In Vivo Topological Analysis of Ste2, a Yeast Plasma Membrane Protein, by Using β -Lactamase Gene Fusions

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Gene fusions were constructed between Ste2, the receptor for the *Saccharomyces cerevisiae* α -factor, and β la, the secreted form of β -lactamase encoded by the *bla* gene of pBR322. The Ste2 and β la components were linked by a processing fragment (P) from the yeast killer preprotoxin containing a C-terminal lysine-arginine site for cleavage by the Golgi-associated Kex2 protease. Ste2 is predicted to have a rhodopsinlike topology, with an external N terminus and seven transmembrane segments. Fusions to three of the four Ste2 domains predicted to be external resulted in β la secretion from yeast cells. A fusion at a site just preceding the first transmembrane segment was an exception; the product was cell associated, indicating that the first 44 residues of Ste2 are insufficient to direct secretion of β la; translocation of this domain presumably requires the downstream transmembrane segment. Expression of fusions located in two domains predicted to be cytoplasmic failed to result in β la secretion. Following insertion of the preprotoxin signal peptide (S) between the Ste2 and P components of these cytoplasmic fusions, secretion of β la activity occurred, which is consistent with inversion of the orientation of the β la reporter. Conversely, insertion of S between Ste2 and P in an external fusion sharply reduced β la secretion. Complementary information about both cytoplasmic and external domains of Ste2 was therefore provided, and most aspects of the predicted topology were confirmed. The steady-state levels of β la detected were low, presumably because of efficient degradation of the fusions in the secretory pathway; levels, however, were easily detectable. This method should be valuable in the analysis of in vivo topologies of both homologous and foreign plasma membrane proteins expressed in yeast cells.

The use of fusions of N-terminal fragments of a target protein to a C-terminal reporter has proved to be one of the more successful in vivo expression techniques for analyzing membrane insertion topologies of transmembrane proteins in *Escherichia coli*. Its utility was first demonstrated by Manoil and Beckwith (27), who constructed a series of gene fusions between *E. coli* Tsr, an integral membrane protein, and the normally secreted enzyme alkaline phosphatase (PhoA); bacteria containing such fusion proteins expressed high levels of alkaline phosphatase activity only if the site of fusion was located in a domain of Tsr predicted to be periplasmic. Fusions located in putative cytoplasmic domains of Tsr exhibited little or no alkaline phosphatase activity. The gene fusion method has since been used in the topological analysis of several *E. coli* membrane proteins, and β -galactosidase (*lacZ*; 17, 26) has been used as a cytoplasmically active reporter complementary to PhoA. The β -lactamase product of the *bla* gene of pBR322 is an especially useful reporter in *E. coli*, since it is capable of positively identifying both external and cytoplasmic fusion sites (8). The results are usually interpreted on the basis of the signal/stop-transfer hypothesis of membrane protein insertion (3, 16). This hypothesis assumes that the two-dimensional topology in polytopic transmembrane proteins is determined by the sequential and alternate insertion of each membrane-spanning segment as either a signal peptide (C terminus outside) or a stop-transfer region (C terminus inside) without reference to downstream sequence. The validity of this interpretation is enhanced if both internal and external fusion sites are positively identified and the resulting topological model is internally consistent.

Although several *Saccharomyces cerevisiae* membrane proteins have been cloned and their topologies have been predicted from the genomic sequence (11, 12, 19, 29, 34, 36), only one, 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is located in the endoplasmic reticulum (ER), has so far proved amenable to thorough in vivo topological analysis by gene fusion (33). Successful applications of gene fusion techniques to topological analysis of yeast plasma membrane proteins have so far been limited to in vivo analysis of the most C-terminal segment of the Ste3 α -factor receptor (12) and in vitro expression systems (1, 20). Notwithstanding the utility of such studies, a broadly applicable in vivo approach would, in addition to avoiding artifactual problems associated with in vitro systems, be more rapid and flexible. The present paper describes such a system employing a modification of the target-reporter gene fusion technique to allow processing and secretion of the reporter.

Ste2, the product of the *STE2* gene, an integral plasma membrane protein that is the receptor for the yeast α -factor mating pheromone, was the target protein chosen for this study. Its predicted topology is homologous to that of the bacteriorhodopsin class of membrane receptors, containing seven clearly predictable transmembrane segments, and it couples to a homologous G protein (Fig. 1; 9, 29). The cytoplasmic location of the C-terminal domain of Ste2 is implied by the α -factor-dependent degree of phosphorylation occurring within this domain (31). The deduced orientation of the N terminus is external (Fig. 1), as in rhodopsin and all G protein-coupled receptors of this type, even though this N-terminal sequence is not preceded by and does not contain a recognizable signal sequence.

