The yeast homolog of H β >58, a mouse gene essential for embryogenesis, performs a role in the delivery of proteins to the vacuole

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The highly conserved and ubiquitously expressed mouse gene H β >58, identified through insertional mutagenesis, has been shown to be essential for early postimplantation development in mouse, but the mechanism by which it acts is unknown (Radice et al. 1991; Lee et al. 1992). We report here the identification of a yeast gene related to the H β >58 gene and provide biochemical and genetic evidence for its function within the cell. The gene, *PEP8*, plays a role in the delivery of proteins to the vacuole. Disruption of the gene did not affect cell viability. However, the disruptants were shown to have a defect in the processing of the soluble vacuolar proteases but not the membrane vacuolar hydrolases. The processing defect appeared to be a consequence of the inability of the soluble vacuolar hydrolase to reach the vacuole. Although a small amount of the vacuolar precursors was mis-sorted to the extracellular medium, mis-sorting did not appear to be the primary defect in these cells. Pep8p was identified by epitope tagging of the protein. Biochemical fractionation indicated that the protein was peripherally bound to membranes. Immuno-gold electron microscopy indicated that the Pep8p localized to vacuolar membranes. Complementation experiments with the mouse H β >58 cDNA revealed that a Pep8p-H β >58 fusion protein in which the carboxy-terminal 85 amino acids of Pep8p were replaced by the carboxy-terminal 115 amino acids of H β >58 was functional. The results reveal a conserved post-Golgi step in the delivery of proteins to the vacuole/lysosome and that the primary defect in the mouse embryos that carry homozygous mutations in H β >58 may be the failure of proteins to be correctly delivered to the lysosome by this pathway.

[Key Words: Mouse gene; yeast homolog; H β >58; vacuole; lysosome]

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Proteins that enter the secretory pathway destined for the cell surface or the extracellular medium transit sequentially through the different compartments by vesicular budding and fusion in a process that appears to be highly conserved from yeast to man (Rothman and Orci 1992). Proteins destined for the lysosomes or vacuole, however, are directed away from this bulk flow by targeted vesicular budding and fusion by a mechanism that appears to be quite different in mammals and yeast. The major pathway that targets soluble proteins to mammalian lysosomes relies on the presence of mannose-6-phosphate residues on the targeted protein that are recognized by a mannose-6-phosphate receptor (MPR) (Varki and Kornfeld 1981; Waheed et al. 1981). Proteins exit the Golgi complex in vesicles to an acidic prelysosomal compartment where the receptor is released and recycled while the lysosomal or prelysosomal protein

proceeds further to the lysosome (Griffith et al. 1988). In yeast, targeting appears to be quite different in that it occurs independent of glycosylation (Winther et al. 1991; Nebes and Jones 1992). The signal that targets proteins to the vacuole lies within short peptide stretches of the proteins (Valls et al. 1987, 1990; Klionsky et al. 1988).

Evidence also exists in mammalian cells, however, for an MPR-independent pathway of lysosomal targeting. In I-cell disease (mucopolipidosis II), in which the phosphorylation of mannose to mannose-6-phosphate residues is greatly impaired, cells from several tissues of these patients have near normal levels of lysosomal enzymes (Owada and Neufeld 1982; Waheed et al. 1982). Recently, several workers have shown the association of several prelysosomal proteins with microsomal membranes in an MPR-independent fashion that is also suggestive of an alternate receptor for lysosomal protein sorting (McIntyre and Erickson 1991, 1993; Rijnboutt et al. 1991; McIntyre et al. 1994). In addition to this, the ability of mammalian cathepsin L to target to the yeast vacuole correctly (Nishimura and Kato 1992) and the

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ability of the yeast carboxypeptidase Y vacuolar sorting signal to target proteins to the *Xenopus* lysosome (Rottisch and Lehle 1991) are strong indications that other glycosylation-independent vacuolar sorting signals exist and are probably conserved along the evolutionary ladder. The protein machinery involved in this sorting and delivery pathway would also be expected to be conserved.

