth; sec62(ts), *sec62*^{ts} strain RDM50-94C at 24°C; sec62(ts)/YCP, RDM 50-94C transformed with vector YCP33GAL at 24°C; sec62(ts)/YCP7, RDM 50-94C transformed with the *Dtrp1* construct YCP33GAL7 at 37°C; sec62(ts)/RSB, RDM 50-94C transformed with the *SEC62* construct RSB 482 at 37°C; sec62^{-}/YCP7, haploid *sec62::HIS3* derivative of knockout strain RPD 99 rescued by *Dtrp1* construct YCP33GAL7; sec62^{-}/RSB, haploid *sec62::HIS3* derivative of RPD 99 rescued by wild-type *SEC62* construct RSB 482.

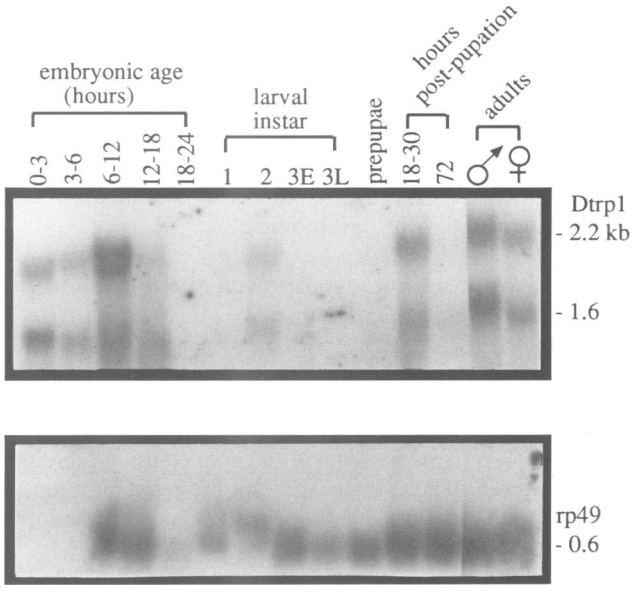

Fig. 6. Developmental profile of *Dtrp1* mRNA expression in *Drosophila*. Poly(A)$^{+}$ RNA was isolated from the indicated developmental stages and hybridized against the *Dtrp1* cDNA. Embryonic age is indicated in h. Larvae: 1, first instar; 2, second instar; 3E, early third instar; 3L, late third instar. Prepupae were obtained 120 h after laying; pupae are shown as h post-pupation; (♂) male adults and (♀) female adults. A probe for the ribosomal protein 49 gene (rp49) was included as a loading control.

gene expression was performed in the fly. A Northern blot which examines the expression of *Dtrp1* during the life cycle of the fly is shown in Figure 6. The *Dtrp1* gene encodes two different messages, of ~2.2 and ~1.6 kb, that are expressed concomitantly at various stages of development. Expression of both messages occurs throughout the embryonic stage, with a clear peak occurring in

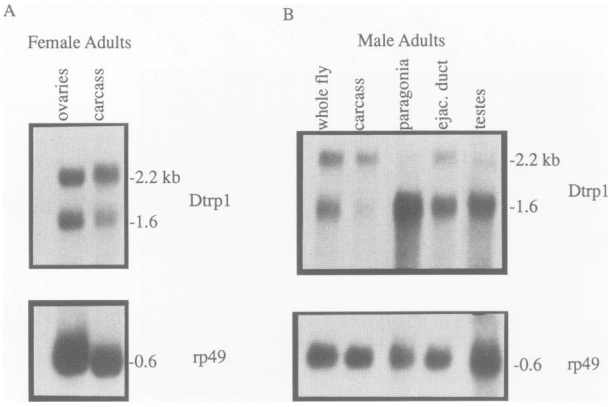

Fig. 7. Northern analysis of *Dtrp1* expression in the reproductive systems of wild-type male and female adult flies. Poly(A)$^+$ samples were hybridized with the *Dtrp1* cDNA and rp49 was included as a control for loading. (**A**) Expression in the female reproductive system; (**B**) expression in the male reproductive system. For both males and females, carcass refers to the remaining fly carcass after dissection of the reproductive system.

6–12 h embryos, followed by a diminution throughout the rest of the embryonic period. Little expression is seen during the larval stages, although a slight rise does occur at the second instar. Another major peak of expression is seen at 18–30 h post-pupation, again followed by diminished expression in later pupal stages. Both messages are expressed in adults; however, the kinetics of appearance and relative amounts of expression of the two messages differ somewhat between male and female flies (unpublished results). These data on expression therefore suggest that the *Dtrp1* messages are subject to complex temporal regulation throughout development.

