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Secretion of *Saccharomyces cerevisiae* Killer Toxin: Processing of the Glycosylated Precursor

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Killer toxin secretion was blocked at the restrictive temperature in *Saccharomyces cerevisiae* *sec* mutants with conditional defects in the *S. cerevisiae* secretory pathway leading to accumulation of endoplasmic reticulum (*sec18*), Golgi (*sec7*), or secretory vesicles (*sec1*). A 43,000-molecular-weight (43K) glycosylated protoxin was found by pulse-labeling in all *sec* mutants at the restrictive temperature. In *sec18* the protoxin was stable after a chase; but in *sec7* and *sec1* the protoxin was unstable, and in *sec1* 11K toxin was detected in cell lysates. The chymotrypsin inhibitor tosyl-L-phenylalanyl chloromethyl ketone (TPCK) blocked toxin secretion in vivo in wild-type cells by inhibiting protoxin cleavage. The unstable protoxin in wild-type and in *sec7* and *sec1* cells at the restrictive temperature was stabilized by TPCK, suggesting that the protoxin cleavage was post-*sec18* and was mediated by a TPCK-inhibitable protease. Protoxin glycosylation was inhibited by tunicamycin, and a 36K protoxin was detected in inhibited cells. This 36K protoxin was processed, but toxin secretion was reduced 10-fold. We examined two *kex* mutants defective in toxin secretion; both synthesized a 43K protoxin, which was stable in *kex1* but unstable in *kex2*. Protoxin stability in *kex1 kex2* double mutants indicated the order *kex1* → *kex2* in the protoxin processing pathway. TPCK did not block protoxin instability in *kex2* mutants. This suggested that the *KEX1*- and *KEX2*-dependent steps preceded the *sec7* Golgi block. We attempted to localize the protoxin in *S. cerevisiae* cells. Use of an in vitro rabbit reticulocyte-dog pancreas microsomal membrane system indicated that protoxin synthesized in vitro could be inserted into and glycosylated by the microsomal membranes. This membrane-associated protoxin was protected from trypsin proteolysis. Pulse-chased cells or spheroplasts, with or without TPCK, failed to secrete protoxin. The protoxin may not be secreted into the lumen of the endoplasmic reticulum, but may remain membrane associated and may require endoproteolytic cleavage for toxin secretion.

Type 1 killer *Saccharomyces cerevisiae* cells contain M₁ double-stranded RNA (dsRNA), a cytoplasmic plasmid encapsulated in virus-like particles. The virus-like plasmid contains genes coding for a secreted 11,000-molecular-weight (11K) protein toxin and an unidentified immunity protein determining resistance to this toxin, which confer the killer (K₁⁺), immune (R₁⁺) phenotype of killer strains (see references 3 and 18 for recent reviews).

The system allows a genetic approach to be taken toward understanding protein secretion in this simple eucaryote. The toxin resembles many secreted proteins in that it is made as a larger precursor. The primary translation product in vitro is a 32 to 36K preprotoxin. This precursor is processed cotranslationally in vitro

by dog pancreas microsomal membranes to a 43K glycoprotein, a protoxin with a 1.6K amino-terminal signal sequence removed (1, 2). A 43K glycoprotein protoxin is also found in vivo in pulse-labeled killer cells (2, 4), and the protoxin has a precursor-product relationship to the secreted toxin in a pulse-chase experiment. M₁ dsRNA killer plasmid mutants which are defective in protoxin processing and are unable to secrete toxin have been described (4).

In this paper we examine processing and secretion of toxin in mutants defective in nuclear genes that control secretion, and we show toxin secretion to be on a pathway defined by the secretory *sec* mutants of Novick and Schekman (11) and the *kex* mutants of Wickner and Leibowitz (9, 19). In addition, we examine the

effect of tunicamycin and protease inhibitors on toxin processing and attempt to localize the protoxin in producing cells.

MATERIALS AND METHODS

Yeast strains and media. The diploid *S. cerevisiae* T158C/S14a (ATCC 46427) was used as a typical K₁ killer strain (4). The secretory mutant strains HMSF1 *sec1-1*, HMSF6 *sec7-1*, and HMSF176 *sec18-1* were obtained from R. Schekman. These temperature-sensitive, nonkiller *sec* mutants were crossed with a killer strain, A8207B [KIL-k₁] α *his4C-864*; the killer diploid sporulated, and the asci dissected. All spores were killer (K⁺ R⁺), two per tetrad were temperature-sensitive mutants, and one temperature-sensitive K⁺ R⁺ mutant was taken from each cross for use. All four spores from a *sec1-1* cross gave a 43K protoxin when cell lysates were immunoprecipitated with antitoxin antiserum after pulse-labeling with [³⁵S]sulfate for 20 min at 24°C.

The following *kex* mutant strains were used and all were [KIL-k₁]; a *kex2-1 ura⁻ ade⁻*; a *kex1-3 ade⁻*; S90 a *kex2 ade2 ural*; S91 α *kex1-1 arg1 thr1*; 3413-10B a *kex1-1 kex2-1 ural thr1*; 3413-8A a *kex1-1 kex2-1 ade2 his7 arg1 thr1*. All but the first two strains were from the laboratory of Reed Wickner.