Results and Discussion

The PEP8 gene is related to H< β >58, a mouse gene essential for embryogenesis

We have been interested in the pathways and events that lead to the delivery of proteins to the vacuole. With this intention the *pep* (peptidase-deficient) mutants of *Saccharomyces cerevisiae* were isolated as defective in the vacuolar protease carboxypeptidase Y (CpY) using a simple colorimetric plate assay (APE test) and placed into 17 complementation groups (Jones 1977). These are among the >50 complementation groups that appear to play a role in vacuolar biogenesis and the sorting and delivery of proteins to the vacuole (Jones 1984; Rothman et al. 1986; Robinson et al. 1988). Strains bearing the *pep8* mutation showed a relatively weak CpY-negative phenotype. The *PEP8* gene was isolated from a yeast genomic library (Rose et al. 1987) by complementation of the *pep8-6* mutation. The initial 11-kb *pep8* complementing fragment was demonstrated to contain the *PEP8* gene by subcloning a 7-kb *SpeI* fragment in pRS306, integrating the plasmid into a *pep8 ura3* strain (BJ7000) by transformation after linearization at the *SnaBI* site of the fragment, crossing the integrant with a strain that was *PEP8 ura-3* (BJ5412), and showing through tetrad analysis that the insert had integrated at a site closely linked to *PEP8*. Among the 36 tetrads dissected, 34 were parental ditype, segregating *2Pep⁺Ura⁺:2Pep⁺Ura⁻*. The 1.7-kb *SnaBI*–*XbaI* fragment (Fig. 1a), which subcloning and complementation studies indicated to contain the *PEP8* gene, was sequenced and found to contain a single open reading frame (ORF) encoding 379 amino acids that displayed no predicted signal sequences, membrane spanning domains, or other recognizable motifs (GenBank accession number L11623). Examination of the GenBank and EMBL data bases, using the BLAST program (Altschul et al. 1990), identified the H< β >58 gene, a mouse gene shown to be essential for embryogenesis but whose mode of action is unknown (Radice et al. 1991; Lee et al. 1992). The *PEP8* ORF and the H< β >58 gene product exhibited 44% identity (67% similarity if conservative changes are taken into consideration) at the amino acid level over the entire lengths of the proteins (Fig. 2).

Disruption of the PEP8 gene leads to a defect in the processing of soluble but not membrane hydrolases

To investigate the role of *PEP8* and examine whether it was essential for cell viability, the *PEP8* gene was disrupted (Fig. 1b) in a diploid, the diploid was sporulated,

Figure 1. (a) Restriction map of the 7-kb *SpeI* fragment. The 7-kb *SpeI* fragment from the initial 14-kb complementing fragment was restriction mapped in detail; the *PEP8* ORF is shown. (Sp) *SpeI*; (Sh) *SphI*; (X) *XbaI*; (C) *ClaI*; (R) *EcoRI*; (B) *BamHI*; (S) *SnaBI*; (M) *MluI*; (H) *HindIII*; (V) *EcoRV*; (Bc) *BclI*. (b) Disruption of the *PEP8* gene. The *HindIII* fragment of *PEP8* was removed from plasmid pMX8 that contained the *MluI*–*XbaI* fragment in pRS316 and replaced by a 1.1-kb *HindIII* fragment containing the *URA3* gene as a selectable marker. The *EcoRI*–*XbaI* fragment of the disrupted plasmid p8MU4 was used to transform a diploid strain that was homozygous for *ura3* (BJ7339).

and tetrads dissected. Each ascus gave four surviving spores, indicating that the *PEP8* gene was not essential for viability. No differences in growth were apparent between the *pep8* and *PEP8* spores.

The *pep8* disruptant appeared to have normal vacuoles as seen by light microscopy using the vacuole-specific dye 6-carboxyfluorescein diacetate (Preston et al. 1989). The disruptants were able to grow at 37°C and, on glycerol medium, were able to tolerate elevated levels of metal ions and to grow on medium at pH 7.5. In addition, diploids that were homozygous for *pep8* lacked any sporulation defects. Because most of the other vacuolar biogenesis mutants isolated exhibit a defect in one of the above phenotypes, it appears from these studies on the *pep8* disruptants that vacuolar biogenesis per se was not affected in these mutants (Banta et al. 1990; Wada et al. 1990; Woolford et al. 1990; Preston et al. 1991; Robinson et al. 1991; Raymond et al. 1992).

Expression of *PEP8* on a high copy plasmid, pRS425, also failed to bring out any observable effect on cell viability or any discernible effect on vacuolar protein delivery as seen by the CpY plate assay.