We have previously demonstrated that β la, the mature β -lactamase encoded by the *bla* gene of pBR322, is efficiently secreted from yeast cells when it is expressed from a

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FIG. 1. Proposed topology of Ste2 and sites of reporter fusions. The external N terminus, seven predicted transmembrane segments, and the cytoplasmic C terminus are shown. There are six fusion sites, at the *HpaI*, *DraI*, *NsiI*, *StyI*, *ClaI*, and *PstI* sites indicated. The sequences resulting from fusion at Val-44, Phe-98, Ala-206, Leu-235, Ser-259, and Ala-369 to P-βla in pSte2-PxB constructs are shown at the bottom. The *PstI* linkers inserted into both Ste2 and ppTox during construction result in the insertion of one to four Ala residues between Ste2 and P-βla, as indicated by the boxes.

yeast promoter and preceded by a yeast signal peptide (S) and an intervening processing region (P) derived from K₁ preprotoxin, ppTox (6, 10). βla is attached to the C terminus of P at a site for cleavage by the Kex2 endopeptidase (18, 28), so that processing in the Golgi leads to secretion of βla modified by the N-terminal addition of an aspartyl-prolyl-glycyl tripeptide. βla functions as a monomer, lacks natural N-glycosylation sites, and is completely stable in the culture supernatants of yeast cells grown on appropriately buffered media (10). Moreover, βla remains active and stable in yeast cells when fused to the C termini of a variety of short secreted peptides or when cytoplasmically expressed (10, 39). Since a modified colorimetric assay permits facile detection of βla at the picogram level (10), we anticipated that direct C-terminal fusions to βla would allow topological analysis of yeast plasma membrane proteins. However, when such direct fusions to Ste2 were expressed in yeast cells, we could not detect the products of any fusion that disrupted the normal Ste2 topology. Destruction of aberrant membrane proteins in the ER of mammalian cells is well documented (2, 30, 38), and such mechanisms almost certainly exist in yeast cells, where many components of the ER salvage pathway have been identified (30, 32).

Hoping that at least part of this destruction might be a post-Golgi event involving targeting to the vacuole, we modified our approach by constructing a series of gene fusions between Ste2 and βla bridged by the Kex2-cleavable ppTox fragment P. Indeed, expression of Ste2-βla fusions bridged by this fragment led to production of detectable βla. Fusions at sites predicted to be extracellular yielded secreted βla activity, whereas fusions at sites predicted to be cytoplasmic yielded βla activity that remained associated with cells. Moreover, insertion of S, the preprotoxin secretion signal, between the Ste2 and P components of these

cytoplasmic fusions resulted in secretion of βla activity. Thus, reciprocal information about periplasmic and cytoplasmic domains of Ste2 was obtained, and an internally consistent topology that agreed with predictions was derived.

MATERIALS AND METHODS

Strains and media. *E. coli* AKEC 28 (24) was used for plasmid selection and propagation. *S. cerevisiae* DJ213-7-3 (*MATa ste2-10::LEU2 ura3 bar1-1 cry1 ade2 his4-580 lys2 trp1 tyr1 sup4-3ts*) and DJ240-8-3 (*stp22-7 BARI*, otherwise isogenic to DJ213-7-3), both obtained from Duane Jenness, were the standard host strains used to test expression of Ste2-βla fusions. Strain BFY113 (*MATa kex2::TRP1 can1 his3 leu2 trp1 ura3 ade2*), obtained from Robert Fuller, was used to demonstrate Kex2-dependent cleavage of fusion proteins. Restoration of Kex2 function to BFY113 was achieved by transformation with the plasmid pKEX2-H, a YEp6 derivative containing *HIS3* and the *KEX2* coding region and promoter (39). pKEX2-T is a derivative carrying the *TRP1* gene (39). Transformants of strains containing fusion protein expression plasmids were selected by uracil prototrophy and maintained on synthetic complete medium lacking uracil (35).

Construction of p20SPnB and p20SPxB expression vectors and of direct Ste2-βla fusions. p20TB is a derivative of YEp352 (a pUC derivative carrying the 2μm origin and the *URA3* gene; 22) with an expression cassette (Fig. 2A) inserted at its multiple cloning site. This cassette consists of promoter and terminator fragments of the *PGK* gene flanking a fusion of T, a DNA fragment encoding the first 233 residues of K₁ preprotoxin (ppTox; 6), to B, a DNA fragment encoding βla (10). βla is the mature sequence of β-lactamase preceded by an Asp-Pro-Gly tripeptide (Fig. 2B, i). The C terminus of T is the Lys Arg-233 site for cleavage by the Kex2 endopeptidase. Cleavage at this site during expression of p20TB results in secretion of βla (10). p20SPB (Fig. 2B, i) is identical to p20TB except that the *PstI*-to-*AccI* fragment encompassing codons 35 to 176 of ppTox is deleted and an in-frame *PstI* linker is inserted, leaving the S (signal) and P (processing) ppTox fragments separated by a single alanine residue (10). The sequences of S and P are shown in Fig. 2G. Insertion of an *XhoI* linker at the *BstEII* site of p20SPB produced the xB fragment in p20SPxB; βla is changed to xβla, and its N terminus is changed to Asp Pro Gly His Asn Ser Ser Cys (Fig. 2B, ii). The Asn Ser Ser N-glycosylation site was introduced to provide an additional indicator of βla secretion. Insertion of an *NsiI* linker at the same *BstEII* site in p20SPB produced p20SPnB (Fig. 2B, iii). The P-βla fusion is no longer in frame, but insertion of Ste2 fragments in place of P produced direct Ste2-βla fusions (Fig. 2E).