Growth medium was the minimal medium described previously (4) supplemented as appropriate with 0.5% yeast extract–0.5% peptone–2% glucose. For some pulse-labeling experiments, a sulfate-free minimal medium (4) was used plus 0.25 mM homocysteine thiolactone as a sulfur source (11).

dsRNA isolation and in vitro translation. M₁ dsRNA was isolated from killer strain T158C/S14a and denatured and translated in a rabbit reticulocyte lysate system as described previously (4). EDTA-stripped, micrococcal nuclease-treated dog pancreas microsomal membranes, prepared by the Shields and Blobel (15) procedure, were obtained from Gordon Shore. Tetra-caine was obtained as Pontocaine from Winthrop Laboratories; trypsin was obtained from Boehringer Mannheim Corp.

Immunoprecipitation of toxin and toxin precursor. Cultures were labeled with [³⁵S]sulfate (carrier-free, New England Nuclear Corp.) or [³⁵S]methionine (Amersham Corp.), and cells and medium were obtained by centrifugation. Growth medium was concentrated with ultrafiltration cones (CF-25; Amicon Corp.). Cells were lysed with glass beads, and the lysates and medium concentrates were immunoreacted with antitoxin antiserum and protein A-Sepharose as described previously (4).

Immunoreactants were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and fluorography as described previously (4). To estimate the intensity of protoxin and toxin bands, films were scanned with a densitometer (Ortec), and the areas under the peaks were estimated by weighing. To correct for sample variability in gel lanes, protoxin levels were normalized relative to a stable, nonspecific protein migrating at approximately 48K on the gels. To determine protoxin half-lives in mutants, decay curves from at least two pulse-chase experiments were plotted, and half-lives were estimated and averaged.

Protease inhibitors were tosyl-L-phenylalanyl chloromethyl ketone (TPCK), *N*-carbobenzoxy-L-phenyl-

alanyl chloromethyl ketone, phenylmethylsulfonyl fluoride, tosyl-L-lysyl chloromethyl ketone, tosyl-L-arginyl-methylester, and chymostatin. They were obtained from Sigma Chemical Co. Inhibitors insoluble in water were dissolved in dimethyl sulfoxide before use as described previously (5).

RESULTS

Killer toxin processing and secretion in *sec* mutants. Novick and Schekman have isolated and characterized a series of conditional-lethal mutants defective in protein secretion and cell surface growth (11). At the restrictive temperature, these mutants accumulate secretory organelles and can be ordered into a pathway that is remarkably similar to that seen in mammalian cells (10).

We studied killer toxin processing and secretion in three such mutants that accumulate endoplasmic reticulum (*sec18*), Golgi (*sec7*), or secretory vesicles (*sec1*) at the restrictive temperature. To examine toxin processing and secretion in *sec* mutants, we constructed *sec* [KIL-k₁] strains by standard genetic techniques. The strains had normal K⁺ R⁺ killer phenotypes at 24°C but failed to grow at 37°C.

The *sec* killer strains were grown at 24°C, switched to 37°C for 1 to 1.5 h, pulse-labeled for 10 min, chased, and the label in toxin and precursors examined (Fig. 1 and 2). No detectable toxin was secreted at 37°C in the *sec1* strain (Fig. 1A, lanes d, e, and f), although a wild-type strain (T158C/S14a) secreted toxin under similar growth and labeling conditions (Fig. 1A, lanes a, b, and c). A similar blockage of toxin secretion at 37°C was found in *sec7* and *sec18* strains (data not shown). We examined toxin precursors that had accumulated at the *sec* blocks by immunoreaction of cell lysates with antitoxin serum. In all cases, a 43K protoxin was detected after a pulse (see Fig. 1B, lane a for *sec1*; Fig. 2, lanes b and f for *sec7* and *sec18*, respectively). The protoxin was identical in size to that seen in these mutants at 24°C and to the protoxin from a wild-type strain (data not shown). Pulse-chase experiments indicated that in *sec18* the protoxin was stable after a 60-min chase at 37°C (Fig. 2, lanes g and h), whereas for *sec7* the protoxin was unstable with a half-life of approximately 45 min (Fig. 2, lanes c and d). In the *sec1* strain, the protoxin was more labile, with a half-life of approximately 15 min (Fig. 1B, lanes b and c). In *sec1*, but not *sec18* or *sec7*, cell lysates 11K toxin was also found (see Fig. 1B), although no toxin or protoxin was secreted. Protoxin in a wild-type strain at 37°C was less stable than in the *sec1* strain. Protoxin was not detectable after a 30-min chase (data not shown), indicating a half-life of less than 10 min. By 30 min at 37°C,

