

# Intracellular Retention of Newly Synthesized Insulin in Yeast Is Caused by Endoproteolytic Processing in the Golgi Complex

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**Abstract.** An insulin-containing fusion protein (ICFP, encoding the yeast prepro- $\alpha$  factor leader peptide fused via a lysine-arginine cleavage site to a single chain insulin) has been expressed in *Saccharomyces cerevisiae* where it is inefficiently secreted. Single gene disruptions have been identified that cause enhanced immunoreactive insulin secretion (*eis*). Five out of six *eis* mutants prove to be vacuolar protein sorting (*vps*) $\delta$ , *vps35*, *vps13*, *vps4*, and *vps36*, which affect Golgi $\leftrightarrow$ endosome trafficking. Indeed, in wild-type yeast insulin is ultimately delivered to the vacuole, whereas *vps* mutants secrete primarily unprocessed ICFP. Disruption of *KEX2*, which blocks intracellular processing to insulin, quantitatively reroutes ICFP to the cell surface, whereas loss of the Vps10p sorting receptor is without effect. Secretion of unprocessed ICFP is not based on a dominant secretion signal in the  $\alpha$ -leader peptide. Although

insulin sorting mediated by Kex2p is saturable, Kex2p functions not as a sorting receptor but as a protease: replacement of Kex2p by truncated secretory Kex2p (which travels from Golgi to cell surface) still causes endoproteolytic processing and intracellular insulin retention. Endoproteolysis promotes a change in insulin's biophysical properties. B5His residues normally participate in multimeric insulin packing; a point mutation at this position permits ICFP processing but causes the majority of processed insulin to be secreted. The data argue that multimeric assembly consequent to endoproteolytic maturation regulates insulin sorting in the secretory pathway.

**Key words:** multimerization • assembly • secretory protein • trafficking • sorting by retention

## Introduction

We are interested in better understanding the principles governing insulin trafficking in the eukaryotic secretory pathway. In this study, we have employed heterologous protein expression in the yeast, *Saccharomyces cerevisiae*. One might imagine that a confounding factor in this system would be the absence of specialized processing enzymes, PC1 and PC2, that higher eukaryotes normally employ for endoproteolytic processing to insulin from its protein precursor (Steiner, 1998). However, this problem is circumvented by the synthesis of a chimeric protein in which the signal peptide and leader sequence of yeast prepro- $\alpha$  factor is fused (via a single dibasic site that is recognized and processed by the Kex2p endoprotease, which acts in the yeast Golgi complex [Fuller et al., 1989]) to a single chain insulin (comprised of insulin B and A chains that are contiguous, linked by a short noncleaved peptide). Upon en-

doproteolytic cleavage to liberate the  $\alpha$ -leader peptide, a crystallizable insulin is produced with a structure essentially isomorphous with that of authentic two-chain insulin (Thim et al., 1986; Hua et al., 1998; Kjeldsen et al., 1998).

Despite this, various insulin and/or insulin-like growth factor-containing constructs have been found to be secreted inefficiently from yeast (Steube et al., 1991; Kjeldsen et al., 1997); these constructs are apparently subject to quality control in the secretory pathway. Indeed, the poor secretion of a variety of heterologous secretory proteins from *S. cerevisiae* is generally attributed to the recognition and retention of these proteins in the endoplasmic reticulum (ER)<sup>1</sup> (Kowalski et al., 1998), typically via binding of ER molecular chaperones (Ellgaard et al., 1999). Accumulated proteins that do not satisfy quality control requirements for export are gen-

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<sup>1</sup>Abbreviations used in this paper: CPY, carboxypeptidase Y; *eis*, enhanced immunoreactive insulin secretion; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ICFP, insulin-containing fusion protein; *vps*, vacuolar protein sorting.

erally degraded by ER-associated degradation (ERAD) (Brodsky and McCracken, 1997; Kopito, 1997).

By contrast, studies of insulin storage in the regulated secretory pathway of mammalian cells generally support a model known as “sorting by retention” (Kuliawat and Arvan, 1994). The model suggests that endoproteolytic processing of the insulin precursor, resulting in polymeric assembly of the processed insulin product (Huang and Arvan, 1995), prevents its rapid (constitutive-like) secretion and facilitates its intracellular retention (Arvan and Castle, 1998). Recently, by introducing the PC1 cDNA into regulated secretory cells that do not normally express this endoprotease, we found that conversion to insulin (by endoproteolysis) improves the efficiency of intracellular hormone storage. Specifically, to the extent that the insulin precursor remains unprocessed it is very rapidly secreted, whereas the fraction effectively processed to insulin is retained intracellularly with remarkable efficiency (Kuliawat et al., 2000). Based on these findings and the existence of detailed structural information (Dodson and Steiner, 1998), insulin seemed like a suitable substrate for further studies of quality control and sorting in *S. cerevisiae* (an organism particularly amenable to such studies) (Gillece et al., 1999; Wang and Chang, 1999). In particular, we have been interested to learn if the reportedly inefficient insulin secretion occurs primarily in relation to ER quality control events or perhaps in relation to processing or other events that take place more distally in the secretory pathway.

Here, we describe an insulin-containing fusion protein (ICFP) that is secreted inefficiently from *S. cerevisiae*. However, instead of ERAD we find that the single chain insulin is sorted in the Golgi complex and is retained intracellularly and eventually degraded within the endosomal/vacuolar system. Remarkably, we find that this sorting requires *KEX2* and is dependent on endoproteolytic processing to form mature insulin; either loss of this cleavage or a point mutation that is known to affect the nature of insulin crystal packing causes dramatically augmented secretion of ICFP or insulin, respectively. These data appear consistent with a model in which insulin’s biophysical properties participate in its sorting in the secretory pathway.

## Materials and Methods

### Strains

The strains employed in this study are described in Table I. Strain L5145 (*MAT $\alpha$* , *kss1*, *ura3-52*, *leu2-3,112*, *his3*, *trp1<sup>Δ63</sup>*, *ade2*, *GAL+*) and L5141 that is MAT $\alpha$  but otherwise isogenic to L5145 (from the lab of G. Fink, Whitehead Institute, Boston, MA). Unless stated otherwise, all strains used in this study are isogenic to L5145. To construct the *pep4 $\Delta$*  strain called PA13 (Table I), a deletion construct marked with *hisG-URA3-hisG* (pAS173 cut with EcoRI and XhoI) was used for transformation (Chang and Fink, 1995). Pop out of the *URA3* marker was obtained by selection on 5-FOA (Boeke et al., 1987). *pep4 $\Delta$*  was confirmed phenotypically by loss of carboxypeptidase Y (CPY) processing to the mature form. A strain (L5145/pMI316) expressing ICFP from a single chromosomal locus was generated by transformation of L5145 with a yeast integration plasmid (pRS304) bearing *GALI-ICFP*; the plasmid was linearized with StuI for integration at *ura3*. A *kex2 $\Delta$*  strain was generated by transformation with a BamHI fragment from pKX::HIS3-(S) (Redding et al., 1991). A *vps1 $\Delta$* ::*LEU2* strain was obtained by transformation with pCRK3A (Rothman et al., 1990). A *vps10* strain was obtained by transformation with XhoI and BamHI fragment of pEM10-103 (Marcusson et al., 1994).