Construction of Ste2-nB (direct) and Ste2-PxB fusions. The entire coding sequence of the *STE2* gene, as a 1.7-kb *HindIII* fragment derived from pWS2 (obtained from D. Jenness), was inserted into the *HindIII* site of the plasmid YEpDT-GAL (24), producing pDTGSte2 (Fig. 2D). The 8-bp *PstI* linker 5' GCTGCAGC 3' was introduced at the *HpaI*, *DraI*, and *ClaI* sites in the *STE2* segment of pDTGSte2 after blunt ends were created, if necessary, with the Klenow fragment of DNA polymerase I. The 12-bp *PstI* linker 5' CGGCTGCA GCCG 3' was similarly introduced into the *STE2* *SryI* site. Plasmids pDTGSte2(Pst-44), pDTGSte2(Pst-98), pDTGSte2(Pst-235), and pDTGSte2(Pst-259) were thus generated. *STE2* contains one *PstI* and two *NsiI* sites (Fig. 2D); these sites and all of the *PstI* sites introduced into *STE2* are in the

same reading frame. An *EcoRI* site immediately flanks the N-terminal *STE2 HindIII* site in these constructions; N-terminal *EcoRI-NsiI* or *EcoRI-PstI* *Ste2* fragments of variable length, from pDTGSte2 or its derivatives, were inserted into p20SPnB, giving direct *Ste2-βla* fusions (pSte2-nB; Fig. 2E). Insertion into p20SPxB (Fig. 2B, ii) gave fusions pSte2-44-PxB, etc. (Fig. 2F). Each construct was numbered according to the *Ste2* codon at the fusion site. Sequences at the fusion sites were determined by using the primer (5') CC GCT GTT GAG ATC CAG, complementary to codons 24 to 29 of mature β -lactamase. All sequences were as predicted. The position of each *Ste2-PxB* fusion within the framework of the proposed *Ste2* topology and the sequence at each fusion site are shown in Fig. 1. A variable number of Ala residues derived from the *PstI* linkers employed were inserted between *Ste2* and P.

Construction of *Ste2-SPB* fusions. An *XhoI* linker (5' CCTCGAGG 3') was inserted into the *EcoRI* site (blunted with mung bean exonuclease) located at the 3' end of pPGK in p20SPB (Fig. 2B). A second linker (*PstI*; 5' GCTGCAGC 3') was inserted into this *XhoI* site after filling with the Klenow polymerase I fragment, producing p21SPB (Fig. 2C). An open reading frame from this *PstI* site overlaps the N terminus of the ppTox signal after five codons (Ala Ala Arg Gly Asn MET; Fig. 2G). The SPB fragment was excised from p21SPB by partial *PstI* digestion followed by complete digestion with *BglII* and was used to replace the (*PstI*-to-*BglII*) PxB fragment in plasmids pSte2-98-PxB, pSte2-235-PxB, and pSte2-369-PxB, producing the equivalent pSte2-SPB fusions (Fig. 2G).

Preparation of cell extracts and assay of β -lactamase activity. Cells were harvested from late-exponential-phase cultures (2×10^7 to 4×10^7 cells per ml) growing in SC-Ura medium buffered with 100 mM NaPO_4 (pH 6.8). β -Lactamase is stabilized in culture supernatants above pH 5.5. Cell extracts were prepared, and β -lactamase activities in samples (20 μl) of culture supernatants and cell extracts were determined by using a microtiter plate assay (10). The limits of detection in unconcentrated culture media and in solubilized whole-cell extracts corresponded to 40 and 5 pg of β -lactamase per 10^6 cells, respectively.

Immunoblotting. Proteins (50 to 100 μg total) in yeast cell extracts were denatured by heating them in gel loading buffer (0.5 M Tris, 20% [vol/vol] glycerol, 1% [wt/vol] sodium dodecyl sulfate, 1% [vol/vol] β -mercaptoethanol; pH 6.8) for 30 min at 37°C and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to nitrocellulose membranes, and after incubation with immunoadsorbed rabbit anti- β -lactamase antisera (5 Prime-3 Prime Inc., West

Chester, Pa.), *Ste2-βla* fusion proteins were detected by incubation with ^{125}I -labeled protein A (0.1 mCi/ml; NEN Research Products, Boston, Mass.) followed by autoradiography.

RESULTS

Activity of direct *Ste2-βla* fusions. In transformants of strain DJ213-7-3 expressing pSte2-369-nB, a direct βla fusion (Fig. 2E) within the presumptively cytoplasmic C terminus of *Ste2*, we were able to detect cell-associated β -lactamase activity at a level of 1 to 2 ng/ 10^7 cells and a weak signal in Western blots (immunoblots) for a fusion protein of the expected size (data not shown). However, we were unable to detect either activity or immunoreactive protein in cell extracts or membrane preparations from cells of the same strain expressing the equivalent *Ste2-98* or *Ste2-206* direct fusions. We surmised that any *Ste2* fusion lacking parts of its transmembrane segments was being efficiently retained in the ER and destroyed by surveillance systems for aberrant membrane proteins. It also seemed likely that any fraction of the fusion proteins escaping the salvage mechanism and reaching the Golgi was being targeted directly to the vacuole or suffered endocytosis and vacuolar destruction rather than accumulating on the cell surface. We therefore inserted P, containing a C-terminal Kex2 processing site, between the target and reporter in the hope of rescuing the βla reporter component of any fraction of the fusion proteins reaching the Golgi compartment where Kex2 is active (18, 23, 28). P incorporates codons 177 to 233 of the yeast K_1 killer preprotoxin (ppTox; 6, 10) and contains three N-glycosylation sites and a second internal potential Kex2 processing site (Fig. 2G; 39). We also inserted an N-glycosylation site at the N terminus of the βla reporter, changing it to x βla ; core N glycosylation of secreted x βla occurs at this site with reasonable efficiency (50 to 60%); this has no detectable effect on either the efficiency of secretion or the activity or stability of x βla in culture supernatants (10). The modified fusion constructs are called pSte2-PxB (Fig. 2F).

