A Role For Lte1p (a Low Temperature Essential Protein Involved in Mitosis) in Proprotein Processing in the Yeast Secretory Pathway*

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We previously identified six single gene disruptions in Saccharomyces cerevisiae that allow enhanced immunoreactive insulin secretion primarily because of defective Kex2p-mediated endoproteolytic processing. Five *eis* mutants disrupted established VPS (vacuolar protein sorting) genes, The sixth, LTE1, is a Low Temperature (<15 °C) Essential gene encoding a large protein with potential guanine nucleotide exchange (GEF) domains. Lte1p functions as a positive regulator of the mitotic GTPase Tem1p, and overexpression of Tem1p suppresses the low temperature mitotic defect of *lte1*. By sequence analysis, Tem1p has highest similarity to Vps21p (yeast homolog of mammalian Rab5). Unlike TEM1, LTE1 is not restricted to mitosis but is expressed throughout the cell cycle. Lte1p function in interphase cells is largely unknown. Here we confirm the eis phenotype of *lte1* mutant cells and demonstrate a defect in proalpha factor processing that is rescued by expression of full-length Lte1p but not a C-terminally truncated Lte1p lacking its GEF homology domain. Neither overexpression of Tem1p nor 13 other structurally related GTPases can suppress the secretory proprotein processing defect. However, overexpression of Vps21p selectively restores proprotein processing in a manner dependent upon the active GTP-bound form of the GTPase. By contrast, a vps21 mutant produces a synthetic defect with *lte1* in proprotein processing, as well as a synthetic growth defect. Together, the data underscore a link between the mitotic regulator, Lte1p, and protein processing and trafficking in the secretory/endosomal system.

A genetic screen has previously been performed with yeast mutants by random gene disruptions (by insertion of *lacZ*/*LEU2*) to identify genetically engineered cells exhibiting enhanced immunoreactive insulin secretion (eis),² derived from an integrated GAL1 promoter-driven insulin-containing fusion protein called ICFP, Ref. 1. The ICFP is comprised of the leader and propeptide of the yeast alpha-mating factor contiguous with a single chain insulin, separated by a Kex2p cleavage site. Cleavage at the Kex2p-processing site causes insulin to be delivered ultimately to the vacuole in a manner that requires specific insulin structural features (2). From 90,000 transformants that were originally screened, six independent gene disruptions were identified. Each of the six *eis* mutants were found to secrete slightly to dramatically increased amounts of unprocessed ICFP, and indeed, a kex2Δ mutant exhibited maximal immunoreactive insulin secretion (1). Upon further analysis, five of the six *eis* mutants were found to be vacuolar protein sorting mutants vps8, vps35, vps13, vps4, and vps36, which affect protein trafficking and thereby impair protein processing in the Golgi ⇔ endosomal system (e.g. Ref. 3). Such an outcome can be explained because the ability of Kex2p and other secretory protein processing enzymes (such as Ste13p and Kex1p) to function properly requires their continuous recycling between Golgi and endosomal systems (4).

In this article, we report that *eis4* is caused by disruption of the gene known as LTE1 (5). LTE1 is a low temperature essential gene product that is required at <13 °C to allow yeast cells to advance through the spindle pole checkpoint during mitosis, in which the small GTPase Tem1p is essential (6). Lte1p is a positive regulator of Tem1p function (7) and overexpression of TEM1 rescues the cold-sensitive growth arrest of *lte1* cells (8). Lte1p has a large, modular-appearing primary structure including small GEF homology domains embedded in the C- and N-terminal regions (9). Overexpression of *lte1-mini* (truncated to lack these domains) can suppress the cold-sensitive growth arrest of *lte1D* cells, raising the possibility that Lte1p might not be a GEF for Tem1p (10); however, other analysis supports the hypothesis that GEF function of Lte1p activates Tem1p (11). Importantly, while Tem1p expression becomes significant only during mitosis (telophase), Lte1p is stably expressed throughout the cell cycle (12), suggesting other possible roles for Lte1p in interphase cells.