### Plasmids and Molecular Biology

pAK966 is a 2- $\mu$  plasmid marked with *URA3* in which the *GALI* promoter controls expression of ICFP inserted between the EcoRI and XbaI sites of pYES2 (Invitrogen). pMI306 and pMIGLC are *TRP1*-marked centromeric plasmids (pRS314; Sikorski and Hieter, 1989) bearing ICFP driven by the *GALI* or *GPD1* promoter, respectively. pMI306 was created by excising an  $\sim$ 1-kb XbaI-SpeI fragment (containing *GALI-ICFP*) from pAK966 and subcloning it into the SpeI site of pRS314. pMI316 was created by excising an  $\sim$ 1-kb Sall-NotI fragment (containing *GALI-ICFP*) from pMI306 and subcloning it into the respective sites of pRS304 marked with *URA3* (Sikorski and Hieter, 1989). pMIGLC was created by excising an  $\sim$ 0.6-kb BamHI-XbaI fragment from pAK966 and subcloning it into the respective sites of pUC19 and then excising an  $\sim$ 0.6-kb BamHI-Sall fragment from the resulting plasmid and subcloning it into the respective sites of pRSGLC, a *TRP1*-marked centromeric plasmid that employs the *GPD1* promoter (from Dr. J. Warner, Albert Einstein College of Medicine) (Warner, 1991). pASZ-SL-GM was created by excising an  $\sim$ 1-kb XbaI fragment (containing *GALI-ICFP*) from the commercial pSL1180 shuttle vector (Amersham Pharmacia Biotech) and subcloning it into pASZ11, an *ADE2*-marked centromeric plasmid (Stotz and Linder, 1990). pHSP150<sub>frag</sub>-insulin was constructed as follows. Single chain insulin was amplified from pAK966 by PCR using two primers: TATCCATGGCTGCAGCCAA-GAGATTTCGTTAACCAACAC (which introduces a PstI site, underlined) and GTCGACTCTAGAGCCTGCGG (which introduces an XbaI site, underlined). After digestion with PstI and XbaI, the  $\sim$ 200-bp PCR product was subcloned into pSL1180 (Amersham Pharmacia Biotech) and then excised at PstI and ClaI and subcloned again into the respective sites of pKTH4536 (from Dr. M. Makarow, University of Helsinki, Helsinki, Finland) (Simonen et al., 1994). The resulting plasmid contains the *HSP150* promoter and encodes the HSP150 signal and a portion of the leader peptide up to amino acid 63, linked via KR to single chain insulin; the 3' ClaI site immediately precedes the alcohol dehydrogenase terminator. An  $\sim$ 3-kb fragment containing all of these regions was excised by BamHI and finally cloned into the BamHI site of pRS315, a *LEU2*-marked centromeric plasmid (Sikorski and Hieter, 1989). pKX::HIS3-S(y), a plasmid for complete gene replacement of *KEX2* with *HIS3* (*kex2 $\Delta$ 2::HIS3-S*), and pAB23, a *URA3*-marked 2- $\mu$  plasmid in which the *TDH3* promoter drives the overexpression of secretory Kex2p, were from Dr. R. Fuller (University of Michigan, Ann Arbor, MI), a plasmid for replacement of the HindIII-XbaI region of *PEP4* with *TRP1* (*pep4::TRP1*) was from Dr. A. Cooper (University of Missouri at Kansas City, Kansas City, MO); the plasmid was cut with SacI and XhoI before integration/disruption. The H90T mutant in ICFP (mutation of His5 of the insulin B-chain) was made by a 369-bp EcoRI-HindIII PCR-amplified fragment containing the relevant CAC(His) $\rightarrow$ ACC (Thr) mutation, which was used to replace the matching EcoRI-HindIII fragment of ICFP in pYES2.

### Filter Blot Assays

To detect secretion of insulin or its precursor (defined as “immunoreactive insulin secretion”), colonies or patches were replica plated onto nitrocellulose filters placed on top of plates containing 2% galactose as the sole carbon source. After 24–40 h, cells were washed off the filter with PBS plus 0.1% Tween-20. The filter was then incubated with a 1:500 dilution of guinea pig antiinsulin serum (Linco Research) followed by peroxidase-conjugated goat anti-guinea pig secondary (1:2,500; Jackson ImmunoResearch Laboratories) and finally detected by ECL. To detect CPY secretion after overnight growth of the cells, the filter was incubated sequentially with a 1:4,000 dilution of mouse monoclonal anti-CPY (Molecular Probes) and a peroxidase-conjugated goat anti-mouse secondary antibody (1:3,000; Bio-Rad Laboratories).

### Metabolic Labeling and Immunoprecipitation

Cells were grown to exponential phase in minimal medium supplemented with required amino acids but without methionine and cysteine using either 2% galactose or 2% glucose as appropriate and then sedimented at 3,000 g for 5 min and resuspended at 4 OD<sub>600</sub>/ml in the same medium plus 1 mg/ml BSA. Insulin contains six cysteine residues and the  $\alpha$ -leader peptide contains one methionine. For pulse labeling, [<sup>35</sup>S]cysteine (NEN Life Science Products) was added at 50  $\mu$ Ci per OD<sub>600</sub> for the times indicated. For chase, the cells were sedimented and resuspended in synthetic complete (SC) medium plus 10 mM methionine and cysteine. At various chase times, 1-ml aliquots were transferred to ice, and 10 mM sodium azide and a protease inhibitor cocktail (0.25  $\mu$ g/ml aprotinin, 0.1 mM leupeptin, 10

Table I. Strains Used in This Study

L5145*	<i>MATα kss1 ura3-52 leu2-3, 112 his3 trp1<sup>Δ63</sup> ade2 GAL+</i>
L5141*	<i>MATα kss1 ura3-52 leu2-3, 112 his3 trp1<sup>Δ63</sup> ade2 GAL+</i>
PA13 <sup>‡</sup>	<i>MATα kss1 ura3-52 leu2-3, 112 his3 trp1<sup>Δ63</sup> ade2 GAL+ pep4Δ::URA3</i>
DI3G <sup>‡</sup>	<i>MATα kss1 ura3-52 leu2-3, 112 his3 trp1<sup>Δ63</sup> ade2 GAL+ pep4Δ::URA3 vps35::LEU2</i>
YB108 <sup>‡</sup>	<i>MATα kss1 ura3-52 leu2-3, 112 his3 trp1<sup>Δ63</sup> ade2 GAL+ pep4Δ::TRP1 vps35::LEU2</i>
DI4E <sup>‡</sup>	<i>MATα kss1 ura3-52 leu2-3, 112 his3 trp1<sup>Δ63</sup> ade2 GAL+ pep4Δ::URA3 vps13::LEU2</i>
DIV3H <sup>‡</sup>	<i>MATα kss1 ura3-52 leu2-3, 112 his3 trp1<sup>Δ63</sup> ade2 GAL+ pep4Δ::URA3 vps4::LEU2</i>
DVI4A <sup>‡</sup>	<i>MATα kss1 ura3-52 leu2-3, 112 his3 trp1<sup>Δ63</sup> ade2 GAL+ pep4Δ::URA3 vps8::LEU2</i>
DVIIIIC <sup>‡</sup>	<i>MATα kss1 ura3-52 leu2-3, 112 his3 trp1<sup>Δ63</sup> ade2 GAL+ pep4Δ::URA3 vps36::LEU2</i>
PA11D <sup>‡</sup>	<i>MATα kss1 ura3-52 leu2-3, 112 his3 trp1<sup>Δ63</sup> ade2 GAL+ pep4Δ::URA3 vps10::LEU2</i>
YB96 <sup>‡</sup>	<i>MATα kss1 ura3-52 leu2-3, 112 his3 trp1<sup>Δ63</sup> ade2 GAL+ pep4Δ::URA3 kex2::HIS3</i>
TBY102 <sup>§</sup>	<i>MATα ura3-52 leu2-3, 112 his3-D200 trp1-Δ901 lys2-801 suc2-Δ9 sec18-1 GAL+</i>

\*As described in Materials and Methods.

<sup>‡</sup>This study.

<sup>§</sup>Brigance et al., 2000.

μM pepstatin, 5 mM EDTA, 10 μM E64, 1 mM DFP, 0.02% BSA) were added immediately. Cells were then sedimented at 3,000 g for 5 min, and the supernate-containing secreted proteins were collected for further analysis. The cells were resuspended in 100 μl breaking buffer containing 6 M urea, 1% SDS, 1 mM EDTA, 50 mM Tris, pH 7.5. In Fig. 10 B, urea and SDS were replaced by 1% NP-40 in the breaking buffer. The cells were lysed by vortexing with glass beads and then boiling for 4 min (although in Fig. 10 B the boiling step was omitted). Samples were diluted 10-fold to a final concentration of 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, debris was pelleted in a microcentrifuge, and immunoprecipitations were normalized to acid precipitable counts per minute. Samples were precleared with 20 μl Zysorbin for 30 min (Zymed Laboratories). Media and cell lysates were then immunoprecipitated with 10 μl guinea pig antiinsulin using 20 μl Zysorbin as a secondary immunoadsorbent. Immunoprecipitates were routinely digested with PNGase F (New England Biolabs, Inc.) and analyzed by tricine-urea-SDS-PAGE (Schagger and von Jagow, 1987), fluorography or PhosphorImaging, and quantitation using ImageQuant software (Molecular Dynamics).