Activity and subcellular location of β -lactamase components of *Ste2-PxB* fusions. We predicted that *Ste2-PxB* fusions which reach the Golgi anchored to the luminal face of the membrane by their *Ste2* component would be processed to release secreted β -lactamase, while those fusions anchored to the cytoplasmic face would not. This is schematically represented for the *Ste2-98-PxB* (luminal) and *Ste2-235-PxB* (cytoplasmic) fusions in Fig. 3A and C, respectively. β -Lactamase activities of transformants containing the various pSte2-PxB fusion plasmids are shown in Table 1. In the wild-type tester strain DJ213-7-3, only cells transformed by

FIG. 2. Construction of *Ste2-βla* fusions. All of the expression cassettes shown were inserted into the multiple cloning site of YEp352 (22) and are flanked by the *PGK* promoter and terminator fragments shown in p20TB. *Ste2* segments (dark stippling) and the βla and x βla reporters (light stippling) are indicated. (A) p20TB. The *PGK* expression cassette encompasses T, the first 233 codons of K_1 preprotoxin (ppTox), fused at its KR-233 site to B, the *BamHI*-to-*BglII* fragment which encodes βla . (B) i, p20SPB, produced from p20TB by insertion of a *PstI* linker at the *AccI* site in T followed by deletion of the *PstI* fragment internal to ppTox. ii, p20SPxB, the product of insertion of the *XhoI* linker AACTCGAGTT at the filled-in *BstEII* site of p20SPB. The N-terminal extensions of the βla and x βla reporters (DPG and DPGHNSC, respectively), produced by Kex2 cleavage, are illustrated. iii, p20SPnB, the product of insertion of the *NsiI* linker TGCCTAGTGCA at the filled-in *BstEII* site of p20SPB. (C) p21SPB, produced from p20SPB by sequential insertion of *XhoI* and *PstI* linkers at the *EcoRI* site. (D) pDTGSte2, constructed by insertion of the *HindIII* fragment encoding *STE2* from pWS2 into pDTGAL (24). The sites at which *PstI* sites were introduced (Fig. 1) are indicated. (E) Direct *Ste2-βla* fusions resulting from insertion of *EcoRI-PstI* or *EcoRI-NsiI* fragments from pDTGSte2 into p20SPnB cut with *EcoRI* and *NsiI*. (F) *Ste2-PxB* fusions resulting from insertion of the same fragments from pDTGSte2 into p20SPxB cut with *EcoRI* and partially cut with *PstI*. (G) *Ste2-SPB* fusions. The *PstI-BglII* fragment encompassing PxB in the *Ste2-PxB* fusions was replaced with the *PstI-BglII* fragment of p21SPB encompassing SPB. Amino acid sequences of the S and P fragments are shown below in standard one-letter code. Kex2 cleavage sites and N-glycosylation sites are shown in boldface type.

FIG. 3. Schematic representations of fusion product topologies and potential Golgi processing. The PxB and PB reporters are shown as P-KR-xβla and P-KR-βla, where KR represents the Kex2 cleavage site at the C terminus of P. The Kex2 protease is represented as a membrane-anchored enzyme with its active site directed towards the lumen. (A) Ste2-98-PxB fusion, illustrating potential release of secreted xβla by Kex2 cleavage. (B) Ste2-98-SPB fusion, illustrating how S, by acting as a stop-transfer sequence, may render the PB reporter cytoplasmic. (C) Ste2-235-PxB fusion, illustrating the cytoplasmic location of the reporter. (D) Ste2-235-SPB fusion, illustrating how S, by acting as a signal, exposes the reporter to Kex2, leading to βla secretion.

construct Ste2-98-PxB, Ste2-206-PxB, or Ste2-259-PxB secreted amounts of βla detectable by activity assay. The site of fusion between Ste2 and PxB in each of these constructs is predicted to be just prior to or within an external loop (Fig. 1). Transformants containing the three remaining Ste2-PxB fusion protein constructs failed to secrete βla (Table 1).

According to the topological model of Ste2, the fusion sites of Ste2-235-PxB and Ste2-369-PxB should be cytoplasmic, while the fusion site of Ste2-44-PxB should be external. However, the N-terminal domain of Ste2 up to Val-48 does not contain a recognizable signal sequence. Translocation of this region of the protein into the lumen of the ER, therefore, is presumably dependent on insertion of at least the first membrane-spanning segment of Ste2 into the ER membrane (15, 16). In the absence of the first membrane-spanning segment, membrane association and translocation of the Ste2-44-PxB fusion protein would not be predicted, suggesting that this fusion protein should remain cytoplasmic and therefore cell associated, as we found. These results, therefore, are entirely consistent with the topology predicted for Ste2 based on hydropathy analysis and analogy to bacteriorhodopsin (Fig. 1; 9).