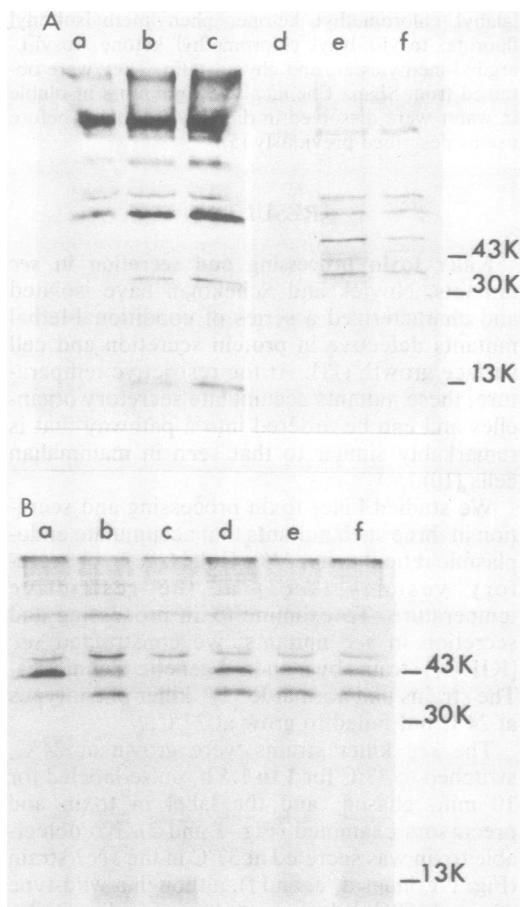


FIG. 1. Synthesis of protoxin and secretion of killer toxin in *SECI* and *sec1* killer strains of 37°C. Strains were grown at 24°C, switched to 37°C for 1 h, labeled with [³⁵S]sulfate for 10 min, and chased with cold sulfate for up to 1 h at 37°C. (A) Secreted proteins were obtained from the growth medium, and after concentration they were applied to an SDS-polyacrylamide gel, electrophoresed, and a fluorogram of the gel obtained. Lanes a through c (in *SECI*) chase duration: lane a, 0 min; lane b, 30 min; lane c, 60 min. Toxin is seen in lanes b and c as the band just below the 13K marker. Lanes d through f (in *sec1*) chase duration: lane d, 0 min; lane e, 30 min; lane f, 60 min. (B) Cells were lysed and proteins immunoreacted with antitoxin antiserum. The immunoreactive species were electrophoresed on an SDS-polyacrylamide gel, and a fluorogram was made. Lanes a through c, *sec1*; lanes d through f, *sec1* treated with TPCK during labeling. Chase duration: lanes a and d, 0 min; lanes b and e, 30 min; lanes c and f, 60 min. The protoxin band migrated at 43K.

secreted 11K toxin was clearly visible in the growth medium (Fig. 1A, lane b).

Novick and Schekman (11) showed that acid phosphatase and invertase, accumulated in the secretory vesicles in a *sec1* mutant at 37°C, were

secreted after lowering the culture temperature from 37°C to the permissive temperature of 24°C and in the absence of further protein synthesis. In a similar experiment, a *sec1* killer strain was grown at 37°C and pulse-labeled with [³⁵S]sulfate. After the pulse, protein synthesis was inhibited with cycloheximide, the culture was transferred to 24°C, and toxin synthesis was examined (Fig. 3). Protoxin made at 37°C decreased with time at 24°C (Fig. 3A) and, in contrast to the situation at 37°C, this decrease in protoxin coincided with an increase in the amount of secreted 11K toxin (Fig. 3B). Note also that several other secreted proteins made at 37°C were chased out at 24°C under these conditions.

Effect of tunicamycin on protoxin glycosylation and toxin secretion. Glycosylation of protoxin was previously identified in vitro by endoglycosidase H susceptibility (2). To determine the importance of glycosylation for toxin maturation, we examined the effect of tunicamycin (7) on in vivo protoxin synthesis and processing. A culture of killer strain (T158C/S14a) was incubated with tunicamycin, pulse-chased with [³⁵S]sulfate, and samples of cell lysate and growth medium taken for analysis of protoxin and secreted toxin. The results (Fig. 4) showed that tunicamycin inhibited synthesis of the 43K protoxin, whereas a 36K species, immunoreactive with antitoxin serum, was detected in cell lysates in the presence of the drug (Fig. 4A, lane d). This 36K precursor, presumably devoid of any *N*-asparaginyl-linked polysaccharide, was similar in size to the preprotoxin synthesized in

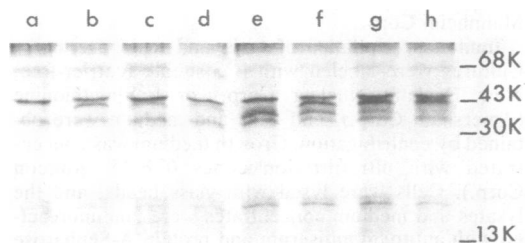


FIG. 2. Processing of protoxin in *sec7* and *sec18* strains at 37°C. Strains were grown at 24°C, shifted to 37°C for 1.5 h, labeled with [³⁵S]sulfate for 10 min, and chased with cold sulfate for 60 min at 37°C. Cells were lysed, immunoreacted with antitoxin antiserum, electrophoresed, and a fluorogram obtained. Lanes a through d (in *sec7*) chase duration: lane a, 0 min, preimmune serum; lane b, 0 min, antitoxin serum; lane c, 30 min; lane d, 60 min. Lanes e through h (in *sec18*) chase duration: lane e, 0 min, preimmune serum; lane f, 0 min, antitoxin serum; lane g, 30 min; lane h, 60 min.