Examination of the processing of the three soluble vacuolar proteases, CpY, proteinase A, and proteinase B, in the *pep8* disruptant indicated defects in processing as observed by an accumulation of precursor forms on Western blots (Fig. 3). CpY appeared to be the most affected; the defects in both proteinase A and proteinase B were less marked, although clearly evident.

Because the final step in the processing of these proteases is in the vacuole, we sought to determine whether the defect in processing of CpY (which had the most

Yeast homolog of mouse II β58 gene

Pep8	...MsiFFKp	piDIElIFdn	eEsRKhvDia	Trssnssyys	mkEslpvYeD	GESlgGiltVL	57
Hβ58	msfLggFFGp	icEIDVaLnd	gEtRKmaEmk	Tedgkv....	..EkhyLFyD	GESvsGKvnl	54
Consen	-----FF-P	---I-----	-E-RK-----	T-----	--E-----D	GES--G-V-L	
Pep8	rvrds.sskvd	HLGikVsvIG	sIDMLkshgs	gnssskkvts	stssssnsgs	vdvrvnsvdq	116
Hβ58	afkqpgkrlE	HqGlrIefVG	qIELFnd...			...Ksnthe	87
Consen	-----K---	H-GI-----G	-I-----	-----	-----	---K----	
Pep8	FlcqsylDlcp	aGELqhsGSF	pFlrldlsKr	YESYkGkNVd	vaYvkvTVm	RksTDiskik	176
Hβ58	FvnlvkELal	pGELtqSrSY	dFeImqvekp	YESYiGanvr	lrYFlKVTiv	RrlTDLvkey	147
Consen	F-----L--	-GEL--S-S-	-F-F----K-	YESY-G-NV-	--Y--KVT--	R--TD--K--	
Pep8	rFwylvlnsv	tteptnltsan	etkattndia	ggnyaadnas	dntqtkstqg	eaadVnvlvp	236
Hβ58	dL.....					...iVhqlat	156
Consen	-----	-----	-----	-----	-----	---V-Q---	
Pep8	ishsNNepkp	VrLDIGIEnc	LHIEFEVaks	qysLKEVIVG	rIYFLltRlr	LkHMElsLIt	296
Hβ58	ypdvNN...s	LkMEVGIEdc	LHIEFEY-KS	kyhLKDVIVG	kIYFLlvRik	LqHMElqLlk	213
Consen	----NN----	-----GIE-C	LHIEFEY-KS	-Y-LK-VIVG	-IYFLl-R--	I-HMEL-LI-	
Pep8	rEssGlqtSn	vmtdsTairY	EIMDGssVKG	EtIPIRLFLs	GydlTEnMs.	.cnyEnVknY	354
Hβ58	kEitGigpSt	tteteTiaky	EIMDGapVKG	EsIPIRLFLa	GyDpTETmrd	vnnkksVryF	273
Consen	-E--G--S-	-----T--Y	EIMDG--VKG	E-IPIRLFL-	GyD-TP-M--	----F-V---	
Pep8	LsLVlIDEEd	RRYFKQsEIt	LYRtr....				379
Hβ58	LnLVlVDEEd	RRYFKQqEIt	LWRkapeklr	kqrtnfhqrf	espsdqasae	qpem	327
Consen	L-LV--DE--	RRYFKQ-EI-	L-R-----	-----	-----	----	

Figure 2. Sequence alignment of Pep8p with the Hβ58 gene product. The entire lengths of the two proteins were aligned using the PILEUP and PRETTY programs using the GCG sequence analysis software.

severe defect) was a consequence of its inability to reach the vacuole and whether the precursor was being mistargeted to the extracellular medium. This was especially likely, as an independent mutant screen that was more specifically examining vacuolar protein secretion in yeast yielded several *vpt* mutants; and one of them, *vpt4*, was subsequently shown to be allelic to *pep8* (Robinson et al. 1988). Spheroplasts of *pep8* disruptants were pulse-labeled and chased for up to 30 min, separated into intracellular and extracellular fractions, and analyzed for the presence of CpY by immunoprecipitation. The P1 and P2 forms correspond to the endoplasmic reticulum (ER) and Golgi-modified precursors of the enzyme and indicate that the transit through the ER and Golgi is normal. A small amount of the P2 form of CpY was detected in the extracellular medium (Fig. 4), indicating that this was being mistargeted to the extracellular me-