The 1.6 kb message is expressed specifically in the male reproductive system

We pursued our studies of *Dtrp1* expression in adult flies by focusing on the male and female reproductive systems. In the female reproductive system, the 2.2 and 1.6 kb messages are expressed at essentially equivalent levels (Figure 7A). However, there is noticeably preferential expression of the 2.2 kb message in the remaining female carcass, reflecting the overall preponderance of this message seen in whole female flies (unpublished results).

Dtrp1 mRNA derived from the male reproductive system revealed a strikingly different pattern of accumulation. Thus, expression of the 1.6 kb message is confined almost exclusively to the reproductive system (Figure 7B), with little or none seen in the carcass. Differential expression of the two messages is also observed within the reproductive system itself. Both messages are expressed in ejaculatory duct; however, expression of the smaller message is strongly favored, in contrast to the pattern seen in females. In addition, in both the testes and the paragonial glands only the 1.6 kb message is expressed. Significantly, these are the first tissues to be analyzed in which expression of the two *Dtrp1* messages has not been concomitant. We believe that what appears to be very low level expression of the 2.2 kb message in testes and paragonia, and of the 1.6 kb message in carcass, is likely to be sample contamination arising from the dissection process rather than true expression of these messages. The extremely high level of the 1.6 kb message in the paragonial glands seems particularly interesting given the specialized function of these glands. Paragonia are the accessory glands of the male reproductive system. They secrete specialized peptide components that elicit specific behavioral and physiological responses upon transfer to a female (for reviews see Leopold, 1976; Chen, 1984; Chen *et al.*, 1988). The glands themselves are lobe-like structures that branch off the ejaculatory duct. Each lobe is composed of two distinct secretory cell types: ~1000 'main' cells and 43 'secondary' cells (Bertram *et al.*, 1992). The specific expression of the 1.6 kb *Dtrp1* message at such high levels in this major secretory tissue strongly implicates the protein encoded by this message in a specialized secretory protein translocation process in paragonia, and potentially in testes and ejaculatory duct.

Discussion

The work presented here describes the initial characterization of a new *Drosophila melanogaster* gene, which we have named *Dtrp1*. The *Dtrp1* gene product has been demonstrated to exhibit both structural and functional similarity to the protein encoded by the *S.cerevisiae SEC62* gene, and provides the first evidence for a Sec62p homolog in higher eukaryotic cells. A series of elegant experiments by Schekman and colleagues have shown that *SEC62* encodes an integral ER membrane protein that functions as an essential component of the yeast protein translocation machinery. Genetic tests have indicated that the *SEC61*, *SEC62* and *SEC63* genes act together to facilitate polypeptide translocation across the ER membrane (Rothblatt *et al.*, 1989). Biochemical studies have revealed that Sec62p is one subunit of a dynamic protein complex in the ER membrane, and that Sec62p transiently contacts polypeptides in an initial, but required, step of the translocation process (Deshaies *et al.*, 1991; Musch *et al.*, 1992; Sanders *et al.*, 1992). A structural and mechanistic model has been proposed for the yeast translocon (Sanders and Schekman, 1992) wherein Sec62p, as part of the Sec62p–Sec63p subcomplex, contacts nascent polypeptides early in the translocation process in an ATP-independent manner. ATP hydrolysis then somehow acts to effect the transfer of the chain from Sec62p to Sec61p, initiating the final stage of the process — movement across the ER membrane. However, up to this point no evidence for analogs of Sec62p and/or Sec63p had been obtained in other eukaryotic cells. Owing to its ubiquitous distribution in both prokaryotes and eukaryotes, it has been anticipated that Sec61p (along with other proteins such as TRAM; Görlich and Rapoport, 1993) is likely to be the principal constituent of the protein-conducting channel in the ER membrane of all eukaryotic cells (reviewed by Rapoport, 1992; Sanders and Schekman, 1992; Gilmore, 1993).

We show here that the *Dtrp1* cDNA derived from *Drosophila* is able to function in place of the *SEC62* gene in yeast. In the rescue of *sec62*^{ts} mutants, growth was restored to levels close to that seen with the wild-type *SEC62* gene itself (Figure 3). The ability of *Dtrp1* to rescue a *SEC62* gene knockout provided an unambiguous demonstration that *Dtrp1* is capable of substituting the viability function normally provided by *SEC62* in yeast.