Beginning at the G₁ → S transition of the cell cycle, the secretory pathway focuses on the bud site and expands the bud that...
shall become a daughter cell (13). Lte1p localization (which is regulated via phosphorylation, Ref. 9) is normally confined to buds growing from secretory activity, at least in part by virtue of interactions with plasma membrane-associated Ras2p (10) and buds growing from secretory activity, at least in part by virtue of interactions with plasma membrane-associated Ras2p (10) and perhaps by being bound to other cell polarity proteins in the bud such as Kel1p and Kel2p (14). Even so, we found it surprising to discover lte1 in a screen revealing secretory protein processing mutants. In the present study, we have sought to confirm a secretory protein processing defect in lte1 mutant cells. The present work highlights the importance of the Lte1p C-terminal region (that includes a GEF domain) in proprotein processing (1). Strain 773 is the mutant of L5145/pMI316 that includes the entire LTE1 gene. From pPD34, a ~6.0 kb Ncol-Sphl fragment containing the LTE1 coding and downstream sequences (but lacking the promoter) was excised and subcloned into shuttle vector pSL1180. A cDNA fragment encoding an initiator methionine and triple Myc tag was fused in-frame directly before the start codon of LTE1. The expression of triple Myc-LTE1 was induced by transforming yeast cells with pYL9DEV, which was constructed by digesting the PCR products with BamH I and SalI followed by subcloning into the respective sites of pRS426. The TDH3 promoter was used to drive the expressed Lte1p. 

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—Standard yeast media and genetic manipulations were as described (15). Strain L5145/MI316 expresses ICF from a single chromosomal locus driven from the GAL1 promoter (1). Strain lte1-773 is the eis4 mutant of L5145/pMI316 displaying enhanced immunoreactive insulin secretion after random mutagenesis and screening (1). The other strains employed in this study, unless stated otherwise, are isogenic to either W303 or L3852 (Table 1).

**Plasmids and Molecular Biology**—pRSGLC is a TRP1-marked centromeric plasmid (pRS314, Ref. 16) bearing the TDH3 promoter (17). The pPD34 plasmid, as previously described (8), carries a ~9-kb yeast genomic DNA fragment that includes the entire LTE1 gene. From pPD34, a ~6.0 kb Ncol-Sphl fragment containing the LTE1 coding and downstream sequences (but lacking the promoter) was excised and subcloned into shuttle vector pSL1180. A cDNA fragment encoding an initiator methionine and triple Myc tag was fused in-frame directly before the start codon of LTE1. A ~4.5-kb fragment including the coding sequence and terminator was excised with SacI and inserted with appropriate orientation at the site in pYES2 (InVitrogen) to produce pYL9, a 2-µm yeast plasmid that includes the entire LTE1 gene. From pPD34, a ~6.0 kb Ncol-Sphl fragment containing the LTE1 coding and downstream sequences (but lacking the promoter) was excised and subcloned into shuttle vector pSL1180. A cDNA fragment encoding an initiator methionine and triple Myc tag was fused in-frame directly before the start codon of LTE1. The expression of triple Myc-LTE1 was induced by transforming yeast cells with pYL9DEV, which was constructed by digesting the PCR products with BamH I and SalI followed by subcloning into the respective sites of pRSGLC. Other plasmids employed in this study are described in Table 2.

## TABLE 1
Yeast strains used in this study

| Strains | Genotype | Source |
|---------|----------|--------|
| W303    | MATa ade2-1 canl-100 his3-11 leu2-3,112 trpl-1 ura3-52 | 30 |
| L3852   | MATa his3-200 lys2-201 leu2-3,112 ura3-52 ade2 | 3 |
| L5145   | Mata his3-200 lys2-201 leu2-3,112 ura3-52 ade2 | 3 |
| L4      | MATa ade2-1 canl-100 his3-11 leu2-3,112 trpl-1 ura3-52 | This study |
| H4      | MATa ade2-1 canl-100 his3-11 leu2-3,112 trpl-1 ura3-52 | This study |
| lte1Δ   | MATa ade2-1 canl-100 his3-11 leu2-3,112 trpl-1 ura3-52 | This study |
| vps21Δ  | MATa ade2-1 canl-100 his3-11 leu2-3,112 trpl-1 ura3-52 | This study |
| vps8Δ   | MATa ade2-1 canl-100 his3-11 leu2-3,112 trpl-1 ura3-52 | This study |
| VPS21 lte1Δ | MATa his3-200 lys2-201 leu2-3,112 ura3-52 ade2 lte1Δ URA3 | This study |
| vps21Δ lte1Δ | MATa his3-200 lys2-201 leu2-3,112 ura3-52 vps21Δ ΔLYS2 lte1ΔΔURA3 | This study |
| vps9Δ   | MATa ade2-1 canl-100 his3-11 leu2-3,112 trpl-1 ura3-52 vps9ΔΔURA3 | This study |
| VPS9A lte1Δ | MATa ade2-1 canl-100 his3-11 leu2-3,112 trpl-1 ura3-52 vps9ΔΔURA3 | This study |
| BFY106-4D | MATa ade2-1 canl-100 his3-11 leu2-3,112 trpl-1 ura3-52 lte1ΔΔURA3 | 3 |
| LM23-3az | MATa ade2-1 canl-100 his3-11 leu2-3,112 trpl-1 ura3-52 lte1ΔΔURA3 | 20 |