### Genetic Screen

L5145/pMI316 cells bearing integrated *GALI-ICFP* were transformed with a mutagenized yeast genomic library with random insertions of *lacZ/LEU2* (Burns et al., 1994). *Leu*<sup>+</sup> transformants were replica plated to nitrocellulose filters atop plates containing 2% galactose as the sole carbon source. Filter blot assays for immunoreactive insulin secretion were performed as described above. Out of 90,000 transformants screened, 31 colonies were picked that displayed enhanced immunoreactive insulin secretion (*eis*) over the untransformed parental strain. After patching and rescreening, 24 of these were reconfirmed. After backcross to the parental ICFP-containing strain followed by sporulation and tetrad analysis, nine of these colonies showed a 2:2 distribution of *eis* linked to the *LEU2* insertion, indicating single gene disruption. These clones were designated sequentially *eis1* to *eis9*. After transformation with pRSQ, the disrupted genes were recovered as plasmid DNA (Burns et al., 1994). The identity of disrupted *eis* genes was determined by sequencing genomic DNA adjacent to the insertion.

### Indirect Immunofluorescence

Indirect immunofluorescence was performed as described (Preuss et al., 1991) with the following modifications. In brief, cells growing exponentially in 2% galactose-containing medium were fixed with 4% formaldehyde in 0.1 M potassium phosphate, pH 6.5. Cells were spheroplasted with oxalyticase (Enzogenetics) and permeabilized briefly with methanol and acetone. Cells were then incubated overnight with antiinsulin at 1:500 followed by a Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Cells were viewed with an Olympus IX70 microscope; images were collected digitally and analyzed using Adobe Photoshop<sup>®</sup> software.

### Sucrose Gradient and DTT Accessibility Assays

For in vitro analyses of intracellular ICFP and insulin, cells expressing ICFP from the *GPD1* promoter were employed. Cells were labeled as de-

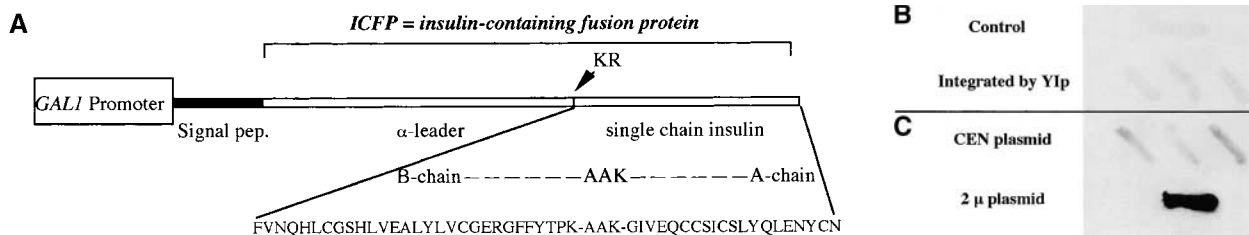
scribed above. For sucrose gradient analysis, cells were lysed with glass beads in a buffer containing 50 mM NaCl, 2 mM ZnSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, and 25 mM MES, pH 5.0. The cell lysate (2 ml) was then loaded onto a discontinuous sucrose gradient comprised of 2 ml each of 15 and 30% sucrose in the same buffer. The velocity gradients were centrifuged at 100,000 *g*<sub>av</sub> for 90 min. Gradient collection was simplified to two fractions comprising the upper half (including all of the load and the upper half of the 15% sucrose layer) and the lower half (including the lower half of the 15% sucrose layer and all of the 30% sucrose layer). From equal aliquots of each half, sucrose was diluted and the samples were immunoprecipitated with an antiinsulin antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

The disulfide accessibility assay was modified from Huang and Arvan (1995). In brief, cells were lysed and immunoprecipitated in a buffer containing 170 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM Tris, pH 7.5. The insulin immunoprecipitates were washed once in a buffer containing 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 10 mM Tris, pH 7.5. After a brief water wash, parallel samples were either employed as untreated controls or treated first with 20 mM DTT in water at 37°C for 30 min followed by incubation with 29 mM iodoacetamide. Subsequently, all samples were incubated with 1,000 U of endoglycosidase H at 37°C for 1 h. Samples to be used as untreated controls were boiled in SDS-gel sample buffer in the presence or absence of 20 mM DTT and finally incubated at 37°C for 30 min in the presence of 41 mM iodoacetamide before gel loading. The experimental sample was not further treated with DTT but brought to 41 mM iodoacetamide and incubated at 37°C for 30 min before boiling in SDS-gel sample buffer.

## Results

### Insulin Undergoes Retention and Degradation in the Yeast Secretory Pathway

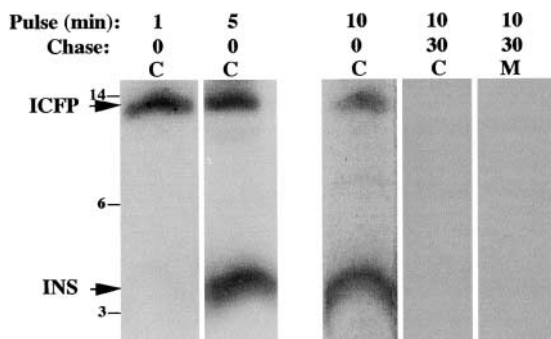
The ICFP cDNA was constructed as a chimera encoding the signal and leader peptides of prepro-α factor fused via a single dibasic (KR) cleavage site to a polypeptide in which the B chain (first 29 residues) and A chain of insulin are made contiguous by an alanine-alanine-lysine linker (Fig. 1 A). (For simplicity in this paper, we refer to this single chain insulin simply as insulin or mature insulin in reference to its endoproteolytic liberation from the ICFP precursor.) When ICFP driven by a *GALI* promoter (Fig. 1 A) was expressed as a single copy integrated in the genome (YIp), secretion of immunoreactive insulin (defined as secretion of protein blotable with antiinsulin antibody; described in Materials and Methods) was at the lower limits of detection when yeast were grown on galactose as the sole carbon source (Fig. 1 B). To examine further the expression of ICFP, cells were pulse labeled with [<sup>35</sup>S]cysteine for 1 min followed by immediate lysis, insulin im-



**Figure 1.** (A) Structure and expression of the ICFP. (B) Yeast expressing ICFP either in single copy (YIp), low copy (CEN plasmid), or high copy (2  $\mu$  plasmid) were grown as patches and replica plated onto nitrocellulose filters atop galactose-containing plates. After 40 h, the filters were washed to remove cells and immunoblotted with antiinsulin antibody and a peroxidase-conjugated secondary antibody.

munoprecipitation, deglycosylation of the  $\alpha$ -leader with PNGase F, and analysis by tricine-urea-SDS-PAGE. (Note that all cysteine residues are conserved between ICFP and mature insulin.) In this case, an intracellular ICFP band migrating at  $M_r \sim 14$  kD was observed (Fig. 2 A, left lane). In a parallel sample that was labeled for 5 min (Fig. 2 A, right lane), a second smaller band was produced that comigrated with an authentic insulin standard. These data suggested that ICFP may undergo relatively rapid proteolytic processing to mature insulin (described further below).