Furthermore, the product of the *Dtrp1* gene probably performs this rescue by functioning in a manner analogous to that of Sec62p, i.e. by cooperating in the transport of secretory protein precursors across the ER membrane. Such an assertion is supported by the Western data showing that unglycosylated ppαF is converted to glycosylated pro-α-factor (gpαF) in the *Dtrp1*-rescued knockouts (Figure 5). This conversion can only occur upon the transfer of ppαF into the ER lumen where such core glycosylation takes place.

Although the sequence similarity between Sec62p and DTRP1 is limited to the transmembrane domains and the proximal C-terminal region, rescue of yeast viability by DTRP1 suggests that the overall structural and functional integrity of these two proteins has been largely conserved throughout evolution. Schekman and colleagues (Deshaies and Schekman, 1989; Rothblatt *et al.*, 1989) have shown that the efficiency of transfer mediated by Sec62p is somewhat dependent on the relative signal sequence hydrophobicity of the polypeptide in question. Thus, global structural features of both Sec62p and the polypeptide to be translocated may play a significant role in the interaction between the two proteins. The conservation of features between Sec62p and DTRP1, such as the predicted α-helical structures of the N- and C-terminal domains and their highly charged termini, along with the sequence similarities already noted, undoubtedly contribute to the ability of DTRP1 to functionally substitute for Sec62p. Further functional studies in both yeast and flies should provide details of the interaction of DTRP1 with other ER membrane proteins and with nascent polypeptides. It would be particularly interesting to know if DTRP1 interacts with the Sec61p–Sec63p subcomplex in yeast, as does Sec62p, whether it contacts polypeptides in the same ATP-independent manner of Sec62p, and whether similar Sec61 and Sec63 proteins are present in *Drosophila*. Moreover, the discovery of a Sec62p analog in higher eukaryotes should aid in the solution of whether the two known targeting pathways differ with respect to their usage of translocon components.

The observation that *Dtrp1* gene expression is regulated both temporally and spatially in the fly provides a novel and unexpected future direction for research in the field of protein translocation. The *Dtrp1* gene encodes two spliced mRNA species of ~2.2 and ~1.6 kb, with a putative protein product of ~46 kDa encoded by the 2.2 kb mRNA. Primer extension analysis has strongly suggested that both mRNAs are derived from the same transcription start site; the two messages are apparently generated by alternative splicing in the 3′ portion of the ORF and/or the 3′ untranslated region (unpublished results). In contrast, *SEC62* encodes a single unspliced 1.0 kb mRNA which produces a 32 kDa protein (Deshaies and Schekman, 1989). In addition, the developmental expression pattern of the *Dtrp1* gene is quite complex, with three separate peaks in mRNA levels occurring prior to the adult stage. Interestingly, maximal expression is confined, for the most part, to the embryonic and pupal period, developmental time points during which complex growth and differentiation events are taking place. Analysis of *Dtrp1* expression in adult males and females led us to the most intriguing result of our molecular analysis: namely that in males the 1.6 kb message is expressed exclusively in the reproductive system. Expression of this smaller transcript is by far the highest in the paragonial glands, which are specialized secretory organs somewhat equivalent to the mammalian prostate gland. Mutational analysis of Sec62p has demonstrated that the C-terminal domain is likely to contact nascent polypeptides during translocation (Deshaies and Schekman, 1990). Since this region also shows the highest degree of sequence similarity between DTRP1 and Sec62p, by analogy the C-terminal domain of DTRP1 is likely to contact peptide precursors. We hypothesize that the alternative splicing which apparently occurs at the 3′ end of the precursor mRNA to generate the 1.6 kb message (see above) may result in the production of a protein in which the specificity of the C-terminal domain has been altered. This specialized isoform might then function in the transport of a subset of secretory peptides or proteins specific for the male reproductive system. This selectivity feature appears realistic given the observations that yeast Sec62p is the initial component of the translocon to exhibit cross-links to a translocating polypeptide (Musch *et al.*, 1992): its position early in the pathway may provide an initial recognition step. Should this be confirmed by further work, it would be the first demonstration that specificity of transfer may sometimes be conferred by different isoforms of translocation apparatus proteins, resulting in a titration of the specificity to fit a particular cellular or tissue environment. Previous observations of variable translocation efficiencies on different substrates in studies that utilized mutants in one or other of the targeting pathways (Rothblatt *et al.*, 1989; Green *et al.*, 1992; Stirling *et al.*, 1992) provide strong experimental support for such a possibility. Such variability may well reflect differential requirements for translocation components by subsets of precursor molecules. Given that such tissue specificity has not been described previously, our observations represent a promising new direction for protein translocation research.