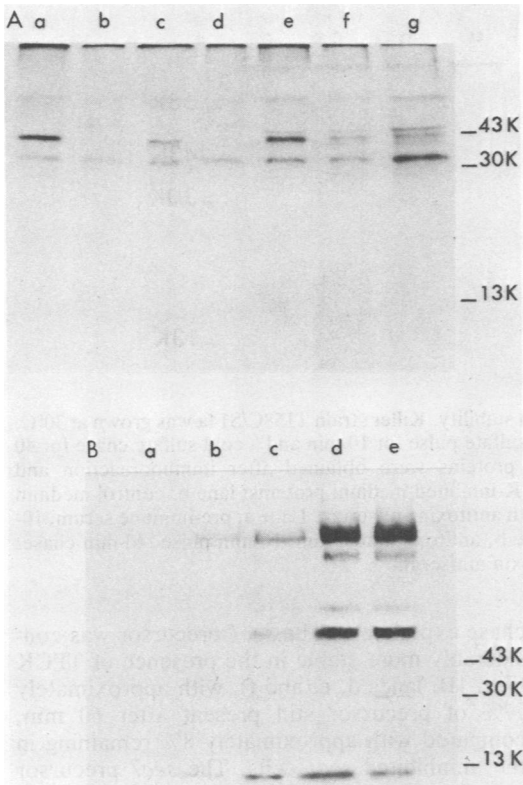


FIG. 3. Protoxin decay and toxin secretion after a switch of a *secl* strain pulse-labeled at 37 to 24°C in the presence of cycloheximide. The *secl* strain was grown at 37°C for 1 h and pulse-labeled with [³⁵S]sulfate for 10 min at 37°C. Cycloheximide (120 μg/ml) was added with 10⁻⁴ M sodium sulfate, and the culture was transferred to 24°C. (A) Cells were lysed, immunoreacted with antitoxin antiserum, and immunoreactants electrophoresed and fluorographed. Lane a, 10-min pulse at 37°C, antitoxin antiserum; lane b, 10-min pulse at 37°C, pre-immune serum; lane c, 30-min chase at 24°C, antitoxin antiserum; lane d, 30-min chase at 24°C, pre-immune serum; lanes e through g, antitoxin antiserum, 15-, 45-, and 60-min chase at 24°C, respectively. (B) Medium was taken, concentrated, electrophoresed, and fluorographed. Lane a, 10-min pulse at 37°C; lanes b through e, 15-, 30-, 45-, and 60-min chases, respectively, at 24°C with cycloheximide. Toxin appears as a 11K band just below the 13K marker.

vitro (Fig. 4A, lane c). Although no 43K protoxin was detected in the presence of tunicamycin, a small amount of 11K toxin was secreted (Fig. 4B, lanes a and c) which was approximately 10% of that produced in the absence of tunicamycin (Fig. 4B, lanes b and d). Immunoreaction of secreted proteins showed that the 11K toxin was the only secreted cross-reactive component (Fig. 4B, lanes a and b).

Decreased secretion of toxin in the presence

of tunicamycin could result from instability of the unglycosylated protoxin. However, in a pulse-chase experiment, the kinetics of unglycosylated protoxin decay were measured and found to be identical to those of the glycosylated

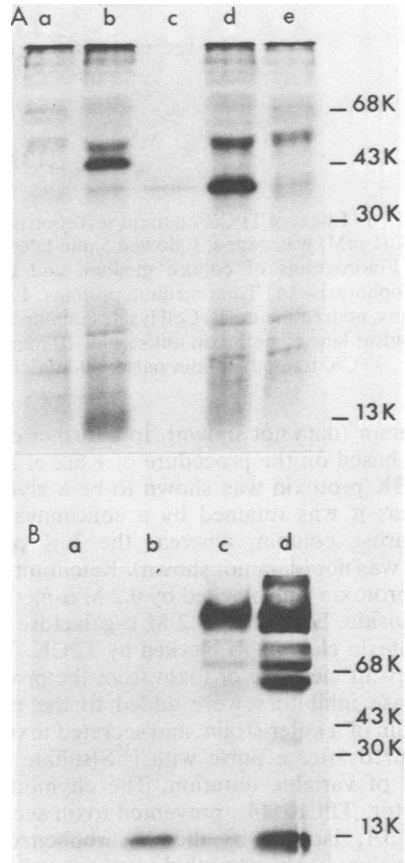


FIG. 4. Effect of tunicamycin on protoxin glycosylation and toxin secretion in vivo. Killer strain T158C/S14a was incubated at 30°C for 30 min with tunicamycin at 15 μg/ml. The culture was labeled with [³⁵S]sulfate for 10 min and, where indicated, chased for a further 27 min with cold sulfate. (A) Cell lysates were prepared and immunoreacted with antitoxin antiserum, electrophoresed, and a fluorogram obtained. Lanes a and b, lysates of untreated control cells, 10-min pulse, pre-immune and antitoxin antiserum, respectively; lane c, in vitro translation of denatured M dsRNA immunoreacted with antitoxin antiserum; lanes d and e, tunicamycin-treated cell lysates, 10-min pulse, immunoreacted with antitoxin and pre-immune sera, respectively. (B) Growth medium concentrated, immunoreacted with antitoxin serum, electrophoresed, and fluorographed. Lanes a and c, culture fluid from pulse-chased tunicamycin-treated cells, immunoreacted and total secreted protein, respectively; lanes b and d, culture fluid from pulse-chased untreated control cells, antitoxin immunoreacted and total secreted protein, respectively.