To understand the poor secretion of immunoreactive insulin, cells expressing ICFP on a centromeric plasmid were pulse labeled for 10 min and then either lysed or chased for 30 min. Although both ICFP and single chain insulin bands were detected intracellularly immediately after pulse labeling (Fig. 2 B, left lane), secretion of radiolabeled ICFP or processed insulin at 30 min was virtually undetectable (Fig. 2 B, right lane). Moreover, after a 30-min chase essentially no immunoprecipitable forms remained



**Figure 2.** In wild-type yeast, ICFP is processed to single chain insulin but is not secreted efficiently. (A) Yeast expressing ICFP driven by a *GALI* promoter on a high copy plasmid were pulse labeled with [ $^{35}$ S]cysteine for the times indicated without further chase. (Note that in this strain the *GALI* promoter is not particularly strong.) Cells were lysed and insulin immunoprecipitated and digested with PNGaseF before nonreducing SDS-PAGE. Note that the initially synthesized precursor is  $\sim 14$  kD, which is processed to an  $\sim 5$ -kD band comigrating with authentic insulin (INS). (B) Yeast expressing ICFP driven by a *GALI* promoter on a low copy (CEN) plasmid were pulse labeled for 10 min with [ $^{35}$ S]cysteine and either lysed or chased for a further 30 min in complete medium. Note that after chase, insulin-immunoprecipitable bands are undetectable in either the medium or cell lysate. C, cells; M, medium.

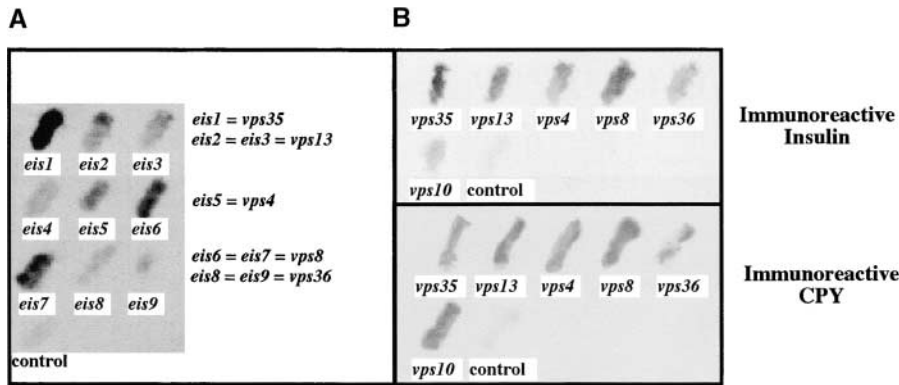
intracellularly (Fig. 2 B, middle lane), suggesting relatively rapid intracellular degradation.

### Gene Disruptions That Enhance Immunoreactive Insulin Secretion

The foregoing results suggested some form of quality control of insulin secretion. To examine this further, a genetic approach was undertaken. Using yeast expressing ICFP from a single copy integrated in the genome (see Fig. 1 B) and a mutagenized genomic library containing random insertions of *lacZ* and *LEU2* (Burns et al., 1994), 90,000 transformants were screened by the Western blot assay to yield nine *eis* mutants displaying enhanced immunoreactive insulin secretion (as described in Materials and Methods). The nine *eis* mutants corresponded to six distinct gene disruptions, five of which were identified as *VPS35*, *VPS13*, *VPS4*, *VPS8*, and *VPS36*. These genes have been shown previously to regulate trafficking in the Golgi $\leftrightarrow$ endosome $\leftrightarrow$ vacuole pathway (Stack and Emr, 1993; Bryant and Stevens, 1998). *vps35* and *vps8* appeared to produce the greatest enhancement of immunoreactive insulin secretion, *vps4* and *vps13* produced an intermediate enhancement, and *vps36* produced only a slight increase (Fig. 3 A).

### Most ICFP Advances through the Golgi Complex into the Endosomal/Vacuolar System

Even though enhanced immunoreactive insulin secretion was observed in several *vps* mutants acting in the distal secretory pathway, ER to Golgi transport is known to be a rate-limiting step in the trafficking of chimeras containing the prepro- $\alpha$  leader (Elliott et al., 1989). We therefore employed a standard assay (Finger et al., 1993; Loayza et al., 1998) to examine whether ICFP is subject to ERAD. The ICFP was expressed in a *sec18* strain in which traffic to the Golgi complex is blocked at the nonpermissive temperature. After shifting to 37°C, cells were pulse labeled, lysed at varying times of chase  $\leq 1.5$  h, and analyzed by immunoprecipitation with antiinsulin, PNGase F digestion, and tricine-urea-SDS-PAGE. Strikingly, no endoproteolytic processing of ICFP was observed in the *sec18* background (Fig. 4, A and B) in contrast to wild-type yeast (see Fig. 2 A). Furthermore, unlike what has been observed for ERAD substrates (Plemper and Wolf, 1999) the ICFP molecule was completely stable throughout the 90-min time course. Thus, no evidence was obtained for ERAD of the insulin-containing chimera.



**Figure 3.** Identification and characterization of *eis* mutants that yield enhanced insulin secretion. (A) Filter blot assay for immunoreactive insulin secretion from the nine colonies obtained by genetic screening, which represent disruptions of six independent genes. Five of these proved to be genes in the *vps* pathway as indicated. (B) Five *eis/vps* mutants and a *vps10*-null mutant were replica plated and tested

by filter blot assay for immunoreactive insulin secretion (top panel) and CPY secretion (bottom panel). Note that like the *eis/vps* mutants obtained by genetic screen *vps10* dramatically increases CPY secretion, but the effect on ICFP secretion is minimal.

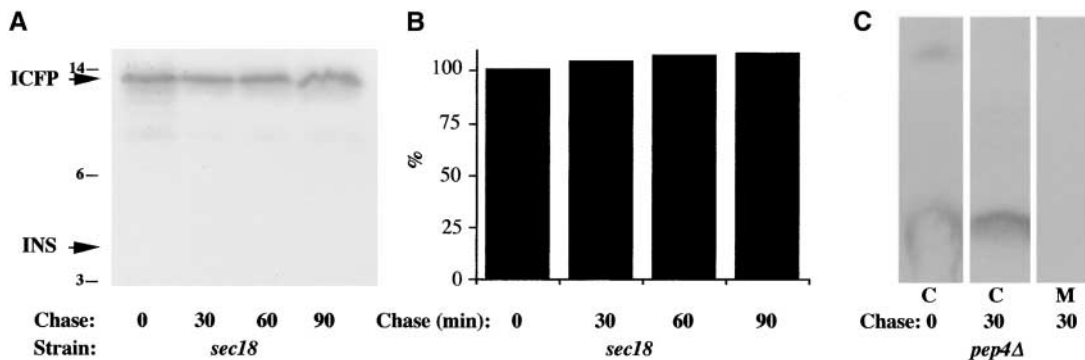
We next tested directly whether immunoreactive insulin was degraded in the endosomal/vacuolar system by examining ICFP expression in a *pep4Δ* strain (deficient for vacuolar protease activity) (Ammerer et al., 1986). In this case, fully processed intracellular insulin was stabilized dramatically, although no increase in insulin secretion was observed (Fig. 4 C). Indeed, insulin clearly accumulated in the vacuole (identified under Nomarski optics) as judged by indirect immunofluorescence in *pep4Δ* cells (Fig. 5, top panels). Furthermore, in an *eis5/vps4 pep4* double mutant (known to accumulate an enlarged prevacuolar compartment [Bryant and Stevens, 1998]) staining was concentrated in a bright spot next to the vacuole (Fig. 5, bottom panels), indicating accumulation in the prevacuolar compartment. These data prove that immunoreactive insulin is retained intracellularly by trafficking from the Golgi complex through the endosomal system to the vacuole.

### Is *Vps10p* the Insulin Sorting Receptor?

Several groups have described previously the yeast Golgi complex as a regulatory site from which foreign, mutant, or even wild-type versions of certain yeast proteins may be recognized for delivery to the endosomal/vacuolar system

(Hong et al., 1996; Luo and Chang, 1997; Roberg et al., 1997; Li et al., 1999; Liu and Culotta, 1999). In at least some of these cases, the recognition and/or targeting machinery appears to be saturable upon substrate overexpression (Hong et al., 1996). We tested whether insulin delivery to the endosomal/vacuolar system was saturable by expressing ICFP at low copy on a centromeric plasmid (CEN) or at high copy on a 2- $\mu$  plasmid. Indeed, higher immunoreactive insulin secretion was observed in a dosage-dependent manner (Fig. 1 C). Even greater secretion was observed when ICFP expression was driven by the strong constitutive *GPD1* promoter (see below).