## TABLE 2
Plasmids used in this study

| Plasmid | Description | Source |
|---------|-------------|--------|
| pSL2105 | CEN URA3 GAL-STE3-myc on YCp50 | 22 |
| pYpt51  | 2µ URA3 VPS21 on pSEY8 | 31 |
| pYpt52  | 2µ LEU2 YPT52 on YEp13 | 31 |
| pYPT53  | 2µ URA3 YPT52 on pSEY8 | 31 |
| pYpGlu181-TEM1 | 2µ LEU2 TEM1 on YEp13 | 31 |
| pBB80   | 2µ LEU2 SEC4 on YEp24 | 31 |
| pAC77   | 2µ LEU2 YPT7 | 31 |
| pNS455  | 2µ URA3 GAL-YPT32 on YEp52 | 33 |
| pNS5198 | 2µ URA3 GAL-YPT31 on YEp24 | 33 |
| pRBI594 | 2µ LEU2 CDC42 on Yep531 | 34 |
| pRBI596 | 2µ TRP1 RHO2 on pYO324 | 34 |
| pRBI592 | 2µ TRP1 RHO1 on pYO324 | 34 |
| pYO324-RHO4 | 2µ TRP1 RHO4 on pYO324 | 35 |
| YepRas1 | 2µ LEU2 RAS1 | 36 |
| YepRas2 | 2µ LEU2 RAS2 | 36 |
| pYPT6  | CEN URA3 YPT6 on YIp5 | 37 |

* L3852Δ, vps21Δ, lte1ΔΔURA3 are isogenic to L3852.
The single point mutant Vps21p-S21L was made by using the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) with plasmid-encoded wild-type VPS21 as a template and a pair of primers: GGTGAGGCAGCAGTTGGTAAATTGTCAATAGTCTAAGG and CCTTAGGACTATTGACATTATTTCAACACTGTGCTGTCCC. The single point mutant Vps21p-Q66L was made with the same kit employing TGGGACACTGCTGGGCTAGAGAGATTTGCATCTTTAGCA and TGCTAAAGATGCAAATCTCTCTAGCCCAGTGTTGTCCCA as mutagenic primers.

**Filter Blot Assays**—To assay secretion of insulin-containing peptides, cell patches were replica-plated onto nitrocellulose filters placed on top of plates containing 2% galactose as sole carbon source. After 24–40 h, the filter was washed and processed for immunoblotting with guinea pig anti-insulin and a peroxidase-conjugated secondary antibody as previously described (1). CPY secretion from cells was detected by filter overlay and overnight growth of the cells as described (18) using mouse mAb anti-CPY (Molecular Probes, 1:4000 dilution) and a peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, 1:10000 dilution).

**Bioactive Alpha Factor and Proalpha Factor Secretion**—Assay for secretion of bioactive alpha factor was done as described (19). MATα cells (0.015 OD_{600}/1.5 μl) were spotted onto a thin lawn of MATα bar1 cells (LM23–3az; Ref. 20) on synthetic complete medium. After 1–2 days at 30 °C, halos surrounding patches of cells were visualized by placing plates directly on a scanner and converting to digital images. Halo areas were quantified using Image Quant v5.2 ( Molecular Dynamics, Inc.).

To examine secretion of proalpha factor, cells were grown to mid-log phase in synthetic complete medium without methionine and cysteine. Cells were harvested and resuspended at 0.8 OD_{600}/ml in fresh medium with 1 mg/ml bovine serum albumin. 500 μCi of ^{35}S amino acid mixture (Expre^{35}S^{35}S, PerkinElmer Life Sciences) was added to each sample, and incubation continued for 50 min at room temperature with shaking. Medium was separated from cells by centrifugation at 19,500 × g for 5 min. SDS was added to the medium to a final concentration of 1%, and samples were boiled for 3 min. Secretion of alpha factor-containing peptides was recovered by immuno-
precipitation with the RW1 rabbit polyclonal anti-alpha factor antibody (kind gift of Dr. D. Shields, Albert Einstein College of Medicine, Bronx, NY) and analyzed by SDS-15%-PAGE and fluorography.