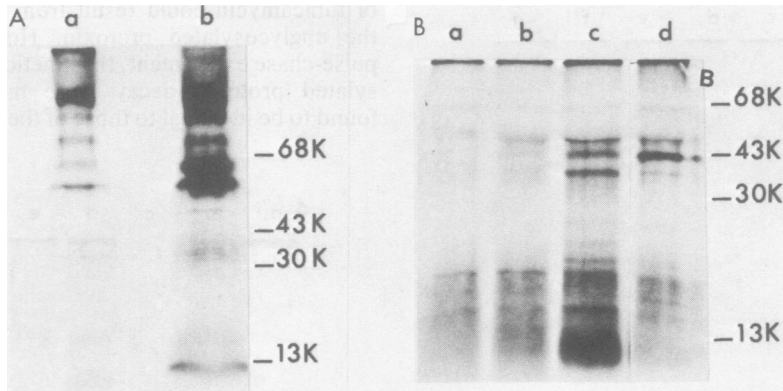


FIG. 5. Effect of TPCK on toxin secretion and protoxin stability. Killer strain T158C/S14a was grown at 30°C, TPCK (1 mM) was added, followed 5 min later by a [35 S]sulfate pulse for 10 min and a cold sulfate chase for 40 min. Fluorograms of culture medium and cell lysate proteins were obtained after immunoreaction and electrophoresis. (A) Total medium proteins. Lane a, TPCK-inhibited medium proteins; lane b, control medium proteins, no treatment. (B) Cell lysates immunoreacted with antitoxin antiserum. Lane a, pre-immune serum, 10-min pulse; lane d, antitoxin antiserum, 10-min pulse; lane b, antitoxin antiserum, 10-min pulse, 40-min chase; lane c, TPCK treated, 10-min pulse, 40-min chase, antitoxin antiserum.

precursor (data not shown). In a further experiment based on the procedure of Katz et al. (6), the 43K protoxin was shown to be a glycoprotein, as it was retained by a concanavalin A-Sepharose column, whereas the 36K preprotoxin was not (data not shown). Retention of the 43K protoxin was blocked by 0.2 M α -methyl-D-mannoside, but not by 0.2 M D-galactose.

Protoxin cleavage is blocked by TPCK. To try to prevent cleavage of toxin from the protoxin, protease inhibitors were added to the growth medium of a killer strain, and secreted toxin was measured after a pulse with [35 S]sulfate and a chase of variable duration. The chymotrypsin inhibitor, TPCK (14), prevented toxin secretion (Fig. 5A, lane a) as did *N*-carbobenzoxyl-L-phenylalanyl chloromethyl ketone, although toxin was secreted in the presence of phenylmethylsulfonyl fluoride, tosyl-L-arginyl-methyl-ester, tosyl-L-lysyl chloromethyl ketone or chymostatin (data not shown). Although TPCK blocked toxin secretion, it did not prevent secretion of many medium proteins (Fig. 5A, compare lanes a and b). Examination of cell lysates from pulse-labeled TPCK-treated cells, after a chase period sufficient for labeled protoxin to have disappeared from untreated cells, showed that the protoxin persisted and remained cell-associated, which is consistent with TPCK blockage of protoxin cleavage and nonsecretion of protoxin (Fig. 5B, compare lanes b and c). Toxin-sized material was also found in TPCK-inhibited cells. This material may arise from degradation of protoxin on extraction or may imply a more complex mode of action of TPCK.

At the restrictive temperature, the *sec1* mutant had an unstable toxin precursor in a pulse-

chase experiment. The *sec1* precursor was considerably more stable in the presence of TPCK (Fig. 1B, lanes d, e, and f), with approximately 77% of precursor still present after 60 min, compared with approximately 8% remaining in the uninhibited *sec1* cells. The *sec7* precursor was also more stable in the presence of TPCK, with no significant decay after 60 min, compared with some 40% remaining in the uninhibited *sec7* cells (results not shown).

Protoxin synthesis in *kex* mutants. The products of two nuclear genes, *KEX1* and *KEX2*, appear essential for protoxin processing. Mutations in these genes lead to loss of ability to secrete toxin while retaining normal M dsRNA plasmids (9, 12, 19). We examined *kex1* and *kex2* mutants for toxin precursors in a pulse-chase experiment. The result (Fig. 6, lanes a and c) indicated that both mutants were able to make a 43K protoxin. In *kex1*, the protoxin was relatively stable after a chase, with 42% remaining after a 40-min chase. In *kex2*, the protoxin was unstable, with less than 10% remaining after a 40-min chase. In neither case was secreted toxin or precursor detected, although in *kex1* a very diffuse band of lower molecular weight than the toxin was detectable by immunoreaction with antitoxin antiserum.

To order these mutations in the protoxin processing pathway, we used two *kex1 kex2* double mutants in an experiment similar to that seen in Fig. 6. In both experiments, the same result was obtained, the protoxin was stable after a chase, with greater than 60% remaining after 60 min (data not shown) which suggested the order *kex1* \rightarrow *kex2*. In a further experiment, we tested whether the unstable protoxin in *kex2* mutants