In addition to its receptor function in sorting of vacuolar proteases (Marcusson et al., 1994; Cooper and Stevens, 1996), *Vps10p* has been proposed to function as a Golgi-based sorting receptor for targeting misfolded secretory proteins to the endosomal system for vacuolar degradation (Hong et al., 1996; Durr et al., 1998; Holkeri and Makarow, 1998; Jorgensen et al., 1999). To test whether *Vps10p* plays a role in preventing immunoreactive insulin secretion, Western blot assays were used to compare the secretion of immunoreactive insulin and CPY in a *vps10*-null mutant versus the *eis/vps* mutants. As shown in the bottom panel of Fig. 3 B, all of the *vps* mutants exhibited



**Figure 4.** The ICFP is not subject to ERAD but is subject to degradation by vacuolar proteases. (A) The ICFP was expressed from the *GPD1* promoter in a *sec18-1* mutant strain where the ICFP cannot reach the Golgi complex. Cells were preincubated at the restrictive temperature (37°C) for 10 min and labeled for 5 min. Cells were chased for the times indicated, lysed, and immunoprecipitated with anti-insulin and digested with PNGase F before SDS-PAGE. (B) Quantification by densitometry of the results shown in A. (C) The ICFP was expressed in a *pep4*-null strain. The cells were labeled and chased as in the legend to Fig. 2 B before insulin immunoprecipitation and digestion with PNGaseF followed by SDS-PAGE and fluorography. Note that in a *sec18* strain intracellular ICFP persists, whereas in a *pep4* strain intracellular insulin accumulates.

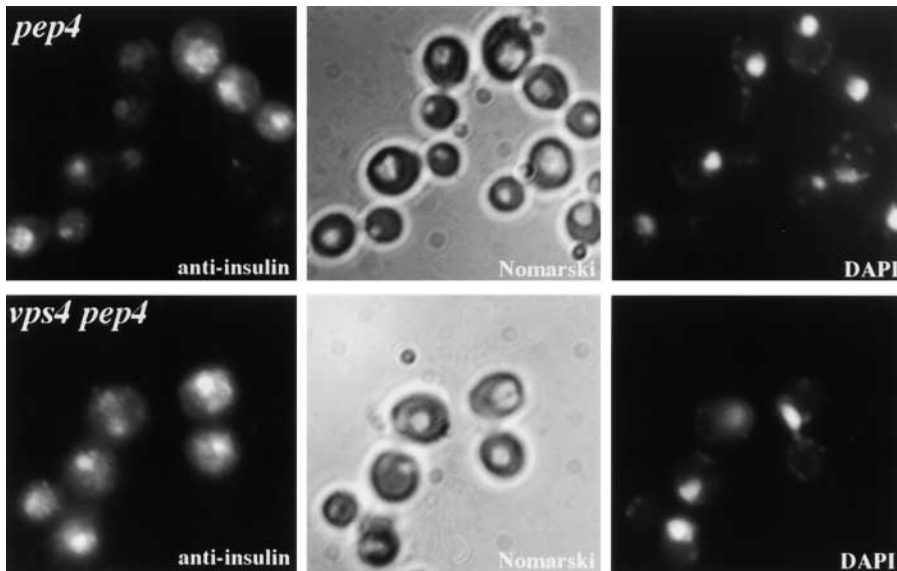


Figure 5. Indirect immunofluorescence localization of insulin in a *pep4* strain and a *vps4 pep4* double mutant. Note that insulin accumulates in the vacuole in a *pep4* mutant and in the prevacuolar compartment in the class E *vps* mutant. Cells were fixed, permeabilized, and insulin labeled with antiinsulin and a Cy3-conjugated secondary antibody.

markedly enhanced immunoreactive CPY secretion, and *vps10* arguably yielded the strongest CPY secretion phenotype. However, enhanced immunoreactive insulin secretion for *vps10* was less than that seen with any other *vps* mutants tested (Fig. 3 B, top panel).

A quantitative analysis of immunoreactive insulin secretion from *vps* mutants was then performed by following [<sup>35</sup>S]cysteine-labeled protein. First, the effect of each of the five *eis/vps* mutations and *vps10* was examined in strains where ICFP expression was driven by a *GALI* promoter on a centromeric plasmid. To improve intracellular recovery, a *pep4* mutation was introduced into each strain. As shown in Fig. 6, each *vps* mutant tested showed an increase in secretion of protein immunoprecipitable with antiinsulin. Quantitation of the data showed that whereas the control strain secreted only ~8% of the total, the *vps36*, *vps4*, *vps13*, *vps8*, and *vps35* strains secreted ~19, ~26, ~41, ~55, and ~76%, respectively. The *vps10* strain showed the lowest level of enhancement over the control, secreting labeled insulin at a level approaching that of *vps36* (~19%).

To quantify the saturability of endosome/vacuole delivery, identical pulse-chase experiments were performed in which ICFP expression was driven by the *GPD1* promoter

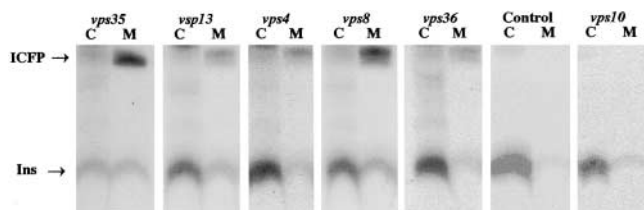


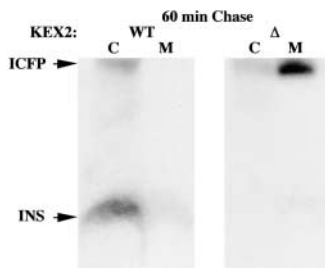
Figure 6. Enhanced immunoreactive insulin secretion in various *eis/vps* mutants is comprised largely of unprocessed ICFP. All strains are *pep4* mutants in order to stabilize the intracellular protein. Cells were pulse labeled for 30 min and chased for 60 min. The cells and media underwent insulin immunoprecipitation and digestion with PNGaseF before analysis by SDS-PAGE and fluorography. Note that secretion of unprocessed ICFP was not detected in the control strain or in a *vps10* mutant.

(which increases ICFP expression 5–10-fold in this strain background; data shown below). This maneuver resulted in an increase in the fractional secretion of protein immunoprecipitable with antiinsulin (~36%). In addition, an assortment of *vps* mutants showed a further increase in fractional secretion (*vps4*, ~47%; *vps1*, ~53%; *vps8*, ~57%; *vps13*, ~64%; *vps35*, ~79%). The one exception was *vps10*, which showed no further increase in fractional secretion (measured at ~27%). Together, these data are inconsistent with a central role of Vps10p as a sorting receptor for ICFP targeting from the Golgi complex to the endosomal system.

### *Kex2p* Is a Saturable Component Conferring Intracellular Retention of Insulin

The observations of proteolytic maturation of ICFP to a form comigrating with authentic insulin (Fig. 2 A and Fig. 4 C) and blockade of this processing in a *sec18* mutant (Fig. 4, A and B) are consistent with the notion that cleavage of ICFP in the Golgi complex is catalyzed by the yeast endoprotease, Kex2p (Fuller et al., 1989). Additionally, a striking finding in most of the *vps* mutants tested was that increased fractional secretion of protein immunoprecipitable with antiinsulin was comprised primarily of unprocessed ICFP. Although these findings are an expected side effect of perturbed Kex2p cycling between Golgi and endosome in *vps* mutants (Bryant and Stevens, 1998), we noted that increased ICFP secretion as a consequence of simple overexpression (in the absence of *VPS* mutations) was also enriched in unprocessed precursor (described further below). These findings raised the possibility that Kex2p itself might be a limiting factor required for insulin sorting.