**Western Blot**—Steady-state levels of mycLte1p, Ste3-Myc, and Kex2p were analyzed in cell lysates prepared from mid-log cultures by vortexing with glass beads, as described previously (21). Sample loading was normalized to lysate protein as measured by BCA protein assay (Pierce). After SDS-PAGE and electrotransfer to nitrocellulose, blots were probed with anti-Myc, anti-Ste3p, and anti-Kex2p (rabbit polyclonal A-14; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-Kex2p (rabbit polyclonal M120D; gift of Dr. R Fuller, University of Michigan) and a peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, 1:5000 dilution). Bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

**Metabolic Labeling and Immunoprecipitation of CPY**—Metabolic labeling was performed using cultures grown to mid-log phase in synthetic complete medium without methionine and cysteine. Cells were resuspended at 1 OD_{600}/ml, labeled with Expre^{35S}S^{35S} at 0.1 mCi per 1 OD_{600} cells for 5 min at room temperature, and chased in the presence of 10 mM unlabeled methionine and cysteine. At various chase times, aliquots were placed on ice in the presence of 10 mM sodium azide. Lysates were prepared and resuspended in radioimmune precipitation assay buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS) for immunoprecipitation with 1 μl of mouse mAb anti-CYP and protein G-Sepharose beads.

**Endocytic Degradation of Ste3p**—As previously described (22), strains carrying pSL2015 (Gal-ST3-myc) were grown to mid-log phase in synthetic complete medium containing 2% galactose as the sole carbon source to stimulate the expression of Ste3p-myc. At time 0, glucose (3%) was added to arrest further Ste3p-myc synthesis. At the indicated times after glucose addition, cells were harvested, and lysate was prepared for SDS-PAGE and Western blotting with anti-Myc antibody.

**RESULTS**

Identification of eis4 as lte1—The insulin secretory phenotype of the eis4 mutant was not as strong as some of the other eis mutants that proved to be vps mutants (1). By sequencing genomic DNA adjacent to the lacZ insertion, the eis4 mutant was found to be caused by disruption of LTE1. Full-length Lte1p is 1435 amino acids, and residues 1292–1315 are critical to the CDC25 homology domain that is thought to have guanine nucleotide exchange activity. We found that the lacZ/LEU2 insertion of eis4 yielded the possibility of expression of an Lte1p gene product C-terminally truncated after residue 783. To test the effect of losing the C-terminal region of Lte1p on protein processing in the secretory pathway, we prepared several plasmid vectors, each bearing an N-terminally Myc-tagged version of Lte1p under control of the GAL1 promoter. We ultimately concentrated on two constructs, one encoding full-length Lte1p and a second encoding an Lte1p_{1012} that was C-terminally truncated after residue 1012. These forms were expressed in cells as stable proteins detected by Western blotting with anti-Myc antibody (Fig. 1A), and the functionality of these proteins was also tested (Fig. 1B, see below).

Haploid yeast on either glucose, galactose, or raffinose plates can grow at 13 °C, whereas either eis4 cells (lte1_{783} data not shown) or lte1_{1012} cells exhibit a significant low temperature growth phenotype regardless of carbon source (middle sector, Fig. 2A). Full-length mycLte1p could restore low temperature growth to lte1_{1012} cells on galactose or raffinose plates, but not on glucose plates in which mycLte1p expression remains repressed (left sector, Fig. 2A). Using serial dilution, full-length mycLte1p expression restored quantitatively normal growth to lte1Δ cells at 13 °C, whereas mycLte1_{1012} could not complement the growth defect (Fig. 2B). Likewise, enhanced secretion of exogenously expressed immunoreactive insulin in lte1_{783} cells was complemented by mycLte1p (Fig. 1B). Thus, whereas C-terminal truncation of Lte1p supports neither low temperature growth nor normal handling of the insulin precursor, mycLte1p is fully functional in both capacities (Figs. 1 and 2).
secretory protein substrate, we checked the ability of \textit{lte1}_{1012} cells to produce bioactive alpha mating factor, as assessed by the ability to cause growth arrest of cells of the opposite mating type (20). When spotted on a lawn of \textit{Mata} cells on galactose plates at 30 °C, wild-type \alpha cells were surrounded by a large halo of growth-arrested \textit{Mata} cells. By contrast, \textit{lte1}Δ cells always exhibited a decrease in halo area (Fig. 3A) that averaged a 35% reduction over numerous experiments. These data suggest that Lte1p assists in the production of bioactive alpha factor as well as in processing the insulin-containing fusion protein (1). Notably, both substrates require cleavage by Kex2p during their maturation within the secretory pathway while proalpha factor requires additional processing events to generate bioactive alpha factor.