dium. However, not all of the P2 precursor form of CpY was secreted. The major part was intracellular. Part of it was processed into the mature-sized enzyme, and part of it remained as P2 precursor form. This is in contrast to the other vacuolar biogenesis/sorting mutants. *PEP3* is a gene required for vacuolar biogenesis/sorting, and cells bearing a disruption in *PEP3* have been shown to secrete large amounts of the P2 precursor of CpY (Preston et al. 1991; Robinson et al. 1991). To determine whether the precursor form that remained intracellular was processed to mature form in *pep3* Δ -bearing cells and compare it with the intracellular processing of *pep8* Δ cells, we carried out a kinetic analysis on the two mutants; the results are shown in Figure 5. No intracellular processing of the P2 form of CpY occurs over the 60-min chase in *pep3* Δ cells, whereas substantial amounts of processing

Figure 3. Western blots of vacuolar proteases CpY, proteinase A (PrA), and proteinase B (PrB). *PEP8* (BJ7384) and *pep8* Δ (BJ7383) were grown in YEPD to stationary phase, and crude extracts were prepared as described in Materials and methods. Five micrograms (for CPY) or 10 μ g (for PrA and PrB) of protein was loaded on SDS-PAGE denaturing gels and, after electrophoresis, transferred to nitrocellulose and probed with the respective antiserum. Detection was carried out using horseradish peroxidase coupled to the secondary antibody with 4-chloro-1-naphthol as the substrate. (p) Precursor; (m) mature form.

Figure 4. Secretion of CpY precursor in *PEP8* and *pep8* Δ cells. Spheroplasts of *PEP8* and *pep8* Δ cells were made as described in Materials and methods; the spheroplasts were washed in WMP-sorbitol medium and resuspended in the same medium containing 1 mg/ml of BSA at an OD_{600} = 5.0. The spheroplasts were pulse labeled for 15 min with 300 μ Ci/ml of Trans- 35 S label and chased for 30 min with excess methionine and cysteine. The spheroplasts were then separated into intracellular and extracellular fractions by centrifugation at 12,000g for 1 min and lysis; immunoprecipitations and SDS-PAGE were carried out as described by Rothblatt and Schekman (1989).

Figure 5. Comparison of intracellular fates of CpY in *pep8Δ* and *pep3Δ* (BJ5579) cells. Spheroplasts made from *pep8Δ* and *pep3Δ* cells were subjected to a 15-min pulse of Trans-³⁵S label and were chased for up to 60 min as described in the Materials and methods. Aliquots taken at the different time points were subjected to lysis, immunoprecipitation, and SDS-PAGE as described.

to mature CpY occur in *pep8Δ* cells. This observation and the fact that very small amounts of CpY precursor are secreted in *pep8Δ* cells suggest that mis-sorting may not be the primary defect in these cells. We could not detect any secretion of the precursor form of proteinase A or proteinase B in the extracellular medium, although the precursor peptides accumulated intracellularly (data not shown).

Vacuolar membrane proteins appear to reach the vacuole by a default pathway different from that followed by the soluble vacuolar proteins (Roberts et al. 1992; Wilcox et al. 1992). We therefore examined the processing of the vacuolar membrane protein alkaline phosphatase in the *pep8* disruptant. The last step in the maturation of alkaline phosphatase occurs in the vacuole and is dependent on proteinase A; maturation of alkaline phosphatase is an indication of its ability to reach the vacuole (Jones et al. 1982; Klionsky and Emr 1989). As seen in Figure 6, there appears to be no apparent defect in the processing of alkaline phosphatase. Even at 0 min of chase (with 20 min of pulse) the major fraction appeared to have reached the vacuole.