Effect of an *stp22-7* mutation on expression of Ste2-β-lactamase fusions. Expression of p20SPxB, in which the Ste2 component of the Ste2-PxB fusions is replaced with the ppTox signal (Fig. 2B), resulted in efficient processing and about 90% secretion of βla at a level 50-fold higher than the highest level seen for any Ste2 fusion (Table 1). Expression of p20SPB gives the same high level of βla secretion (10). It seems apparent, therefore, that the translational efficiency of the Ste2 fusions is low, that Kex2 processing of these fusions is inefficient, or that most of the fusion protein is being

destroyed prior to Kex2 processing in the Golgi. Cotransformation of Ste2-206-PxB transformants of strain DJ213-7-3 with pKEX2-T, which should increase the expression level of Kex2 severalfold above that of wild type, failed to enhance βla expression (not shown), suggesting that Kex2 activity does not limit the efficiency of βla secretion. Ste2 is normally expressed from its own promoter in MATa cells to give about 10,000 functional copies per cell, suggesting that its normal transcript is well translated (25). Thus, destruction of the fusion proteins in the ER seems the most likely cause of low yield. The level of expression of functional Ste2 receptors has not been observed to increase proportionally with gene copy number (25), suggesting that correct membrane insertion may be rate limiting, even for the normal protein.

We tested the effect of the *stp22-7* mutation on expression levels of Ste2-βla fusion proteins. This mutation was isolated as a suppressor of the *ste2-3ts* mutation and allows enhanced expression not only of this missense mutant of STE2 but also of other plasma membrane proteins carrying temperature-sensitive mutations (22a). Transformants of strain DJ240-8-3 (*stp22-7*, otherwise isogenic to DJ213-7-3) containing construct Ste2-206-PxB or Ste2-259-PxB expressed levels of total βla activity 40 to 50% higher than those of comparable transformants of DJ213-7-3 (Table 1). Since a disproportionate fraction of this increase in activity was secreted, an approximately threefold increase in secreted activity was observed. The normal STE22 gene product may increase the proportion of aberrant, membrane-associated proteins destroyed in the ER. The amount of βla activity produced by fusion Ste2-98-PxB was relatively high and was little affected by the *stp22-7* mutation. This suggests either that

TABLE 1. β -Lactamase activities produced by transformants containing Ste2-PxB fusions

Fusion	Yeast strain ^a	β -Lactamase activity ^b		
		Secreted	Cell associated	Total ^c
YEp24	DJ213-7-3	<40	<5	
Ste2-44-PxB	DJ240-8-3	<40	129	129
	DJ213-7-3	<40	169	169
	BFY113	<40	126	126
Ste2-98-PxB	DJ240-8-3	487	255	742
	DJ213-7-3	468	173	641
	BFY113	114	52	166
	BFY113+YEpKEX2 ^d	392	197	589
Ste2-206-PxB	DJ240-8-3	249	234	583
	DJ213-7-3	89	285	374
	BFY113	<40	50	50
Ste2-235-PxB	DJ240-8-3	<40	95	95
	DJ213-7-3	<40	66	66
	BFY113	<40	26	26
Ste2-259-PxB	DJ240-8-3	141	193	334
	DJ213-7-3	53	185	238
	BFY113	<40	56	56
Ste2-369-PxB	DJ240-8-3	<40	450	450
	DJ213-7-3	<40	115	115
	BFY113	<40	129	129
p20SPxB ^e	DJ213-7-3	40,800	3,510	44,310
	BFY113	5,140	8,600	13,740

^a Strains were DJ213-7-3 (*STP22*), DJ240-8-3 (*stp22*), and BFY113 (*STP22 kex2*).

^b All enzyme activities are in picograms of β -lactamase per 10^6 organisms and are means of three replicate determinations carried out on at least two independent transformants. A value of <40 indicates that β -lactamase activity was below the level detectable in unconcentrated culture supernatants. The minimum detectable level of cell-associated β -lactamase activity is approximately 5 pg/ 10^6 organisms. The levels produced by a YEp shuttle plasmid carrying the normal *bla* gene, such as YEp24, were undetectable by these assays.

^c The sums of activities detected in culture supernatants and cell extracts.

^d This plasmid restores a near-normal level of *KEX2* expression (39).

^e These control transformants express the reporter with no Ste2 N-terminal fragment.

there is insufficient Ste2 protein in this severely truncated fusion for recognition by the *STP22*-dependent component of the surveillance mechanism or that because of its structural simplicity, misinsertional or misfolding events involving the Ste2-98-PxB protein are comparatively infrequent.

DJ240-8-3 (*stp22*) transformants containing presumptively cytoplasmic fusion constructs, i.e., Ste2-44-PxB, Ste2-235-PxB, and Ste2-369-PxB, like their *STP22* counterparts, failed to secrete detectable amounts of β la, although a significant increase in cell-associated activity was observed with the last construct (Table 1). The data obtained with strain DJ240-8-3, therefore, confirm those obtained with the *STP22* strain.