Whereas a number of \textit{vps} mutants impair Kex2p-mediated secretory proprotein cleavage via accelerated delivery of Kex2p to a degradative compartment resulting in decreased Kex2p levels, other \textit{vps} mutants can more subtly influence processing enzymes without significant change in Kex2p levels (such as \textit{vps8} (3) or \textit{vps21} (this report)). It is therefore of interest to note that steady state Kex2p levels were not significantly affected by loss of expression of Lte1p (Fig. 3B). There was a small but reproducible increase in the secretion of unprocessed proalpha factor into the medium of \textit{lte1}_{1012} mutant cells, comparable to that of \textit{vps8} cells (Fig. 3C). (For comparison, Fig. 3, B and C also show that \textit{vps21} mutant cells exhibit more proalpha factor secretion without a significant decrease in Kex2p level, whereas \textit{kex2Δ} cells exhibit massive proalpha factor secretion and undetectable Kex2p.)

Because Lte1p exhibits positive regulation of Tem1p, a small GTPase essential for mitosis (7), we wished to determine whether the secretory protein processing defect of \textit{lte1} cells might be secondary to an alteration in cell growth. To check this, we examined whether overexpression of Tem1p could suppress the protein processing defect of \textit{lte1}_{1012} cells. As shown in Fig. 5A, overexpression of Tem1p allowed \textit{lte1} cells to grow like wild type at 13 °C, as previously reported (8). Importantly, however, Tem1p overexpression did not suppress the alpha factor secretion defect of \textit{lte1} (Fig. 4A, left). These data...
suggest that impaired secretory protein processing in \textit{lte1} cells is not secondary to a defect in cell growth.

\textit{lte1} Mutant Cells Do Not Exhibit a \textit{vps} Phenotype—LTE1 is not a known vacuolar protein sorting (VPS) gene (23), but as all other \textit{eis} mutants were known \textit{vps} mutants (1), we checked for secretion of CPY in \textit{lte1} cells (Fig. 5A). Unlike other established \textit{vps} mutants (e.g. \textit{vps4} or \textit{vps23}), \textit{lte1} mutant cells exhibited no augmented CPY secretion by filter overlay assay (Fig. 5A). A more sensitive immunoprecipitation assay from media bathing \textit{lte1} cells also demonstrated inconsequential secretion of newly synthesized CPY (Fig. 5B). We also considered the possibility of delayed delivery of precursor CPY to the endosomal/vacuolar system without frank CPY secretion in \textit{lte1} cells. To check for this, cells were pulse-labeled with $^{35}$S amino acids for 5 min, washed, and chased for various times at 30 °C (Fig. 5C). At 5 min of chase, the P1 form of CPY (in the endoplasmic reticulum) predominated with the P2 (Golgi) and mature (vacuolar) forms already apparent. By 10 min of chase, the majority of newly synthesized CPY had already been processed to the mature form and by 30 min of chase, processing appeared complete. In \textit{lte1}_{1012} mutant cells that exhibit a small alpha factor halo, there was no delay in the appearance of mature CPY, indicating that general protein trafficking within the endosomal system is not substantially perturbed (Fig. 5C). Further, we checked the endocytic internalization and degradation of the cell surface a-factor receptor, Ste3p, using a “GAL-shut-off” assay (22). Specifically, expression of Myc-tagged Ste3p under \textit{GAL1} control is shut off by addition of glucose, and vacuolar delivery and degradation of pre-existing cell surface receptors can be followed by Western blotting with anti-Myc. As shown in Fig. 6, Ste3p was degraded in \textit{lte1}_{1012} mutant cells with normal kinetics. Thus, \textit{lte1} is not a \textit{vps} mutant, showing no global defects in endosomal trafficking.