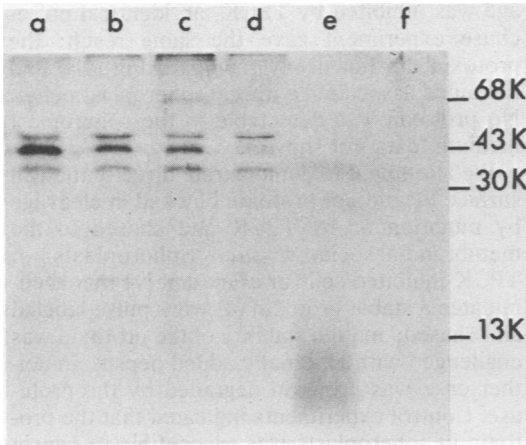


FIG. 6. Protoxin stability in killer expression mutants *kex1* and *kex2*. Cultures were grown at 30°C, pulse-labeled with [³⁵S]methionine for 10 min, and chased with unlabeled methionine (10⁻³ M) for a further 40 min. Cell lysates and growth medium were immunoreacted with antitoxin antiserum, electrophoresed, and fluorographed. Lanes a, b, and e (in *kex1*) lane a, 10-min pulse; lanes b and e, 40-min chase of cell lysate and medium, respectively; lanes c, d and f (in *kex2*) lane c, 10-min pulse; lanes d and f, 40-min chase of cell lysate and medium, respectively.

could be stabilized with TPCK in a pulse-chase experiment. The protoxin remained unstable in two *kex2* alleles under these conditions and implied that the *kex* mutations act before the TPCK-inhibitable step.

Localization of protoxin synthesized in vitro. A series of experiments was aimed at determining whether the protoxin is a secreted protein or an integral membrane glycoprotein. In vitro translation of denatured M₁ dsRNA in a reticulocyte lysate in the presence of dog pancreas microsomal membranes gave a 36K preprotoxin and a glycosylated 43K protoxin (2) (Fig. 7, lane a). Appearance of the 43K protoxin was dependent upon the cotranslational presence of dog pancreas microsomal membranes; only the 36K species was found if the membranes were added post-translationally (Fig. 7, lane g). Treatment of the precursors synthesized in vitro with trypsin showed that the 43K protoxin was relatively resistant to trypsin digestion, although the 36K species was fully degraded (Fig. 7, lanes a, b, and c). One interesting additional feature seen in some trypsin digestion experiments was the appearance of a diffuse immunoreactive 11K band (see Fig. 7, lanes e and f). This band, with the size and antigenic determinants of toxin, presumably results from cleavage of the protoxin at approximately the normal processing site for toxin production. The product, like toxin itself, is relatively resistant to further trypsin proteoly-

sis. Protection of the protoxin synthesized in vitro from trypsin proteolysis was dependent upon the presence of tetracaine (Fig. 7, lanes c and d), a substance known to stabilize microsomal membranes in this system (13). The addition of Triton X-100 to the system before addition of trypsin abolished the protection of the protoxin (Fig. 7, lanes e and f). Apparent complete protection of the protoxin from trypsin degradation indicated that the protoxin either could be an integral membrane protein or could be released within the vesicle lumen with no lysine- or arginine-containing region of the protoxin exposed on the exterior.

Localization of protoxin in vivo. If the protoxin were released within the lumen of secretory vesicles in vivo, it might be secreted transiently during normal toxin synthesis or stably under conditions where toxin cleavage was inhibited. No secreted precursor was found in pulse-labeled cells (4) or in the medium of TPCK-inhibited cells where protoxin accumulated and no toxin was secreted (Fig. 5A, lane a). Because protoxin could be periplasmic and although secreted it was unable to traverse the cell wall, we investigated precursor secretion in spheroplasts

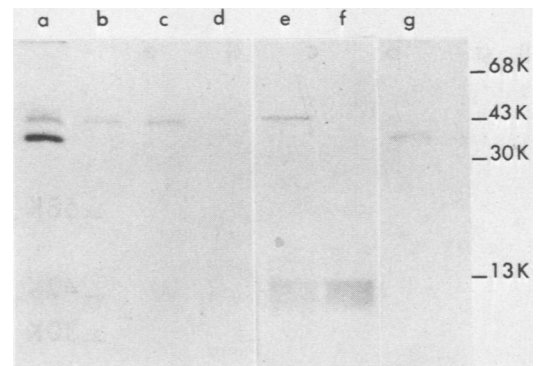


FIG. 7. Stability of glycosylated protoxin to trypsin digestion in an in vitro translation system with dog pancreas microsomal vesicles. Denatured dsRNA was translated in an in vitro system with dog pancreas microsomal vesicles. Translation was stopped with cycloheximide (110 µg/ml), and tetracaine (3 mM) was added and incubated for 5 min at room temperature. Trypsin was added at the indicated concentration and incubated for a further 10 min at room temperature. Incubation was terminated by the addition of SDS (0.25%) and soybean trypsin inhibitor (170 µg/ml). Samples were immunoreacted with antitoxin antibody, electrophoresed, and a fluorogram prepared. Lane a, complete system, no trypsin; lane b, 20 µg of trypsin per ml; lane c, 30 µg of trypsin per ml; lane d, no tetracaine, 30 µg of trypsin per ml; lane e, 30 µg of trypsin per ml; lane f, Triton X-100 (1%) added post-translationally, plus 30 µg of trypsin per ml; lane g, dog pancreas microsomal vesicles added posttranslationally, no trypsin.

that were pulse-chased with [35 S]methionine. The results (Fig. 8B) indicated that the 11K toxin appeared as a secreted protein in spheroplasts, but the protoxin did not. The protoxin remained associated with the spheroplast pellet (Fig. 8A). In spheroplasts where protoxin cleav-