Therefore, we reexamined ICFP expression (driven from a *GALI* promoter on a centromeric plasmid) in a *kex2 pep4* strain. As demonstrated before, in the presence of wild-type levels of Kex2p, virtually all ICFP was processed to single chain insulin, and only a small fraction (<10%) was secreted (Fig. 7, left). However, in the absence of Kex2p not only was there no ICFP cleavage, but the protein was near quantitatively rerouted to the medium (Fig. 7, right).



**Figure 7.** Deletion of *KEX2* causes near quantitative rerouting of ICFP to the medium. The strains are *pep4Δ* mutants in order to stabilize the intracellular protein. Cells were pulse labeled for 30 min and chased for 60 min. The lysed cells

and media underwent insulin immunoprecipitation and digestion with PNGaseF before analysis by SDS-PAGE and fluorography.

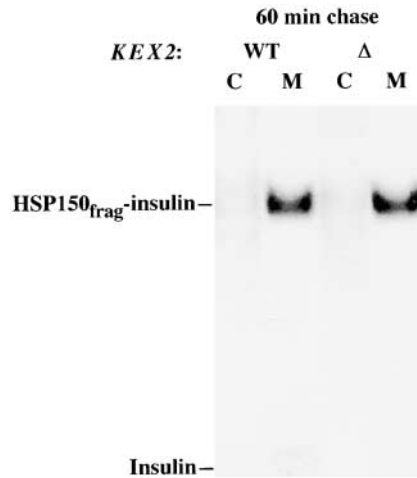
### Secretion of Unprocessed ICFP Does Not Involve a Dominant Secretion Signal in the $\alpha$ -Leader Peptide

All evidence to date indicates that luminal protein targeting from the Golgi complex to the endosomal/vacuolar system requires specific signal recognition, and loss of this sorting results in protein secretion (Marcusson et al., 1994). Nevertheless, we wished to formally exclude the possibility that ICFP secretion might be mediated by a dominant secretion signal in the  $\alpha$ -leader peptide and that loss of this signal upon endoproteolytic cleavage might cause single chain insulin to be delivered to the vacuole. Because published evidence indicates that a fragment of the leader peptide from the secretory protein Hsp150 does not contain a dominant secretion signal (Holkeri and Makarow, 1998), we replaced the  $\alpha$ -leader with this fragment fused to single chain insulin (as described in Materials and Methods). This new insulin-containing chimera was quantitatively secreted (Fig. 8), indicating that secretion of the unprocessed ICFP does not require specific sorting information contained within the  $\alpha$ -leader peptide. Thus, intracellular retention of mature insulin cannot be explained by endoproteolytic removal of a dominant secretion signal.

### Is *Kex2p* the Insulin Sorting Receptor?

Our screen for *EIS* genes did not yield any candidate sorting receptors for insulin because none encoded a protein bearing a luminal domain. However, like the Vps10p sorting receptor *Kex2p* follows a generally similar itinerary in which it is delivered to the endosomal system before recycling back to the Golgi complex (Brickner and Fuller, 1997). Moreover, it has been hypothesized recently in mammalian cells that proteases in the secretory pathway might have the potential to perform double duty as non-catalytic sorting receptors (Cool et al., 1997; Cool and Loh, 1998; Normant and Loh, 1998). Therefore, it was important to test a possible role for *Kex2p* as a sorting receptor for insulin.

Trafficking of *Kex2p* from the Golgi complex into the endosomal system requires specific information included in the COOH-terminal region of the protein: a COOH-terminal deletion removing the serine/threonine-rich domain, the transmembrane domain, and the cytosolic tail domain results in a truncated *Kex2p*, which is catalytically active but behaves as a secretory protein, traveling quantitatively from the Golgi to the cell surface (Brenner and Fuller, 1992). Consequently, this secretory *Kex2p* cannot function as a sorting receptor to convey ligands from the

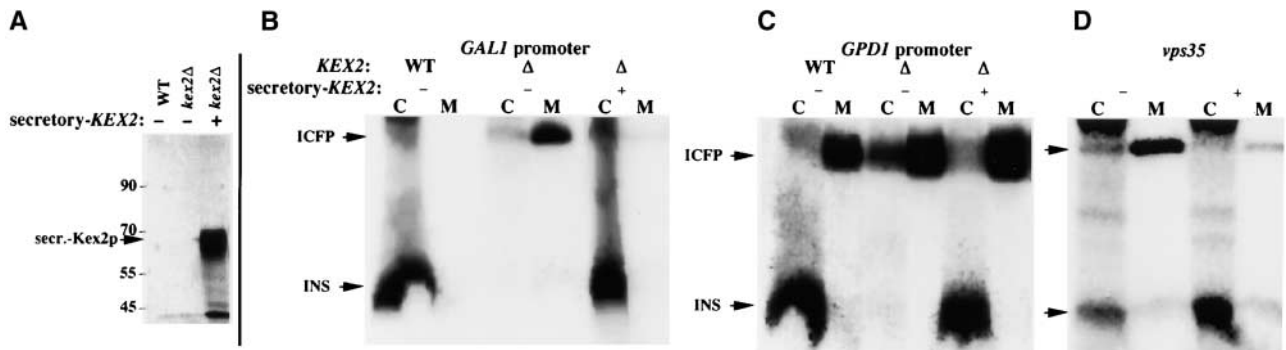


**Figure 8.** Secretion of immunoreactive insulin is not mediated by a dominant sorting signal in the  $\alpha$ -leader. In this case, the  $\alpha$ -leader peptide in ICFP has been replaced by a fragment of the HSP150 leader (as described in Materials and Methods). The strains are *pep4Δ* mutants in order to stabilize the intracellular protein. Cells were pulse labeled for 30 min and chased for 60 min. The lysed cells and media underwent insulin immunoprecipitation before analysis by SDS-PAGE and fluorography.

Golgi to the endosomal system. Therefore, we tested ICFP secretion in a *kex2 pep4* strain expressing secretory *Kex2p*. Confirming previous work, secretory *Kex2p* was massively secreted (Fig. 9 A). Loss of *Kex2p* expression resulted in nearly quantitative ICFP secretion when its expression was driven from a *GAL1* promoter (Fig. 9 B, middle two lanes). Importantly, however, expression of secretory *Kex2p* restored both endoproteolytic processing and intracellular retention of insulin (Fig. 9 B, right two lanes). When the *GPD1* promoter was used to further overexpress ICFP in excess of the ability of secretory *Kex2p* to cleave it, the fractional secretion of protein immunoprecipitable with antiinsulin was markedly increased, and all of the increase was comprised of unprocessed precursor (Fig. 9, C compared with B). Moreover, endoproteolytic processing of ICFP (expressed from a *GAL1* promoter) by secretory *Kex2p* also restored intracellular insulin retention in *vps35* cells (Fig. 9 D). These findings confirm that (a) *Kex2p* is a specific saturable component responsible for intracellular insulin retention, and (b) *Kex2p* acts catalytically as an endoprotease rather than as a luminal sorting receptor for insulin trafficking.