**TABLE 3**

Small GTPases with similarity to Tem1p tested for suppression of protein processing defect in \textit{lte1} mutant cells

| GTPase | % Overall similarity to Tem1p | Type of plasmid* | Vector used for expression |
|--------|-------------------------------|------------------|--------------------------|
| Vps21p | 58                            | Multicopy        | pSEY8                    |
| Vps21p | 58                            | Centromeric      | pRS314                   |
| Ypt52p | 54                            | Multicopy        | YEpl13                   |
| Ras2p  | 52                            | Multicopy        | YEpl13                   |
| Ypt6p  | 51                            | Centromeric      | YEpl13                   |
| Ypt7p  | 50                            | Multicopy        | YEpl13, LEU2             |
| Ypt53p | 55                            | Multicopy        | pSEY8                    |
| Sec4p  | 50                            | Multicopy        | YEpl24                   |
| Rho1p  | 48                            | Multicopy        | pYO324                   |
| Cdc42p | 47                            | Multicopy        | YEpl35                   |
| Rho4p  | 46                            | Multicopy        | pYO324                   |
| Ras1p  | 46                            | Multicopy        | YEpl13                   |
| Ypt32p | 45                            | Multicopy        | YEpl32                   |
| Ypt31p | 45                            | Multicopy        | YEpl24                   |
| Rho2p  | 44                            | Multicopy        | pYO324                   |

* Plasmid sources shown in Table 2.

**FIGURE 7.** Overexpression of \textit{VPS21} rescues the secretory protein processing defect of \textit{lte1}_{1012} mutant cells. A, wild-type (wt) cells or \textit{lte1}_{1012} cells were transformed with plasmid pYP51. The alpha factor halo defect of \textit{lte1} mutant cells (52.5% of that in wild-type cells) was selectively rescued (to 95.3%) by overexpression of \textit{VPS21} (but not any of the other small GTPases listed in Table 3). Note that \textit{VPS21} overexpression did not further enlarge the normal halo of wild-type cells, nor did it normalize the small halo of an unrelated \textit{vps23} mutant. B, filter blot for immunoreactive insulin from wild-type (wt) and \textit{lte1}_{1012} (eis4) mutant cells transformed with vector, or multicycop \textit{VPS21}. Note that the abnormally eis phenotype of \textit{lte1} mutant cells is improved (diminished) upon overexpression of \textit{VPS21}.

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Genetic Interactions between LTE1 and VPS21, the Yeast Homolog of Mammalian Rab 5—While functioning as a positive regulator of Tem1p, Lte1p has also been found to physically associate with Ras2p-GTP in a manner dependent upon the C-terminal GEF-like domain of Lte1p (10). To consider other related GTPases that might be involved in secretory protein processing, a BLASTp search of yeast proteins was performed using full-length Tem1p as the query sequence, yielding the list of small GTPases shown in Table 3. Overall sequence similarity is highest for Vps21p (the yeast homolog of mammalian Rab5), but because of the conserved GTPase domain itself, there is a high degree of similarity with all the GTPases listed. Plasmid vectors for overexpressing each of the yeast gene products shown in Table 3 were transformed into \textit{matα \textit{lte1}} mutant cells to test for suppression of the alpha factor halo defect. As noted above, Tem1p overexpression could not restore a normal alpha factor halo to \textit{lte1} mutant cells (Fig. 4A), and indeed, neither could overexpression of Ras2p nor any of the dozen other GTPases listed in the lower portion of Table 3 (data not shown). However, \textit{VPS21} overexpression, either driven from its own promoter on a high copy plasmid (Fig. 7A) or a strong constitutive (\textit{TDH3}) promoter on a centromeric plasmid (not shown), could rescue the alpha factor halo defect of \textit{lte1} mutant cells. The effect was specific, as overexpression of Vps21p could not increase the halo of wild-type yeast, nor could it restore the halo defect of an unrelated \textit{vps23} mutant (Fig. 7A). The abnormally enhanced immunoreactive insulin secretion of \textit{lte1} cells was also suppressed by overexpression of Vps21p (Fig. 7B).

We then looked for synthetic interaction between \textit{vps21} and \textit{lte1} mutants. Indeed, in a side-by-side comparison on the same plates, while
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**FIGURE 8. Synthetic effects in lte1_1012 vps21Δ double mutant but not lte1_1012 vps9 double mutant.** A, either wild-type yeast (WT) or the mutant MATα tester strains indicated were spotted on a lawn of LM23-3a (MATαbar1) cells in duplicate on a single plate. The results shown here were replicated in three independent experiments. Note that while the single lte1 or vps21 mutants had only modest deficiencies in bioactive alpha factor production; the lte1 vps21 double mutant exhibited a major reduction in halo size ($28.3 \pm 11.3\%$ of that in wild-type cells), indicating a synthetic defect. B, as same as above, except combining lte1 and vps9 mutants. Although the single vps9 mutant has a small halo, its inhibitory effects on production of bioactive alpha factor are not additive to the inhibitory effect of the lte1 mutant.