Pep8p localizes to the vacuolar membrane, suggesting a role at the postsorting stage in the delivery of proteins to the vacuole

The *PEP8* gene was clearly affecting the processing of the soluble proteins of the vacuole. However, it was not

Figure 6. Processing of alkaline phosphatase in *PEP8* and *pep8Δ* cells. Spheroplasts from *PEP8* and *pep8Δ* cells were labeled for 20 min and chased up to 60 min. Lysis, immunoprecipitation, and SDS-PAGE were carried out as described in Materials and methods.

clear to us whether the *PEP8* gene and its encoded protein were acting at the level of sorting or at a stage that was postsorting for delivery to the vacuole. To try and dissect this we needed information on localization of the protein. Our initial attempts to raise polyclonal antisera against a Pep8p-glutathione *S*-transferase (GST) fusion (Smith and Johnson 1988) proved unsuccessful. We therefore tagged the Pep8p with the hemagglutinin (HA) HA1 epitope (Wilson et al. 1984) by introduction of a triple epitope tag at the *Bam*HI site of the *PEP8* gene. The tagged protein was functional, for it complemented the *pep8* defect. To enable growth on rich medium and ease of manipulation, the epitope-tagged *PEP8* gene (*p8HA2*) was also cloned into an integrating vector, *pRS306*, to yield plasmid *p8HA22*. This was integrated into the *ura3-52* locus by transformation of BJ7000 with plasmid *p8HA22* that had been linearized at the *Nco*I site within the *URA3* gene. The additional 60 amino acids increased the expected size of the protein to ~49 kD. A protein of the expected size was observed in extracts made from strains that contained the plasmid but was absent in untransformed cells that lacked the HA-tagged *PEP8*-containing plasmid. Subcellular fractionations indicated that although the protein lacked any membrane spanning stretches, it was not found in the soluble cytoplasmic S100 fraction but in the P100 fraction (Fig. 7). The ability to strip most of the Pep8p off the P100 membrane fractions with 100 mM sodium carbonate at pH 11.0 suggests that the Pep8p is peripherally associated with a membrane fraction (Fujiki et al. 1981). To identify the membrane to which Pep8p localized, we initially carried out immunofluorescence studies on cells expressing the HA-tagged protein. As these studies were unsuccessful, we resorted to immunoelectron microscopy using cryoultramicrotomy and gold-conjugated antibody on cells expressing the HA-tagged or wild-type

Figure 7. Identification and subcellular fractionation of the *PEP8*-encoded protein. Cells that contained an epitope-tagged *PEP8* gene integrated into the *URA3* locus (BJ7518) were grown in YEPD medium to an $OD_{600}=3.0$ – 10.0 , and crude extracts were prepared as described in Materials and methods. The crude extract was separated into (supernatant) S100 and (pellet) P100 fractions by centrifugation at 100,000g for 60 min. A portion of the P100 pellet (250–400 μ g) was extracted with 100 mM sodium carbonate at pH 11.0 for 30 min on ice, followed by another high speed centrifugation at 100,000g to separate the sodium carbonate extractable and insoluble fractions. Each of the fractions (50 μ g of protein) was run on SDS-PAGE and immunoblotted and probed as described in Materials and methods.

protein. Three preparations were made and analyzed: cells bearing the HA-tagged *PEP8* allele with and without anti-HA primary antibody and cells bearing the wild-type *PEP8* allele reacted with anti-HA primary antibody. Data for these three preparations are given in Table 1. There is a substantial increase in number of cells labeled with gold particles, and the number of gold particles per cell when the cells carry the HA-tagged *PEP8* allele and anti-HA primary antibody is present compared with either control sample. About two-thirds of the gold particles localize over the vacuole membrane in the experimental population. In the grid used to generate the numbers in Table 1, for 108 cells, $54/80 = 67\%$ of the gold particles were associated with the vacuole membrane; for 53 cells on a separate grid, the numbers were $29/43$, again 67% . One example is shown in Figure 8. Because the sorting to the vacuole occurs at the late Golgi stage, the localization of Pep8p to the vacuole indicates that it acts at a postsorting stage in the delivery of proteins to the vacuole.