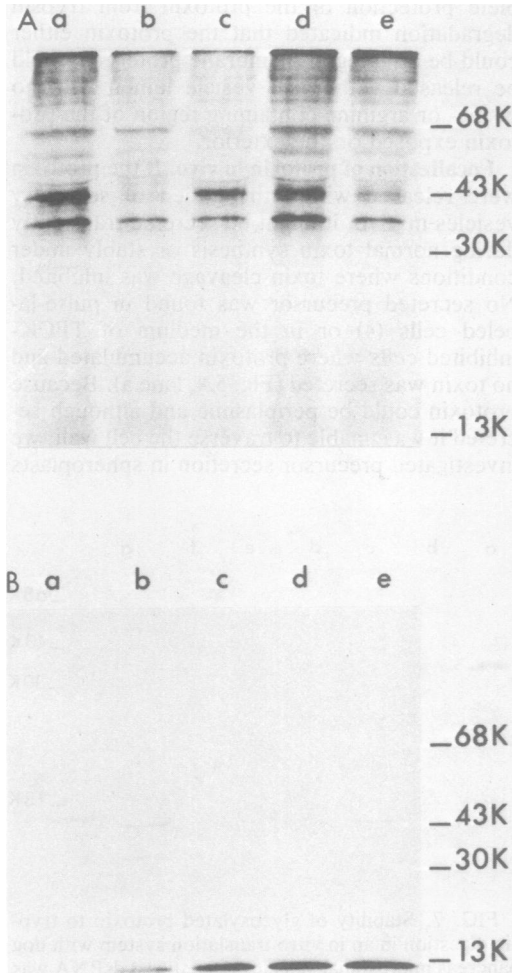


FIG. 8. Location of protoxin and toxin in pulse-labeled spheroplasts. Spheroplasts of T158C/S14a were labeled with [35 S]methionine for 15 min and chased with cold methionine (10^{-3} M) for 40 min. The spheroplasts were obtained by centrifugation. The concentrated medium and lysed spheroplasts were immunoreacted with antitoxin antiserum, and the immunoreactants were electrophoresed and a fluorogram made. (A) Lysed spheroplasts. Lane a, 15-min pulse, antitoxin antiserum; lane b, 15-min pulse, pre-immune serum; lanes c through e, 10-, 20-, and 30-min chase, respectively, antitoxin antiserum. (B) Secreted proteins in growth medium. Lane a, 15-min pulse, pre-immune serum; lane b, 15-min pulse, antitoxin antiserum; lanes c through e, 10-, 20-, and 30-min chase, respectively, antitoxin antiserum.

age was inhibited by TPCK, an identical pulse-chase experiment gave the same result; the protoxin did not decay (compare Fig. 5B) and remained associated with the spheroplast pellet. No protoxin was detectable in the spheroplast medium (data not shown).

We attempted to demonstrate directly the cell surface location of protoxin blocked in cleavage by mutation or by TPCK and chased to the membrane-associated site. Spheroplasts of TPCK-inhibited cells or of mutant N1 that accumulates a stable protoxin (4) were pulse-labeled and chased, and the stability of the protoxin was challenged with externally added pepsin. In neither case was protoxin degraded by the protease. Control experiments indicated that the protoxin in spheroplasts was susceptible to pepsin cleavage after solubilization with SDS.

DISCUSSION

The use of temperature-sensitive *sec* mutants has defined a secretory pathway in *S. cerevisiae* that is similar, in at least major respects, to that in mammals (10). Use of *sec* mutants containing the killer plasmid clearly demonstrates that killer toxin secretion occurs along this pathway. At the restrictive temperature, toxin secretion is blocked in mutants defective in transfer from the endoplasmic reticulum, Golgi, or secretory vesicles.

Glycosylation of the protoxin appears to be complete by the endoplasmic reticulum stage, as protoxin taken from *sec18* at the restrictive temperature is identical in size (as judged by electrophoretic mobility in SDS) to that in *sec18* at the permissive temperature or in a wild-type strain. This suggests that major extension of the core glycosides added at this early stage does not occur during subsequent events in secretion of toxin, which is in contrast to that which occurs with invertase (10). This finding was strengthened by results of *in vitro* processing by dog pancreas microsomal membranes capable of effecting core glycosylation (2) (see above). The protoxin synthesized was again apparently identical in size to that seen *in vivo*. Much of the *in vivo* modification of the protoxin is by di-*N*-acetylchitobiosyl *N*-glycosidic bonding to asparagine residues, as it is sensitive to endoglycosidase H and inhibited *in vivo* by tunicamycin, which blocks *N*-acetyl glucosamine addition to dolichol phosphate (2). However, two observations suggest that this does not account for all of the protoxin modification seen *in vivo*. The unglycosylated preprotoxin synthesized *in vitro* and still containing the amino-terminal signal sequence is similar in size to the precursor produced *in vivo* in the presence of tunicamycin which presumably lacks this signal sequence. It

is also similar in size to the product of endoglycosidase H treatment of the *in vivo* protoxin (2). Perhaps a non-*N*-glycosidic-linked polysaccharide or other component may be added to the protoxin during secretion, accounting for the size difference seen. The *N*-glycosidic-linked polysaccharide moiety of the protoxin seems to be necessary for efficient toxin secretion, as severe (90%) inhibition of toxin secretion was found in the presence of tunicamycin.