Effect of Kex2 on β -lactamase activity of Ste2- β la fusions. Strain BFY113 contains a null mutation in *KEX2*. Analyses of expression of p20SPxB and related β la fusions in this strain demonstrated that apparent Kex2 processing still occurred at 10 to 15% of the level seen in a *KEX2* strain

(Table 1; 39). Similar results have been reported for processing of yeast prepropheromones in *kex2* strains (14, 23), probably because of the presence of a similar enzyme encoded by *YAP3* (14). Secreted β la activities of BFY113 transformants of the Ste2-98-PxB fusion were approximately 25% of those of the DJ213-7-3 transformants, and secretion from the 206 and 259 fusions dropped below detectable levels in this strain, demonstrating that, as expected, Kex2-dependent cleavage is primarily responsible for liberation of β la from these fusion proteins. Since cell-associated β la activities produced by BFY113 transformants containing these fusions were also decreased by 70 to 80%, these cell-associated activities probably also reflect Kex2-processed β la rather than unprocessed, membrane-bound fusion protein. The time required for β la to reach the medium in cells expressing p20SPB is fairly long (about 20 min at 30°C), and a significant fraction of the cell-associated activity is associated with the cell walls (10). This may explain why the fraction of processed β la remaining cell associated increases at lower expression levels (Table 1). Cotransforming BFY113 with Ste2-98-PxB and pKEX2-H elevated the level of activity to that observed in transformants of strain DJ213-7-3 (Table 1), showing that the difference in β la activity observed between BFY113 and DJ213-7-3 is due to *KEX2* activity. With the exception of the activity of the Ste2-235-PxB fusion, levels of cell-associated activity of the presumptively cytoplasmic fusions were unaffected by the *kex2* mutation (Table 1). Since the Ste2-44-PxB fusion is apparently cytoplasmic and the Ste2-369-PxB fusion is correctly folded (see below), it was not surprising that their expression levels were unaffected. Perhaps a small fraction of the cell-associated β la activity of the Ste2-235-PxB fusion results from improper membrane insertion events leading to Ste2- β la molecules that can be cleaved by Kex2. If so, total β la activity could be enhanced by the *stp22* mutation, while the level secreted would still be too low to be detectable in culture supernatants.

Receptor activity of fusion proteins. All transformants containing Ste2-PxB fusion constructs have a *ste2* background and were tested for expression of functional Ste2 derived from the plasmids by use of the zone assay for α -factor sensitivity (24). Activity was detected only for Ste2-369-PxB. Assuming the specific activity of the β la component of the fusion protein to be equal to that of free β la, the expression level of Ste2-369-PxB in strain DJ240-8-3 (*stp22-7*) is 2,000 to 3,000 cell⁻¹, approximately 20 to 30% of the wild-type level of Ste2 (25). As expected if a receptor of normal function were expressed at this reduced level, these transformants gave a near-normal response to α -factor, as judged by zone size and clarity (Fig. 4). Use of heavier inocula produced turbid zones, indicating a response weaker than that of wild type (not shown). DJ213-7-3 (*STP22*) transformants containing the Ste2-369-PxB fusion gave no response to α -factor, presumably reflecting the threefold-lower expression level of the Ste2-369-PxB fusion protein seen in this strain.

Immunoblotting of Ste2-PxB fusion proteins. Since cell extracts obtained from Ste2-44-PxB and Ste2-369-PxB transformants contained β la activities that were not affected by a *kex2*-null mutation, it was anticipated that these activities would reflect the activities of intact fusion proteins. The content and gel mobility of Ste2-P- β la proteins present in transformants containing these plasmids were therefore determined by immunoblotting. The predominant immunoreactive species in transformants containing Ste2-369-PxB was an approximately 80-kDa protein corresponding to the

FIG. 4. Response of transformants containing the Ste2-369-PxB fusion to α -factor. (A) Strain DJ240-8-3 (*MATa stp22 ura3*) transformed with pSte2-369-PxB. (B) Strain DJ147-1-2 (*MATa STP22 ura3*) transformed with pPGK (24) to give a *URA3* phenotype. SC-Ura agar (5 ml) was seeded with 2×10^5 organisms and poured onto an SC-Ura plate, and sterile disks containing 8.5, 2.1, 0.5, or 0.1 μ g of synthetic α -factor (counterclockwise from the top) were placed on top of the overlay. Plates were incubated for 48 h at 30°C.

expected size of the unprocessed Ste2-369-PxB fusion protein (Fig. 5, lane 2). The observed receptor function of this fusion protein indicates that it is correctly inserted into and folded within the ER membrane and is subsequently localized to the plasma membrane. Some smaller-sized species were also present in Ste2-369-PxB-containing extracts, including a species with approximately the same mobility as mature-length β -lactamase (29 kDa). These lower-molecular-mass species probably arise as a result of proteolysis occurring during secretion, turnover, or cell lysis. The predominance of the mature-length β -lactamase species probably indicates that this fragment of the fusion protein, like β -lactamase itself, is relatively resistant to further degradation. The amount of Ste2-369-PxB fusion protein present in cell extracts, estimated from the strength of the Western blot signal (Fig. 5), correlated well with that predicted from β la

FIG. 5. Immunoblot of Ste2-PxB fusion proteins. Lane 1, β -lactamase standard (50 ng of protein, purified as described previously; 10); lane 2, cell extract from strain DJ240-8-3 (*MATa stp22 ura3*) transformed with Ste2-369-PxB; lane 3, cell extract from strain DJ240-8-3 transformed with Ste2-44-PxB. Lanes 2 and 3 were loaded with quantities of extract corresponding to 50 ng of β -lactamase activity as determined by enzyme assay. Numbers at the extreme left represent molecular mass markers (in kilodaltons).