The carboxy-terminal 115 amino acids of Hβ58 can functionally replace the carboxy-terminal 85 amino acids of Pep8p

As shown in Figure 2, alignment of the Hβ58 and Pep8 proteins indicated that significant similarity exists over the entire stretch of the two proteins, with several domains that are highly similar. The Hβ58 gene was identified by insertional mutagenesis; mice that are homozygous for this mutation fail to survive beyond the early stages of postimplantation development. Cells that were homozygous for this mutation were, however, (like the *pep8* disruptants) viable, indicating that although disruption of the gene resulted in embryonic lethality, it did not kill the cells (Lee et al. 1992). The function of this gene has not yet been identified at the cellular level, and we sought to examine whether the Hβ58 gene product would function in yeast or whether the domains could be interchanged. Several in-frame deletions were initially created that removed different domains of *PEP8* (Fig. 9). SHD3 removed domain II, domain III, and part of domain IV; BRV5 removed domain III; and CX2 was a truncation that removed the carboxy-terminal 20 amino acids that formed part of domain IV. None of these proteins was functional nor did they cause a dominant-negative phenotype when expressed in wild-type cells. We expressed the mouse Hβ58 cDNA on a yeast expression vector, pADNS, using the *ADH1* promoter and the

ADH1 terminator downstream (Colicelli et al. 1989). Complementation of the *pep8* defect was not observed, but we were unable to confirm expression of the Hβ58 protein, as antiserum against the Hβ58 protein is currently unavailable. To examine whether the carboxy-terminal domains were functionally homologous, we carried out a domain swap experiment in which the carboxy-terminal 85 amino acids of *PEP8* were replaced with the carboxy-terminal 115 amino acids of Hβ58. The fusion construct was functional in yeast despite the extra 30 amino acids of the Hβ58 protein that are not homologous to Pep8p. The results are especially significant because the truncation of the last 20 amino acids in *PEP8* leads to a nonfunctional protein. These results and the striking similarity between the two proteins at both the nucleotide and the amino acid level indicate that the Hβ58 gene is likely to play a similar role in the delivery of soluble proteins to the mammalian lysosome.

Recent studies have shown that like higher eukaryotic cells, sorting to the vacuole proceeds via a prevacuolar compartment (Vida et al. 1993), an indication that, although the sorting signals are different, the pathway is perhaps quite similar. If *PEP8* and Hβ58 act at a post-sorting stage, then the study presented here probably identifies one of the proteins that act at one of these conserved steps. Further studies will be needed to elucidate the precise role these proteins play in the delivery and how mutation can lead to a partial mis-sorting phenotype. Defects in the subunits of the vacuolar H^+ -ATPase subunits also cause a partial mis-sorting phenotype. However, the *pep8* disruptions did not cause phenotypes similar to those that result from disruptions in the other vacuolar H^+ -ATPase subunits.

Another intriguing consequence of our observations is that the homozygous Hβ58 mutation, which is embryonic lethal in mice, represents the first example of a disruption of a lysosomal protein delivery gene in mice that leads to a failure at such an early stage in development. Defects in lysosomal enzymes are known to lead to abnormal and retarded development in mammals, including man (McKusick and Neufeld 1983). Individuals defective in the phosphotransferase enzyme required for generating the mannose-6-phosphate residues for lysosomal targeting (I-cell disease or mucopolipidosis II) suffer abnormal development but survive through embryogenesis and early adolescence. Defects in Hβ58 are, in contrast, embryonic lethal. This finding is especially surprising because studies with the yeast homolog indicate

Table 1. Immunogold labeling of Pep8p within cells

PEP8 allele	Anti-HA 1° ab ^a	Number of cells	Cells with gold	Total number of gold particles	Number of gold particles/cell				
					0	1	2	3	4
HA-PEP8	yes	48	21	46	27	6	9	2	4
HA-PEP8	no	59	8	10	51	6	2	0	0
PEP8	yes	50	6	6	44	6	0	0	0

^a (1° ab) Primary antibody.

Figure 8. Immunogold localization of the Pep8p on cryosectioned cells making HA-tagged *Pep8p*. Approximately 40% of the cells displayed one to four 5-nm gold particles (arrowheads in *inset*), and 65% of the gold particles localized to the vacuole (V) membrane. Two controls were used: The first was with cells making the HA-tagged protein but not exposed to the HA antibody; the other control utilized wild-type cells exposed to the HA antibody. In both controls ~10% of the cells display gold particles and rarely show more than one gold particle per cell (see Table 1).

that the disruption of the gene leads to only a relatively mild defect in processing and delivery of proteins to the vacuole. More studies will be needed to elucidate the role it plays in development.