### Endoproteolytic Processing of ICFP Causes a Change in the Biophysical Properties of the Insulin Moiety

In mammalian  $\beta$  cells, endoproteolytic processing to generate insulin results in polymerization and intracellular retention (Kuliawat and Arvan, 1994; Kuliawat et al., 2000). To examine the biophysical state of insulin retained in yeast cells, we modified two assays developed originally for mammalian insulin. Multimeric insulin recovered from pancreatic  $\beta$  cells has been detected by rapid sedimentation upon sucrose velocity gradient centrifugation (Michael et al., 1987). Although this sedimentation *in vitro* is not particularly quantitative, it nevertheless represents a feature that is not shared by the insulin precursor (Kulia-



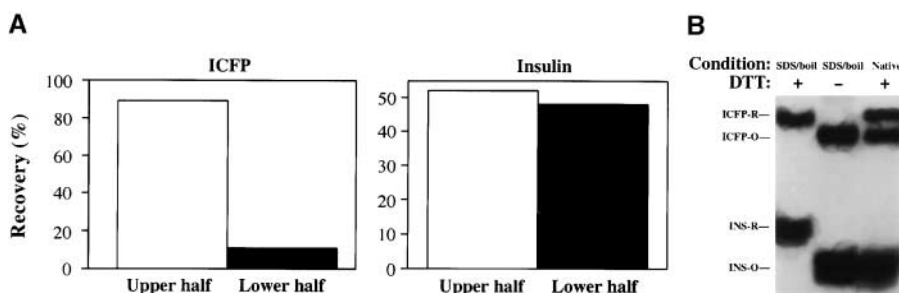
**Figure 9.** Replacement of Kex2p by secretory Kex2p is sufficient to cause intracellular insulin retention. The strains are *pep4Δ* mutants in order to stabilize the intracellular protein. Cells were pulse labeled for 30 min and chased for 60 min. (A) Chase media bathing the indicated strains were collected and immunoprecipitated with an antibody against Kex2p. (B) Intracellularly retained and secreted forms of immunoreactive insulin upon ICFP expression with a *GAL1* promoter on a centromeric plasmid. Note that when truncated secretory Kex2p is expressed in the *kex2Δ* mutant it alters protein targeting to the extent that insulin is made. (C) Intracellularly retained and secreted forms of immunoreactive insulin upon ICFP expression with a *GPD1* promoter on a centromeric plasmid. Note that at the higher levels of ICFP expression ICFP endoproteolytic processing is incomplete and the unprocessed portion is secreted. (D) Intracellularly retained and secreted forms of immunoreactive insulin upon ICFP expression with a *GAL1* promoter in *vps35* cells. In these cells as well, secretory Kex2p expression alters the protein targeting to the extent that insulin is made.

wat and Arvan, 1994). A second approach detects multimeric assembly of mammalian insulin as measured by a loss of accessibility of its disulfide bonds to reduction by DTT under nondenaturing conditions (Huang and Arvan, 1995).

Single chain insulin was examined from *pep4* mutant cells expressing ICFP under control of the strong *GPD1* promoter. Cells were labeled continuously with [<sup>35</sup>S]cysteine for 45 or 60 min; under these conditions, significant amounts of labeled insulin and ICFP precursor coexist. First, we found, using a simplified sucrose velocity gradient (as described in Materials and Methods), that upon processing to insulin about half of the mature molecules exhibited very rapid sedimentation, which was not observed for the ICFP itself (Fig. 10 A). Using a second assay, in the experiment shown in Fig. 10 B insulin immunoprecipitates were resuspended and divided into various aliquots. As a control, one aliquot was denatured in conventional boiling SDS-containing gel sample buffer in the presence of 20 mM DTT and then subsequently alkylated with iodoacetamide. Denaturation allowed DTT to reduce all disulfide bonds in both the unprocessed precursor and mature insulin, causing a marked upward mobility shift for both bands (Fig. 10 B, lane 1). For comparison, a second aliquot was similarly denatured but without reduction, being alkylated only (Fig. 10 B, lane 2). A third aliquot was

tested for disulfide accessibility to 20 mM DTT under non-denaturing conditions and then finally alkylated before SDS-PAGE. As shown, mature insulin was extremely resistant to reduction under nondenaturing conditions (equals 5% reduced), reflecting an inaccessibility of disulfide bonds in its tertiary/quaternary structure, whereas approximately half of the insulin precursor was reduced upon DTT exposure in the identical sample (Fig. 10 B, lane 3). Thus, by two independent assays endoproteolytic processing was associated with a significant change in the biophysical properties of insulin.

The foregoing data raised the question of whether sorting consequent to insulin precursor processing reflects any established features of insulin assembly. It is known that some key residues affecting insulin multimeric interactions lie along the proximal (that is NH<sub>2</sub>-terminal) portion of the insulin B chain. The B1–B8 sequence is notable for exhibiting great conformational flexibility, interconverting in a dynamic equilibrium between extended and more compact ( $\alpha$ -helical) configurations (Renscheidt et al., 1984; Wollmer et al., 1989; Kadima et al., 1992); in particular, the B5 histidine residues in the insulin hexamer play not only an important local role in conformation of the proximal B chain (Bentley et al., 1976; Kim and Shields, 1992) but also make structural contact in neighboring hexamers



**Figure 10.** Structural maturation of insulin upon Kex2p-mediated endoproteolysis. (A) Sucrose velocity gradient centrifugation. (B) Disulfide accessibility of ICFP and mature insulin under denaturing and nondenaturing conditions. Note that in these experiments all samples were exposed to PNGase F (A) or endoglycosidase H (B) at the end of the analysis. In the experiment

shown in B, the enzyme digestion was performed in the complete absence of a reducing agent. The positions of reduced ICFP and insulin standards (ICFP-R and INS-R) and oxidized standards (ICFP-O and INS-O) are shown to the left of the panel.



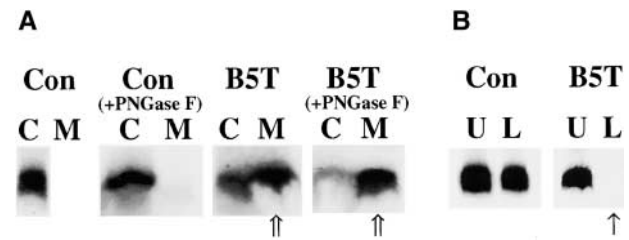
(Baker et al., 1988). Recently, it has been suggested that mutation of B5H can cause structural perturbations in adjacent hexamers, which may affect insulin packing in vivo (Tang et al., 1999). Interestingly, examination of an H90T-ICFP mutant in which the insulin B5H residue is replaced by B5T showed that the majority of processed mutant insulin was secreted rather than being retained intracellularly (Fig. 11 A, open arrows).

The B5T mutation creates a potential NH<sub>2</sub>-linked glycosylation site within insulin in addition to the NH<sub>2</sub>-linked glycosylation sites that are actually used within the  $\alpha$ -leader peptide of ICFP. Notably, however, our studies showed clearly that the B5T insulin is not glycosylated as demonstrated by an identical SDS-PAGE mobility to that of nonmutagenized insulin and a lack of effect of PNGase F digestion (Fig. 11 A). Moreover, whereas the nonmutagenized insulin exhibits multimeric assembly as judged by sucrose velocity gradient centrifugation in vitro (Fig. 10 A and Fig. 11 B), the B5T mutant insulin was unable to sediment from the upper to the lower half of a sucrose velocity gradient (Fig. 11 B), supporting the notion that perturbation of insulin targeting in the secretory pathway is related to structural effects of the threonine mutation itself. Together, these data suggest that intracellular retention of insulin in yeast cells is dependent on insulin structural maturation, which is remarkably similar to recent conclusions about intracellular insulin retention in the regulated secretory pathway of mammalian cells (Kuliawat and Arvan, 1994; Huang and Arvan, 1995; Kuliawat et al., 2000).

## Discussion

It has been reported that improved secretion efficiency of some insulin-containing chimeras can be achieved by replacing an  $\alpha$ -leader peptide with synthetic leader sequences that alter ER residence time, suggesting that ER quality control is an important feature of insulin secretion in yeast (Kjeldsen et al., 1997). However, we have discovered several additional features of the system not typical of ER quality control. First, using the ICFP construct described in Fig. 1 A, simple overexpression caused significantly enhanced secretion of immunoreactive insulin (Fig. 1 C), suggesting that one or more saturable components in the yeast secretory pathway limits insulin secretion. Second, newly-synthesized ICFP, containing a single dibasic cleavage site, was rapidly processed to a band comigrating with authentic insulin (Fig. 2 A) in a manner dependent on arrival in the Golgi complex (Fig. 4 A) and Kex2p activity (Fig. 7). Third, a direct assay for ERAD (in a *sec18* mutant) showed ICFP to be completely stable over a 90-min time course (Fig. 4, A and B), suggesting that it is not a ready substrate for typical ubiquitin-mediated proteasome-dependent proteolysis (Plempner and Wolf, 1999). Fourth, in the absence of Pep4p (vacuolar proteinase A) insulin accumulates not in the ER but in the vacuole (Fig. 4 C) where it is delivered via the endosomal system (Fig. 5). These novel features of ICFP and insulin sorting are summarized in Fig. 12.