TABLE 2. β -Lactamase activities produced by transformants containing Ste2-SPB fusions

Fusion	Yeast strain ^a	β -Lactamase activity ^b		
		Secreted	Cell associated	Total ^c
Ste2-98-SPB	DJ240-8-3	125	160	285
	DJ213-7-3	173	101	274
Ste2-235-SPB	DJ240-8-3	277	230	507
	DJ213-7-3	459	183	632
Ste2-369-SPB	DJ240-8-3	505	213	718
	DJ213-7-3	590	270	860

^a Strains were DJ240-8-3 (*stp22*) and DJ213-7-3 (*STP22*).

^b All enzyme activities shown are in picograms of β -lactamase per 10^6 organisms and are means of three replicate determinations carried out on at least two independent transformants.

^c The sums of activities in culture supernatants and cell extracts.

activity assays (Table 1). This implies that the cytoplasmically located β la, tethered to the membrane at the C terminus of Ste2, retains full activity in yeast cells, a finding that concurs with previous reports on expression of β -lactamase-containing fusion proteins in *E. coli* (8).

Extracts from transformants expressing Ste2-44-PxB did contain a minor immunoreactive species of approximately 40 kDa, the expected size of this fusion protein. However, the majority of immunoreactive material in these transformants appeared to be mature-length 29-kDa β la (Fig. 5, lane 3). Since this apparent processing is not Kex2 dependent, it is presumably occurring nonspecifically, before or after cell breakage. The rather low activities observed suggested that the Ste2 N-terminal fragment targets this fusion protein for destruction by cytoplasmic surveillance mechanisms (4, 21).

β -Lactamase activities of Ste2-SPB fusions. The most utilitarian target reporter gene fusion technologies are those which, like complementary *phoA* and *lacZ* fusions in *E. coli* (26), enable positive identification of domains of integral membrane proteins located on either side of the membrane in which the target protein is situated. With the aim of developing such a reciprocal system in yeast cells, we constructed three Ste2-SPB fusions in which the yeast killer toxin signal sequence (S) is introduced between the Ste2 fragment and the PB reporter (Fig. 2G). In theory, if the preceding Ste2 sequence dictates that the site of fusion is cytoplasmic, as for the Ste2-235-SPB fusion (Fig. 3D), the S signal peptide should act as an internal signal, leading to exposure of the PB reporter on the luminal face of the secretory pathway. In Ste2-235-SPB and Ste2-369-SPB, the introduction of S resulted in secretion of amounts of β la by transformants of strain DJ213-7-3 (*STP22*) or DJ240-8-3 (*stp22*) comparable to or higher than those obtained with transformants possessing Ste2-98-PxB, Ste2-206-PxB, or Ste2-259-PxB (Tables 1 and 2). Insertion of the signal sequence, therefore, apparently led to reorientation of the P- β la component of the resultant fusions from the cytoplasmic to the external compartment, as predicted. β la activities produced by the Ste2-SPB fusions did not appear to be positively affected by the *stp22-7* mutation, suggesting that addition of the yeast killer toxin signal might improve the efficiency of membrane insertion of fusions containing less than the full complement of Ste2 membrane-spanning segments. When the same signal sequence was placed between the Ste2 and P components of Ste2-98-PxB, the most effi-

ciently expressed periplasmic fusion, secretion of β la decreased by 60 to 70%. Reorientation of the PB reporter to the cytoplasm may have occurred (Fig. 3B).

DISCUSSION

This study describes the first demonstration of a gene fusion approach to in vivo analysis of plasma membrane protein topology in *S. cerevisiae*. While analysis of expression of direct fusions between Ste2 and β la was largely fruitless, apparently because of efficient destruction of the fusion products, the introduction of a Kex2-cleavable bridge (P) between the target protein and the reporter resulted in accumulation of secreted β la from those fusions in which the P β la reporter is luminal and therefore exposed to the active site of Kex2 during passage through the Golgi. Although this accumulated activity probably still represented only a few percent of the total products of translation, β la was released at reproducible and readily assayable levels. Analysis of these enzyme activities provided clear-cut, positive evidence of the luminal location of target reporter fusion sites in all three Ste2 loops predicted to be external (fusions at 98, 206, and 259) and gave negative evidence for the location of the two fusion sites at 235 and 369, predicted to be in cytoplasmic domains.