Materials and methods

Materials

Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs and Boehringer Mannheim Biochemicals. Klenow enzyme was a gift of Dr. W. Brown, Carnegie Mellon University (Pittsburgh, PA); 35 S-labeled deoxyadenosine 5'-(α -thio) triphosphate was obtained from New England Nuclear, and Trans 35 S-label was from ICN. 6-Carboxyfluorescein diacetate was obtained from Molecular Probes. Nitrocellulose BA85 was from Schleicher & Schuell. Goat anti-rabbit IgG-horseradish peroxidase conjugate was obtained from Bio-Rad. Monoclonal antibody 12CA5 to the HA epitope HA1 was a gift from Peter Kolodziej (University of California, San Francisco).

Yeast strains and media

The yeast strains used in this study are BJ5412, *MAT α ura3 trp1 lys2 his3*; BJ5579, *MAT α ura3 his1 pep3 Δ ::LEU2*; BJ7000, *MAT α ura3 leu2 pep8-6*; BJ7339, *a/ α ura3/ura3 trp1 + lys2/lys2 his3/his3 leu2 +*; BJ7383, *MAT α pep8 Δ ::URA3 trp1 leu2 his3 lys2*; BJ7384, *MAT α ura3 trp1 leu2 his3 lys2*; BJ7518, BJ7000 containing the epitope-tagged *PEP8* gene integrated at the *ura3* locus.

YEPD and synthetic media were prepared as described by Zubenko et al. (1982); 6-carboxyfluorescein diacetate plates

were prepared as described by Preston et al. (1989); Zn plates (4 mM zinc chloride), Mn plates (3 mM manganese chloride), Ca plates (100 mM calcium chloride), NaCl plates (1 M sodium chloride), and YEPD plates buffered at pH 7.5 were prepared as described by Bachhawat et al. (1993). Wickerham's minimal proline (WMP) medium was prepared as described by Rothblatt and Schekman (1989).

Plasmid constructions and recombinant DNA methods

The parental plasmids used were pRS306 (Sikorski and Hieter 1989), pRS425 (Christianson et al. 1992), pADNS (Colicelli et al. 1989), and PEI.2 (Lee et al. 1992).

The original *pep8* complementing clone in YCp50 was p8c1B. A 7-kb *SpeI* fragment from this was cloned into pRS316 to yield p8SE2 and into pRS306 to yield pSE306.

All other plasmids were derived from p8SE2. p8SXBA3 was a deletion derivative of p8SE2 constructed by digestion of p8SE2 with *XbaI* followed by religation; pMX8 was a deletion derivative of pSXBA3 constructed by cleavage with *MluI* and *XhoI*, blunt-ending with Klenow enzyme, and religating; p8SNX1 was a deletion derivative of p8SXBA3 that was constructed by digestion with *SnaBI* and *XhoI*, followed by blunt-ending with Klenow enzyme and religating. p8MU4, the disruption plasmid, was constructed by digestion of pMX8 with *HindIII* and insertion of a 1.1-kb fragment containing the *URA3* gene into this site. An *XbaI*-*EcoRI* fragment of this plasmid was used to transform the diploid BJ7339 to replace the chromosomal copy with the disrupted copy. The HA1-tagged PEP8 plasmid was constructed by introduction of a *BamHI* fragment containing the HA1 epitope (Tyers et al. 1992) into the *BamHI* site of *PEP8*.

Figure 9. Diagrammatic representation of the different in-frame deletions, truncations, and domain swap constructs used to analyze *PEP8* function. All of the constructs were derivatives of p8SNX1, a plasmid that contained the *SnaBI*–*XbaI* *PEP8*-bearing fragment in pRS316, and their construction has been described in the Materials and methods. Complementation of the *pep8* mutation was verified by the APE test (Jones 1977). Dotted regions indicate H β >58, and the striped and shaded regions indicate *PEP8*. The percentages refer to the identities in amino acids between the indicated domains.

Plasmid pADB36, which contained the H β >58 cDNA in the pADNS expression vector, was constructed by inserting an *EcoRI* fragment that contained the H β >58 cDNA into the *EcoRI* site of the multiple cloning site of pADNS. Plasmid pSHD3 was constructed by an in-frame *HindIII* deletion of p8SNX1 followed by religation; BRV5 was constructed by a *BamHI*-partial *EcoRV* digestion, followed by blunt-ending with Klenow enzyme and religating. CX2 was constructed by digestion with *ClaI* and *XbaI*, blunt-ending with Klenow, and religating; BCX3 was constructed by cloning a *BclI*–*XbaI* fragment of H β >58 into p8SNX1 that was partially digested with *BclI* and *XbaI*.