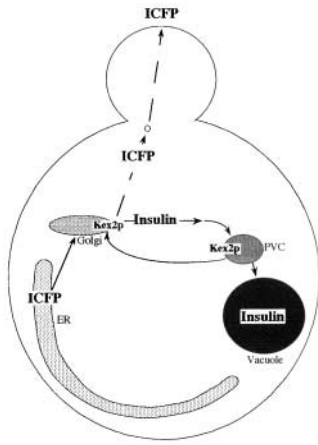
The targeting of insulin expressed from ICFP is decided upon in the Golgi complex (Fig. 12). Initially we thought that vacuolar targeting of insulin might be mediated by a Golgi-based quality control system like that responsible for



**Figure 11.** Intracellular retention of insulin is perturbed in a point mutant changing the insulin B5 histidine to threonine. The strains are *pep4Δ* mutants in order to stabilize the intracellular protein. (A) Intracellularly retained and secreted insulin upon expression of the nonmutagenized ICFP control (Con) or the H90T-ICFP mutant (B5T) with a *GALI* promoter on a centromeric plasmid. Cells were pulse labeled for 30 min and chased for 60 min. The lysed cells and media underwent insulin immunoprecipitation before analysis by SDS-PAGE and fluorography. Note the dramatically increased secretion of the B5T mutant insulin (open arrows). Where indicated, the samples were digested with PNGaseF before SDS-PAGE. Only the insulin bands are shown in order to facilitate direct comparison of insulin secretion efficiency. C, cells; M, medium. (B) Intracellular nonmutagenized insulin control protein (Con) or the mutagenized insulin (B5T) followed by sucrose velocity gradient centrifugation analysis as in the legend to Fig. 10 A. Note that sedimentation of the B5T mutant (arrow) is clearly inhibited. U, top half of gradient; L, bottom half of gradient.

the degradation of a mutant lambda repressor protein fused to invertase (Hong et al., 1996) and a variety of other proteins in the secretory pathway (Chang and Fink, 1995; Luo and Chang, 1997; Beck et al., 1999; Li et al., 1999). Certainly, routing of insulin to the vacuole via the endosomal system provides a rationale for why five out of six *eis* gene disruptions identified in our genetic screen are *vps* mutants (Fig. 3 A) that impair post-Golgi trafficking of CPY (Fig. 3 B) and other vacuole-bound proteins. Nevertheless, there appear to be significant differences in the molecular mechanisms accounting for inefficient secretion of ICFP versus that of other proteins subject to Golgi quality control. First, the vacuolar delivery of the lambda repressor-containing fusion protein occurs primarily when the sequence is mutated to induce misfolding. By contrast, single chain insulin folds similarly to authentic insulin (Thim et al., 1986; Hua et al., 1998; Kjeldsen et al., 1998), and no mutations inducing misfolding have been introduced. Furthermore, all three insulin disulfide bonds appear intact (data not shown), and the band comigrates by nonreducing SDS-PAGE with authentic insulin. Indeed, our current evidence suggest that the unsecreted single chain insulin takes on quaternary structural features (Fig. 10) observed for authentic insulin, which are dependent on a native tertiary structure (Kuliawat and Arvan, 1994; Huang and Arvan, 1995). Second, although Vps10p has been proposed to operate as a quality control receptor in the Golgi complex for certain substrates (Hong et al., 1996; Durr et al., 1998; Holkeri and Makarow, 1998; Jorgensen et al., 1999), *vps10* cells did not show significantly increased secretion of ICFP (Fig. 3 B and Fig. 6). Thus, a Vps10p-mediated mechanism does not appear to contribute significantly to the sorting of insulin in the secretory pathway.

A novel aspect of the present studies was our discovery that deletion of *KEX2* causes quantitative secretion of



**Figure 12.** Model of ICFP and insulin sorting in the secretory pathway of yeast cells. According to this schema, ICFP is quantitatively delivered to the Golgi complex where it may encounter Kex2p. To the extent that the precursor protein is unprocessed by Kex2p, it is delivered to the cell surface via the secretory pathway. To the extent that ICFP is endoproteolytically processed in the Golgi complex, insulin is retained intracellularly and is delivered ultimately to the vacuole. Note that the  $\alpha$ -leader peptide contains no [<sup>35</sup>S]cysteine residues; thus, we have not analyzed the fate of the  $\alpha$ -leader peptide after cleavage from the insulin moiety.

ICFP (Fig. 7). We considered initially the possibility, albeit unprecedented, that the  $\alpha$ -leader peptide might contain a dominant secretion signal, the loss of which would result in intracellular insulin retention. This possibility was excluded from the results of experiments in which the  $\alpha$ -leader was replaced with a fragment of another leader peptide known to lack a dominant secretion signal (Fig. 8). These findings led us to consider a second hypothesis that Kex2p itself might serve as a sorting receptor that causes intracellular insulin retention. However, in either a *kex2*-null strain (Fig. 9 B) or a *vps35* strain (Fig. 9 D), expression of secretory Kex2p is sufficient to cause intracellular insulin retention even though secretory Kex2p itself is delivered from the Golgi complex to the cell surface (Fig. 9 A). Moreover, the degree to which secretory Kex2p (or wild-type Kex2p) conferred intracellular retention was limited to the extent that the precursor protein was processed to mature insulin (Fig. 9 C). This indicates that Kex2p enzymatic activity in the Golgi complex is a saturable limiting component in the molecular sorting of the insulin precursor (Fig. 12). Moreover, when ICFP is mutated to make substitutions at the dibasic cleavage site, secretion of immunoreactive insulin (precursor) is also increased (our unpublished data). Altogether, our data indicate that it is endoproteolysis itself that prevents rapid secretion of the insulin precursor (Fig. 12).

Although our data do not formally exclude the possibility of sorting receptors for processed insulin, no genes encoding proteins with a luminal domain (that could serve as potential sorting receptors) were identified in our genetic screen. The fact that *kex2* was not isolated in the *eis* screen may be accounted for by the fact that in our strain background, *kex2* mutant cells seem to grow more slowly, and the filter blot assay used for screening is indirectly dependent on the size of the colonies secreting ICFP. Likewise, other gene disruptions that adversely affect cell growth would also be overlooked in the screen, and this conceivably could contribute to the fact that we did not recover a potential sorting receptor.

We have proposed in mammalian-regulated secretory cells that endoproteolytic conversion to insulin facilitates

intracellular retention of the newly synthesized protein (Kuliawat et al., 2000). This may occur because homomultimerization limits the ability of insulin to enter post-Golgi transport intermediates leading ultimately to the cell surface (Kuliawat and Arvan, 1992). It seems quite possible that related phenomena could account for insulin trafficking in the secretory pathway of yeast cells. Our evidence supports the idea that there is a change in biophysical properties upon endoproteolytic conversion to the mature insulin species (Fig. 10). Intriguingly, recent proposals for mechanisms that alter post-Golgi targeting of other proteins include homomultimerization facilitated by endoproteolytic cleavage at dibasic sites (Silletti et al., 2000) and ordered luminal protein aggregation (Wolins et al., 1997). Indeed, we find that upon endoproteolytic processing, a B5T mutant insulin is no longer efficiently retained, the majority being secreted instead (Fig. 11 A), and this is correlated with an inability of the protein to form a stable multimeric assembly, as measured by sucrose gradient centrifugation (Fig. 11 B). In conclusion, although the issue remains unresolved of whether a Golgi/post-Golgi membrane-associated receptor plays any role in intracellular insulin targeting, it would appear that precursor endoproteolysis unmasks information required for sorting by retention for the vast majority of, if not all, insulin molecules in the eukaryotic secretory pathway.

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