In interpreting all such fusion experiments, the uncertainty principle must be kept in mind: experimental observation inevitably perturbs the system observed. In gene fusions, the reporter clearly has the potential to affect membrane insertion of the attached target. Nevertheless, our data suggest that the PxB reporter is relatively neutral in its effects. It should be noted, however, that PxB fusions introduce a short, hydrophobic, and potentially α -helical stretch at the N terminus of P that consists of one to four Ala residues derived from the *PsrI* linkers followed by Ile Leu (Fig. 1). The Phe-98 (*DraI*) and Ser-259 (*Clai*) fusion sites occur three and seven residues, respectively, before the predicted C termini of transmembrane segments (Fig. 1). The fusion replaces those residues with Ala Ala Ala Ile Leu, potentially contributing to the fidelity of membrane insertion of the products.

Use of the SP bridge, predicted to result in translocation of the PB reporter from cytoplasmic sites of fusion to the lumen, allowed positive identification of the cytoplasmic fusion sites at 235 and 369. Secreted β -lactamase activities were as high as with any of the luminal PxB fusions lacking the S signal. The marked reduction in secreted β la observed on insertion of S into the Ste2-98-PxB fusion was consistent with the luminal location of this fusion site indicated by the PxB fusion data: the killer toxin signal, when preceded by another signal sequence such as the second membrane-spanning segment of Ste2, may function as a stop-transfer sequence and thus reorient the P- β la reporter to the cytoplasm (Fig. 3B). Such interconversion of signal and stop-transfer functions is sometimes inefficient (13), possibly because of the more-stringent demands for hydrophobicity postulated for stop-transfer segments and possibly because of the dominant role normally played by flanking positive charges in determining topogenicity (7, 37). S is reasonably hydrophobic and does have two positively charged residues near its N terminus but none at its C terminus, where they might enhance its function as a stop-transfer sequence. It is, therefore, probably much more efficient as a signal. An alternative explanation of the effect of insertion of S on expression of the Ste2-98-SPB fusion is that the two additional positively charged residues introduced into the first

extracellular loop of Ste2 (Fig. 1) decrease the efficiency of insertion of the preceding membrane-spanning segment of the Ste2 component and so decrease the amount of Kex2-processed β la released. In practice, insertion of S allowed positive identification of cytoplasmic fusion sites but only corroborated external fusion sites. The results, however, were internally consistent and entirely compatible with the current topological model of Ste2 (9, 29).

The cell association of all products of the fusion at Val-44, just prior to the first transmembrane segment, showed that no cryptic signallike function for this domain was detectable. This is consistent with models in which the normal external location of this domain is dictated by insertion of the first or the first two transmembrane segments. Since the Phe-98 fusion gave the highest secreted activity, the first two transmembrane segments apparently insert with normal topology and reasonable efficiency and should suffice to externalize the N terminus. Direct testing of this inference and testing of the first transmembrane segment in isolation are needed to resolve this issue.

Verification of the in vivo topology of Ste2 was not the only purpose of this investigation. Since Ste2 is one of the few yeast plasma membrane proteins for which a relatively unequivocal putative topology has been proposed, it was chosen as a test system in which to establish the feasibility of using the gene fusion techniques described in this paper for other plasma membrane proteins. Success in this approach is critically dependent not only on normal insertion of membrane protein fusions in the ER, but also on their survival during passage through the secretory apparatus. Subsequent correct targeting and stability are required for detection of fusions not subject to Kex2 cleavage, and failure at this level presumably accounted for the failure of the "direct" β la fusion approach. Northern (RNA) blot analysis (not shown), using the B fragment encoding β la as probe, demonstrated that steady-state transcript levels were comparable in pSte2-98-PxB and p20SPB transformants. However, the total β la activity produced from the Ste2-98-PxB fusion, the most active of the protein constructs tested, was only 2% of the activity produced by a p20SPB transformant (Table 1). Since p20SPB differs from the Ste2 fusions only in lacking the Ste2 fragments (Fig. 2B), it appears that these Ste2 fragments are responsible for loss of most of the translation product prior to the point in the secretory pathway at which Kex2 cleavage occurs. Destruction presumably results from action of the salvage pathway and ER-associated degradation pathways (2, 5, 30, 38), which appear to be efficient in recognizing truncated Ste2 fragments. Only fusion Ste2-369-PxB, in which only 63 amino acids were removed from the C terminus of Ste2, appeared to be relatively stable as a membrane-bound fusion protein. An equivalent C-terminal deletion of Ste2 is stable and functional when expressed from a YE_p plasmid (25).

Several features of the killer toxin- β -lactamase gene fusion approach contribute to overcoming the problem of in vivo fusion protein instability. First, the Kex2-mediated liberation of the β la reporter avoids destruction of fusion proteins in the vacuole due to misrouting or endocytosis. Second, the stability of secreted β la allows accumulation of sufficient enzyme for detection. Third, the sensitivity of the β -lactamase activity assay (10) is such that an expression level of target-reporter fusion protein sufficient for 200 copies per cell per generation to reach the Golgi should be sufficient for the data obtained to be interpretable. The sensitivity of this technique may allow us to analyze the topology of membrane proteins from higher eukaryotes

heterologously expressed in *S. cerevisiae*. Recent advances have also been made, both *in vitro* (37) and via gene fusion technology in *E. coli* (7), in defining elements that control topogenicity in membrane proteins. It would obviously be advantageous to expand these studies into an *in vivo* eukaryotic system. The indirect target-reporter fusion technique we have developed may provide such an opportunity.

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