The *sec* mutants also define when proteolytic cleavage of the toxin precursor occurs in the secretory pathway. In *sec18*, the endoplasmic reticulum-blocked mutant, the precursor was stable after a 60-min chase at the restrictive temperature. In *sec7* and *sec1*, which accumulate Golgi and secretory vesicles, respectively, the precursor was unstable although more stable in *sec7* than in *sec1*. In *sec1*, 11K toxin was detected in cell lysates at the restrictive temperature. The precursor instability in *sec7* and *sec1* can be prevented by the addition of TPCK to the culture. This protease inhibitor was also shown to prevent cleavage of protoxin *in vivo* in a wild-type strain. These results strongly suggest that protoxin cleavage by a TPCK-inhibitible protease does not occur until after the *sec18* block but can occur with low efficiency before the *sec7* Golgi step. The proteolytic processing of proinsulin is thought to occur within the secretion granules formed within the Golgi and to continue as they move to the cytosol (16, 17). This decrease in stability from *sec7*- to *sec1*-blocked mutants would fit this pattern, with only partial activation of protease activity occurring before the *sec7* Golgi block. In principle, examination of other Golgi- and secretory vesicle-blocked *sec* mutants for ability to cleave protoxin may resolve more closely the stage of onset of proteolytic cleavage in the pathway. MdsRNA plasmid mutant N1, which fails to secrete toxin (4), has a phenotype similar to that seen in TPCK-inhibited cells; a stable nonsecreted protoxin is found in pulse-labeled cells. The N1 mutant protoxin may well be a poor substrate for the TPCK-inhibitible endoprotease.

Mutants harboring *kex1* or *kex2* lesions give further information on the protoxin processing pathway. The moderate stability of protoxin in *kex1* mutants and marked instability in *kex2* mutants permits an ordering of these gene products; *kex1 kex2* double mutants have a stable protoxin, suggesting the order *kex1* → *kex2* in the pathway. We can tentatively order the *KEX*-dependent steps in the secretion pathway defined by the *sec* mutants. The finding that TPCK does not stabilize protoxin in *kex2* mutants indicates that instability in *kex2* mutants involves a TPCK-resistant system that fails to act on the protoxin of wild-type or *kex1* cells and suggests

that TPCK acts after the *kex2* step. The *kex1* and *kex2* steps occur after synthesis of the 43K protoxin in the endoplasmic reticulum but before the *sec7* Golgi block when the TPCK-inhibitible protease is active. A more definitive ordering of *kex* mutants awaits the results of appropriate *sec kex* double mutant experiments. The nature of the *KEX*-dependent steps is unknown, but they are essential for toxin secretion. The instability of protoxin in *kex2* mutants may result from susceptibility of the protoxin to degradation by proteases not inhibitible by TPCK. The processing pathway outlined above implies that protoxin regains a TPCK-dependent stability after the *KEX2* step. This could come about through change in the structure or conformation of the protoxin or from its altered location and exposure to proteases in the secretory organelles. One possible defect would be that in *kex2* mutants, protoxin is rerouted to the vacuole instead of to the Golgi, with subsequent degradation by vacuolar proteases. Wild-type cells with correct routing of the protoxin to Golgi would retain a stable protoxin if the TPCK-inhibitible processing protease were prevented from acting.

Neither the *kex1* nor the *kex2* mutation is lethal, so these gene products are not essential for all secreted proteins and may define a secretory subpathway. Mutants in *kex2* secrete an altered set of proteins; some proteins such as killer toxin and α -factor are missing (9, 12, 19), whereas others are secreted as species that are altered in isoelectric point and are of apparent higher molecular weight, as if incompletely processed. The α -factor sex pheromone is also thought to be synthesized as a precursor (8), and presumably both protoxin and pro- α -factor have a common, essential *KEX2*-dependent step.

Two general pathways for toxin secretion are possible. First, the protoxin is secreted into the lumen of the endoplasmic reticulum, and this secreted precursor is cleaved after the *sec18* block into an amino-terminal toxin and a carboxy-terminal remnant which is also secreted or degraded. Second, the protoxin is an integral membrane protein with an amino-terminal portion extending into the lumen of the endoplasmic reticulum and with hydrophilic glycosylated and hydrophobic membrane-inserted regions distal to the toxin sequence. The precursor would be cleaved with the cleavage step necessary for toxin secretion. The remnant carboxy-terminal glycoprotein would remain as an integral membrane protein, probably located on the exterior of the cytoplasmic membrane after vesicle fusion with the membrane (2).

In vitro trypsin protection experiments with microsomal membrane vesicles, although demonstrating protection, did not resolve these pos-

sibilities. Analysis of protoxin location in cytoplasmic mutants that accumulate protoxin (4) in TPCK-inhibited killer cultures and in pulse-labeled cells and spheroplasts gave the same result; the protoxin did not behave as a conventional secreted or periplasmic glycoprotein. Protoxin always remained associated with the cell or spheroplast pellet fraction. The precise location of the protoxin is unknown, but irrespective of its exact location, cleavage of protoxin appears necessary for toxin secretion. Cleavage of proproteins destined for secretion to generate mature, active proteins is of widespread occurrence in eucaryotes (17). Such processed proproteins are thought to be secreted into the lumen of the endoplasmic reticulum. We know of no other example resembling killer protoxin maturation in which cleavage of an apparently membrane-associated proprotein generates a secreted protein.

The possible role of the remnant carboxy-terminal, membrane-associated portion of the protoxin remains unknown. It has been speculated that it may be the immunity component (1, 2), and this remains an attractive possibility.

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