In this paper we have reported the cloning and initial characterization of sequences derived from the *Drosophila melanogaster Dtrp1* gene. The protein encoded by this gene displays both structural and functional homology to the yeast Sec62 protein. This is the first report of such an activity in a system other than *S.cerevisiae*. The investigation of protein translocation in yeast in particular has provided invaluable insights which have allowed several mechanistic hypotheses to be formulated. We anticipate that the ability to perform comprehensive genetic and molecular analyses in the multicellular eukaryote *Drosophila melanogaster* should help lead to the identification of other components of higher eukaryotic translocons, to a knowledge of whether these are also expressed cell and stage specifically, and to an assessment of whether similar or different translocons feature in both the co-translational and post-translational pathways.

Materials and methods

Yeast strains and techniques

S.cerevisiae strains used in this study are listed in Table I. Yeast cultures were grown at 24 or 37°C in YP medium containing 2% yeast extract, 2% peptone and either 4% glucose or 2% galactose/2% raffinose. Tunicamycin (10 μg/ml) was added to appropriate cultures for 60 min, once they had reached the desired optical density, to inhibit asparagine-linked core oligosaccharide addition. Sporulation of liquid cultures and

spore isolation were performed using standard protocols (Campbell and Duffus, 1988; Rockmill *et al.*, 1991). Yeast transformations were performed using a modified LiCl procedure (Hill *et al.*, 1991).

cDNA cloning and analysis

The *Drosophila* 7-1 cDNA was isolated from a wild-type Oregon-R 3–12 h embryonic λgt10 cDNA library (Poole *et al.*, 1985) during a low stringency screen that utilized a 1.2 kb *Sst*I fragment from the viral oncogene v-*ski* (derived from the chicken SK virus; Stavnezer *et al.*, 1986) as a probe. The 7-1 cDNA was excised from the phage clone via digestion with *Eco*RI, and several recombinant plasmids containing the 7-1 cDNA were constructed for use in further analyses using standard methodologies (Maniatis *et al.*, 1982). Single-strand DNA sequencing was performed on a recombinant containing the 7-1 cDNA inserted into the *Eco*RI site of the Bluescript SK^+ phagemid (Stratagene) using a T7 sequencing kit (Pharmacia). The pYES7 construct contains the 7-1 cDNA cloned into the *Eco*RI site of pYES2 (Invitrogen), a high copy number vector designed to allow the inducible expression of genes in *S.cerevisiae* via the *GAL1* promoter. Plasmid YCP33GAL (M.Howell and G.Dean, unpublished results) is a low copy number (*CEN4*, *ARS1*) vector likewise containing the inducible *GAL1* promoter. The 7-1 cDNA was cloned via blunt-end ligation into a *Hin*dIII site downstream of the *GAL1* promoter, thereby creating the recombinant plasmid YCP33GAL7. Plasmid RSB 482 was provided by R.Schekman and contains the complete *S.cerevisiae* SEC62 gene cloned into the *CEN*-containing plasmid pSEYc68 (Deshaies and Schekman, 1989).

Rescue of sec62^{ts} mutant and SEC62 knockout strains

The various recombinant yeast expression constructs described above were used to transform the temperature-sensitive mutant strain, RDM 50-94C. RSB 482 transformants were included as positive controls and non-recombinant expression vector transformants were included as negative controls. Cultures of appropriate RDM 50-94C transformants were grown at 24°C to stationary phase in rich 1% YP medium containing 2% raffinose/2% galactose. These cultures were used to inoculate fresh medium for growth at the restrictive temperature of 37°C. Growth kinetics were monitored during the logarithmic phase by measuring the optical density at 600 nm (OD_{600}) of dilute cell suspensions over time.

Single-copy plasmid YCP33GAL7 was used to transform the diploid *SEC62* knockout strain RPD 99. As before, plasmid RSB 482 was included as a positive control. To obtain haploid progeny, transformants were sporulated in McClary's medium (Campbell and Duffus, 1988) and spores harvested as indicated previously. Vegetative growth was induced by plating spore suspensions onto rich non-selective medium (1% YP; 2% raffinose/2% galactose). Haploid colonies were tested for their ability to grow on appropriate selective medium to test for the successful rescue, or lack thereof, of the *SEC62* knockout by the constructs indicated above.