The 1.7-kb *SnaBI*–*XbaI* fragment was sequenced on both strands using the dideoxy chain termination method of Sanger et al. (1977).

Metabolic labeling, pulse chase, and immunoprecipitations

Cells were grown overnight in WMP medium and were reinoculated to an OD_{600} of 0.5–1.0 and grown for an additional 3 hr. The cells were harvested, washed, and incubated in 100 mM Tris at pH 9.5 containing 40 mM β -mercaptoethanol for 10 min, centrifuged, and resuspended to an OD_{600} = 5.0–10.0 in WMP medium containing 1.3 M sorbitol. Lyticase (Sigma Chemical Co.) was added to 40 U/ OD_{600} of 1, and spheroplasting was carried out for 2 hr. After spheroplasting, the spheroplasts were washed in WMP–sorbitol medium and resuspended in the same medium (in some cases where secretion needed to be checked, with 1 mg/ml BSA) at an OD_{600} = 5.0. The spheroplasts were pulse labeled for 10–20 min with 300 μ Ci/ml of Trans- 35 S label and chased up to the indicated times with excess methionine and cysteine. The spheroplasts were separated into intracellular and extracellular fractions by centrifugation at 12,000 g for 1 min, and lysis, immunoprecipitations, and SDS-PAGE were carried out as described by Rothblatt and Schekman (1989).

Cell lysis and subcellular fractionations

Cells were grown in YEPD medium to stationary phase, harvested, washed, and resuspended in 100 mM Tris/1 mM EDTA buffer at pH 8.0 that contained the protease inhibitors pepstatin (1 mM), leupeptin (10 mM), and PMSF (2 mM). Crude extracts were made by glass bead lysis in a Braun homogenizer, followed by removal of unbroken cells at 10,000g for 20 min. For further fractionation, the crude extracts were separated into P100 and S100 fractions by centrifugation at 100,000g for 60 min. A portion of the pellet (250–400 μ g) was extracted with 100 mM sodium carbonate at pH 11.0 for 30 min on ice followed by another centrifugation at 100,000g to separate the sodium carbonate soluble and insoluble fractions. SDS-PAGE and immunoblotting were carried out as described in Manolson et al. (1989).

Electron microscopy

Cells were submitted for cryoultramicrotomy essentially as described by Tokuyasu (1986). Cells were fixed in 3% paraformaldehyde and 0.01% glutaraldehyde in PBS for 1 hr at 4°C, washed in PBS, and infiltrated with 10% gelatin in 0.1 M phosphate buffer at 4°C for 10 min. The cells were incubated at 4°C for 10 min, fixed with 3% paraformaldehyde (in PBS) for 5 min, washed in PBS, and infiltrated with 2.3 M sucrose in phosphate buffer for 12 hr at 4°C. The cells were mounted on aluminum specimen holders and frozen in liquid nitrogen. Cryosections were cut on a Reichert–Jung Ultracut E equipped with FC4E cryosectioning system. Thin sections (~100 nm) were picked up on 2000 mesh nickel grids. The grids were inverted and placed on 2% gelatin/phosphate buffer (0.1 M Na phosphate buffer at pH 7.4) at 4°C for 15 min, floated onto a drop of PBS, and transferred to a blocking solution containing 1% BSA in PBS for 30 min. The sections were exposed to undiluted HA-tagged antibody for either 2 hr at room temperature or 12 hr at 4°C, washed in PBS, exposed to goat–anti-mouse antibody attached to 5-nm gold particles (Amersham–Auroprobe, diluted 1:100 in PBS) for 1 hr, and washed in PBS. The antibodies were fixed with 2% glutaraldehyde in PBS for 10 min, washed with PBS, fixed in 1% OsO₄ in 0.1 M cacodylate buffer for 10 min, and washed in water (Keller et al. 1984). The cells were stained with 1% uranyl acetate in water for 10 min, dehydrated to 70% ethanol, and infiltrated with LR white for ~30 min. Excess LR white resin was removed with filter paper (Whatman 41). The LR white resin was polymerized under vacuum at 60°C for 12 hr. The specimen was photographed on a Philips 300 at 60 kV.

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