vps21 mutant cells had an alpha factor halo that was smaller than that of wild-type cells, the lte1 vps21 double mutant clearly had a synthetic effect, with an alpha factor halo much smaller than either mutant alone (Fig. 8A). Such a synthetic effect could not be observed with most other vps mutants tested; e.g., lte1 and vps9 (an established GEF for the Vps21p GTPase, Ref. 24) showed no additive inhibition (reduction) of alpha halo (Fig. 8B). Notably, the lte1 vps21 double mutant cells have no major decrease in steady state levels of Kex2p (Fig. 3B), although they exhibit a synthetic increase in the secretion of unprocessed proalpha factor (Fig. 3C). Thus, the data support an interaction of VPS21 with LTE1 in secretory proprotein processing.

**VPS21 Genetically Interacts with Lte1 in Mitosis in a Different Way Than in Secretory Proprotein Processing**—Given the specificity of the Tem1p GTPase for the mitotic spindle pole checkpoint, we had no reason to expect that overexpression of Vps21p, which rescues the alpha factor halo defect of lte1 cells, would impact on low temperature growth of lte1 mutant cells. It was therefore surprising to discover that when VPS21 was expressed from its own promoter on a multicopy plasmid, lte1_1012 mutant cell growth was no longer arrested at 13 °C (Fig. 9A). Also, when VPS21 was expressed from a strong constitutive promoter on a centromeric plasmid, it rescued the growth of lte1Δ cells comparably to that of TEM1 expressed from the same promoter (Fig. 9B).

To explore further the interactions of VPS21 with lte1, we used a semipermissive low temperature of 17 °C to examine growth of vps21 or lte1, or vps21 lte1 double mutant cells. At 25 °C, each of the strains grew like the VPS21 LTE1 wild type (Fig. 10A, left). At 17 °C, the mitotic blockade of lte1 cells was demonstrated as a partial growth defect, while growth of vps21 lte1 double mutant cells was profoundly inhibited (Fig. 10B). Growth of the vps21 mutant alone was not at all inhibited under these conditions (Fig. 10B). These data provide good genetic evidence that the presence of Vps21p, even at endogenous expression levels, limits the mitotic defect of lte1 mutant cells. By contrast, as shown in Fig. 10C, vps9 did not exert any appreciable growth phenotype when combined with the lte1 mutant, suggesting that the GEF activity of Vps9p is not important for Vps21p to play a role in Lte1p function.

To distinguish suppressor effects of Vps21p on the secretory protein processing defect of lte1 cells versus suppression of the low temperature growth arrest of lte1, we compared the effect of overexpressing inactive (persistently GDP-bound) Vps21p-S21L.
cold-sensitive growth arrest of *lte1* cells (8). Tem1p expression is turned on during mitosis, in which it acts as an essential controller of the mitotic exit network. The timing of Tem1p activation by Lte1p occurs when the spindle pole body migrates into the bud. Lte1p localization at the bud appears to be regulated by its interactions with Ras2p (10), its phosphorylation state (11), and interactions with cell polarity proteins (14). However, Lte1p function during S-phase is largely unknown.

We identified *lte1* as *eis4*, from a screen for yeast mutants that exhibit enhanced insulin secretion because of defects in secretory protein processing (1). The *eis* group of mutants appears to work by impairing the recycling of secretory protein processing enzymes (e.g. Kex2p, Ste13p, and Kex1p) between Golgi and endosomal systems (1). In this study we confirm our original observations on *eis4* (which hypothetically produces a protein containing only the first 783 residues of Lte1p). Specifically, we demonstrate that the *eis4* phenotype is complemented by full-length mycLte1p (Fig. 1). Moreover, we demonstrate a proprotein processing defect in *lte1* mutant cells ranging from a complete null to the *lte1* mutant that lacks the C-terminal GEF domain. This truncation mutant also exhibits classic low temperature growth arrest, and under conditions where the truncated Lte1p is stably expressed, it cannot rescue low temperature growth of *lte1* cells (Fig. 2). The *lte1* mutant produces less bioactive alpha factor as judged by a reproducibly smaller alpha factor halo and an increase in the secretion of unprocessed proalpha factor.