RNA isolation and Northern blot analyses

All Northern analyses were performed using poly(A)$^+$ RNA isolated from samples derived from the wild-type Oregon-R strain. Freshly laid embryos were collected and aged at room temperature to the desired developmental stage, then animals were harvested and stored at −70°C. Specific reproductive tissues were dissected in 0.7% saline from freshly collected adults and both these and the remaining carcasses were frozen at −70°C until further use. Poly(A)$^+$ RNA was prepared using a FastTrack mRNA isolation kit (Invitrogen) according to the manufacturer's instructions with the following modifications. Embryos were dechorionated in 1% bleach/1% Triton X-100 and washed extensively with de-ionized water, blotted dry and homogenized. Pupal samples were pulverized using a mortar and pestle at −70°C immediately prior to homogenization. Dechorionated embryos, larvae, pupae and adult samples were homogenized at room temperature in lysis buffer in 10 ml Pyrex homogenizers using a power-driven teflon pestle. The homogenate was extracted once with 1 vol acid phenol/0.2 vol chloroform:isoamyl alcohol (49:1); the aqueous fraction was precipitated with isopropanol overnight at 4°C (Chomczynski and Sacchi, 1987). The pellet was resuspended in lysis buffer and poly(A)$^+$ RNA was then selected according to the FastTrack protocol. RNA samples were glyoxalated (McMaster and Carmichael, 1977), run on 1.2% agarose gels and transferred to nylon blotting membrane (Magna-NT, MSI) according to Maniatis *et al.* (1982). High stringency hybridizations were performed at 68°C using sodium phosphate-based hybridization buffer and washes (Church and Gilbert, 1984). Radiolabeled probes were synthesized by extension of random hexamers using [α-^{32}P]dATP (NEN, 3000 Ci/mmol; Feinberg and Vogelstein, 1983). The 2.0 kb 7-1 cDNA was used as a probe for *Dtrp1* mRNA. Equivalent loading of poly(A$^+$) RNA was monitored by probing for *Drosophila* ribosomal protein 49 (rp49) with a 550 bp *Eco*RI−*Hin*dIII rp49 fragment.

Western analysis

Yeast protein extracts were prepared for Western analysis by harvesting 2 OD_{600} units of the appropriate yeast cultures (for rescued *SEC62* knockout strains, haploid MATα cultures were specifically selected) and resuspending in 1% SDS, 10% sucrose, 10 mM Tris−HCl, pH 8.0, 30 mM DTT, 1 mM EDTA and 0.04 μg/ml bromophenol blue. Samples were boiled for 2 min, vortexed with an equal volume of glass beads (Sigma; 425–600 μm) for 90 s and boiled for 2 min again. Approximately 0.2 OD units of each sample were separated on a 12% SDS−polyacrylamide gel and electrophoretically transferred to PVDF membrane (Millipore). Blotted proteins were probed with a rabbit anti-α-actor polyclonal antiserum (a gift from R.Schekman) diluted 1:10 000. Detection was performed using the Western Light kit (Tropix) and the manufacturer's protocol. The visualization reaction was based on the reaction between a secondary antibody (goat anti-rabbit) conjugated to alkaline phosphatase and the chemiluminescent substrate AMPPD.

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References

Altschul,S.F., Gish,W., Miller,W., Myers,E.W. and Lipman,D.J. (1990) *J. Mol. Biol.*, **215**, 403–410.

Bertram,M.J., Akerkar,G.A., Ard,R.L., Gonzalez,C. and Wolfner,M.F. (1992) *Mech. Dev.*, **38**, 33–40.

Blobel,G. and Dobberstein,B. (1975) *J. Cell Biol.*, **67**, 852–862.

Brodsky,J.L. and Schekman,R. (1993) *J. Cell Biol.*, **123**, 1355–1363.

Campbell,I. and Duffus,J.H. (1988) *Yeast: A Practical Approach.* IRL Press, Oxford, UK.

Cavener,D.R. and Ray,S.C. (1991) *Nucleic Acids Res.*, **19**, 3185–3192.

Chen,P.S. (1984) *Annu. Rev. Entomol.*, **29**, 233–255.

Chen,P.S., Stumm-Zollinger,E., Aigaki,T., Balmer,J., Bienz,M. and Bohler,P. (1988) *Cell*, **54**, 291–298.

Chirico,W.J., Waters,M.G. and Blobel,G. (1988) *Nature*, **332**, 805–810.

Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.

Church,G.M. and Gilbert,W. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 1991–1995.

Connolly,T. and Gilmore,R. (1989) *Cell*, **57**, 599–610.

Deshaies,R.J. and Schekman,R. (1987) *J. Cell Biol.*, **105**, 633–645.

Deshaies,R.J. and Schekman,R. (1989) *J. Cell Biol.*, **109**, 2653–2664.

Deshaies,R.J. and Schekman,R. (1990) *Mol. Cell. Biol.*, **10**, 6024–6035.

Deshaies,R.J., Koch,B.D., Werner-Washburne,M., Craig,E.A. and Schekman,R. (1988) *Nature*, **332**, 800–805.

Deshaies,R.J., Sanders,S.L., Feldheim,D.A. and Schekman,R. (1991) *Nature*, **349**, 806–808.

Feinberg,A. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6–13.

Gilmore,R. (1993) *Cell*, **75**, 589–592.

Gilmore,R., Walter,P. and Blobel,G. (1982) *J. Cell Biol.*, **95**, 470–477.

Görlich,D. and Rapoport,T.A. (1993) *Cell*, **75**, 615–630.

Görlich,D., Prehn,S., Hartmann,E., Kalies,K.-U. and Rapoport,T.A. (1992a) *Cell*, **71**, 489–503.

Görlich,D., Hartmann,E., Prehn,S. and Rapoport,T.A. (1992b) *Nature*, **357**, 47–52.

Green,N., Fang,H. and Walter,P. (1992) *J. Cell Biol.*, **116**, 597–604.

Hartmann,E., Sommer,T., Prehn,S., Görlich,D., Jentsch,S. and Rapoport,T.A. (1994) *Nature*, **367**, 654–657.

Hill,J., Donald,K.A.I.G. and Griffiths,D.E. (1991) *Nucleic Acids Res.*, **19**, 5791.

Ito,K., Wittekind,M., Nomura,M., Shiba,K., Yura,T., Miura,A. and Nashimoto,H. (1983) *Cell*, **32**, 789–797.

Klappa,P., Zimmerman,M., Dierks,T. and Zimmerman,R. (1993) In Borgese,N. and Harris,J.R. (eds), *Subcellular Biochemistry.* Plenum Press, New York, Vol. 21, pp. 17–40.

Kyte,J. and Doolittle,R.F. (1982) *J. Mol. Biol.*, **157**, 105–132.
Leopold,R.A. (1976) *Annu. Rev. Entomol.*, **21**, 199–221.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
McMaster,G.K. and Carmichael,G.G. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 4835–4838.
Meyer,D.I., Krause,E. and Dobberstein,B. (1982) *Nature*, **297**, 503–508.
Musch,A., Wiedmann,M. and Rapoport,T.A. (1992) *Cell*, **69**, 343–352.
Poole,S., Kauvar,L.M., Drees,B. and Kornberg,T. (1992) *Cell*, **40**, 37–43.
Rapoport,T.A. (1992) *Science*, **258**, 931–936.
Rockmill,B., Lambie,E.J. and Roeder,G.S. (1991) *Methods Enzymol.*, **194**, 146–149.
Rothblatt,J.A., Deshaies,R.J., Sanders,S.L., Daum,G. and Schekman,R. (1989) *J. Cell Biol.*, **109**, 2641–2652.
Sanders,S.L. and Schekman,R. (1992) *J. Biol. Chem.*, **267**, 13791–13794.
Sanders,S.L., Whitfield,K.M., Vogel,J.P., Rose,M.D. and Schekman,R.W. (1992) *Cell*, **69**, 353–365.
Stavnezer,E., Barkas,A.E., Brennan,L., Brodeur,D. and Li,Y. (1986) *J. Virol.*, **57**, 1073–1083.
Stirling,C.J., Rothblatt,J., Hosobuchi,M., Deshaies,R. and Schekman,R. (1992) *Mol. Biol. Cell.*, **3**, 129–142.
Toyn,J., Hibbs,A.R., Sanz,P., Crowe,J. and Meyer,D.I. (1988) *EMBO J.*, **7**, 4347–4350.
Walter,P. and Blobel,G. (1981) *J. Cell Biol.*, **91**, 557–561.
Weich,H., Stuart,R. and Zimmerman,R. (1990) *Semin. Cell Biol.*, **1**, 55–63.

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