The data suggest that the C-terminal GEF domain of Lte1p may be involved in its role in secretory protein processing. The phenotype is mild, resulting in no significant change in intracellular Kex2p level, comparable to certain *vps* mutants (Fig. 3). Nevertheless, *lte1* is not a *vps* mutant, exhibiting no defect in the trafficking of vacuolar carboxypeptidase Y (Fig. 5) nor any defect in endocytic internalization and degradation of cell surface Ste3p (Fig. 6). These results are compatible with a growing list of mutants in trafficking genes that affect Golgi-early endosomal trafficking and secretory proprotein processing yet do not produce a *vps* phenotype, such as *tlg2* (and to a large extent, *tlg1* (25), *chc1* (26), as well as *insps* (27, 28)). As one intracellular site of Vps21p function involves Golgi-early endosome trafficking (29), Lte1p may facilitate Vps21p activity at this site; thus *lte1* should be considered a candidate for addition to the foregoing list of mutants.

During S-phase, Lte1p is unlikely to work via Tem1p activation because Tem1p expression is limited during this phase of the cell cycle (12). Indeed, unlike the low temperature growth defect, the secretory protein processing phenotype of *lte1* cannot be rescued by overexpression of *TEM1* (Fig. 4). One might argue that even after multicopy expression (Fig. 4), *TEM1* cannot suppress the alpha factor halo defect of *lte1* mutant cells because cell cycle-dependent regulation limits Tem1p availability. However, the *TEM1* promoter is replaced with the strong constitutive *TDH3* promoter, *TDH3-TEM1* also cannot rescue the alpha factor halo defect of *lte1* cells (not shown). Moreover, neither overexpression of Ras2p, Ras1p, nor 11 other GTPases has any effect on secretory protein processing (Table 3), suggesting that Lte1p is unlikely to work with any of these GTPases in secretory protein processing.
Role of Lte1p in the Yeast Secretory Pathway

By contrast, VPS21, when overexpressed in lte1 mutant cells, restored production of bioactive α-mating factor (as measured by halo assay) and restored to normal the amount of immunoreactive insulin secretion (Fig. 7). This effect was quite specific, insofar as VPS21 overexpression could not increase secretory protein processing in wild-type yeast or in vps mutants bearing unrelated genetic defects (Fig. 7). Moreover, vps21Δ exhibited a synthetic defect in secretory protein processing with lte1, and such a synthetic defect could not be mimicked by deletion of one particular Vps21p GEF, vps9 (Fig. 8). The results provide genetic evidence that LTE1 interacts with VPS21 in a manner that is independent of VPS9. (Intriguingly in lte1 mutant cells, the synthetic defect exhibited by vps21Δ could be mimicked by several other vps mutants tested, including vps4, vps23, and pep12 (not shown), all implying activity in recycling of proprotein processing enzymes through the endosomal system.) Whereas several explanations are possible, a simple hypothesis is that Lte1p might function as a GEF for Vps21p within one endocytic or secretory subcompartment in cells during S-phase. Certainly there is a rapidly expanding number of potential GEFs being identified that appear to support the Vps21p GTPase or its mammalian homolog, Rab 5 (24).

A finding that was surprising (and we believe, quite distinct from that described above) was that overexpression of VPS21, which has not previously been implicated in mitosis, nevertheless rescued low temperature growth in lte1 mutant cells (Fig. 9). This was true both for lte1Δ cells and cells bearing the lte1-1012 truncation. Furthermore, vps21Δ exhibited a synthetic growth defect with lte1 at 17°C, and this genetic interaction with lte1 in mitosis could not be mimicked merely by cutting off VPS9-mediated GEF activity to Vps21p (Fig. 10). We propose that these unusual mitotic effects might derive from structural similarities between Vps21p and Tem1p (Table 3). Whereas we have no reason to believe that Vps21p plays a specific role in mitosis, we hypothesize that Vps21p might compete for certain interaction partners, such as GAP (GTPase-activating) proteins that may tonically inhibit Tem1p by converting the protein from an active GTP-bound form to an inactive GDP-bound form. The recruitment of such proteins to Vps21p might thereby increase the activity of Tem1p, facilitating low temperature mitotic progression in an lte1 background. Alternatively, VPS21 activity in endosomal trafficking might theoretically facilitate the localization of one or more anchoring proteins or regulators of the mitotic exit network, thereby enhancing Tem1p activation at the spindle pole body checkpoint. However, we favor the former hypothesis because rescue of low temperature growth of lte1 mutant cells is also achieved by overexpressing the inactive, persistently GDP-bound, Vps21p-S21L mutant (Fig. 11). Altogether, these findings underscore a secretory protein processing defect of lte1 mutant cells, the rescue of which specifically requires overexpressing an active Vps21p that can achieve the GTP-bound state (Fig. 11).

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