A New Pathway for Protein Export in *Saccharomyces cerevisiae*

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Abstract. Several physiologically important proteins lack a classical secretory signal sequence, yet they are secreted from cells. To investigate the secretion mechanism of such proteins, a representative mammalian protein that is exported by a nonclassical mechanism, galectin-1, has been expressed in yeast. Galectin-1 is exported across the yeast plasma membrane, and this export does not require the classical secretory pathway nor the yeast multidrug resistance–like protein Ste6p, the transporter for the peptide **a** factor. A screen for components of the export machinery has identified genes that are involved in nonclassical export. These findings demonstrate a new pathway for protein export that is distinct from the classical secretory pathway in yeast.

THE classical pathway for secretion of proteins from mammalian cells begins with the translocation of proteins with amino-terminal signal sequences from the cytosol into the lumen of the ER. From the ER, secretory proteins are transported within a series of membrane-enclosed secretory vesicles first to the Golgi apparatus, then through the Golgi stack, and finally to the cell surface (Palade, 1975). There are, however, exceptions to this classical scheme for protein secretion. Mammalian cells produce a variety of proteins that lack a secretory signal sequence, yet they are exported from cells, often after posttranslational processing (reviewed by Muesch et al., 1990; Mignatti and Rifkin, 1991; Rubartelli and Sitia, 1991; Kuchler and Thorner, 1992). Examples of such proteins are interleukin-1β (Rubartelli et al., 1990; Siders and Mizel, 1995), basic FGF (bFGF)[1] (Florkiewicz et al., 1995), thioredoxin (Rubartelli et al., 1992) , and galectin-1 (Cooper and Barondes, 1990). Pharmacological agents such as monensin or brefeldin A, which perturb Golgi function, do not block the release of these proteins (Rubartelli et al., 1990, 1992; Florkiewicz et al., 1995). It is not known if all nonclassically secreted proteins use similar mechanisms of export, but in many cases, cell lysis and cell damage have been ruled out (Cooper and Barondes, 1990; Rubartelli et al., 1990, 1992; Florkiewicz et al., 1995).

Nonclassical protein export has been implicated in many medically relevant contexts. For example, bFGF is a potent tumor-derived angiogenic factor that induces the formation of new capillary blood vessels (reviewed by Folkman and Klagsbrun, 1987). The export of bFGF via a nonclassical mechanism coincides with tumor neovascularization and progression into aggressive fibromatosis (Kandel et al., 1991). The release of angiogenic bFGF is of medical importance, since angiogenesis is also associated with metastasis (reviewed by Fidler and Ellis, 1994), and growth of tumor cells in vivo can be inhibited by anti-bFGF–immunoneutralizing anitbodies (Hori et al., 1991). Thioredoxin, an intracellular disulfide reducing enzyme, is also exported through a nonclassical mechanism (Rubartelli et al., 1992). ADF (adult T cell leukemia–derived factor), now known to be identical to thioredoxin, upregulates the expression of the IL-2 receptor in an autocrine fashion (Tagaya et al., 1989). ADF may be involved in the abnormal proliferation of T cells observed in certain types of leukemogenesis.

Given the existence of nonclassical export in mammalian cells, we have asked if a similar protein export pathway is present in *Saccharomyces cerevisiae* by expressing a mammalian nonclassical export substrate, galectin-1, in yeast. Galectin-1 is a homodimeric lactose-binding lectin that is highly expressed in muscle cells, motor and sensory neurons, thymus, kidney, and placenta, and export of galectin-1 from these tissues can both promote and inhibit cell adhesion (reviewed by Barondes et al., 1994). Furthermore, galectin-1 is expressed and constitutively externalized by CHO cells (Cho and Cummings, 1995). Galectin-1 was chosen for expression because it is small (monomer of 14 kD) and because it lacks posttranslational modifications other than an acetylated amino terminus. Yeast can acetylate the amino termini of heterologous proteins (Barr et al., 1988). Here, we describe the expression of mammalian galectin-1 in yeast. We demonstrate that galectin-1 is

The EMBL/GenBank/DDBJ accession numbers for NCE1, NCE2, and NCE3 are U41658, U41659, and U52369, respectively. Address all correspondence to Ann Cleves, UCSF Box 0534, University of California at San Francisco, San Francisco, CA 94143-0534. Tel.: (415) 476-4095. Fax: (415) 731-3612. e-mail: cleves@cgl. ucsf.edu

1. *Abbreviations used in this paper*: ABC, ATP-binding casette; bFGF, basic FGF; CWP, cell wall protein; hsp, heat shock protein.

exported from yeast cells by a novel nonclassical route and identify genes involved in this process.

Materials and Methods

Media and Reagents

Yeast complex and minimal media have been described (Sherman et al., 1983). Goat anti-rabbit antibodies conjugated to HRP were from Cappel Laboratories (Durham, NC). Protein G–Sepharose was from Pharmacia Fine Chemicals (Piscataway, NJ). ECL detection reagents and hyperfilm were from Amersham Corp. (Arlington Heights, IL).

Plasmids and Genetic Techniques

The *Escherichia coli* strain DH5α (GIBCO BRL, Gaithersburg, MD) was used for plasmid propagation. Plasmids pACY1 and pACY6 are multicopy yeast vectors with *URA3* as the selectable marker containing the rat galectin-1 and rat tag-galectin-1 cDNAs, respectively, under the control of the yeast *ADH1* promoter. Procedures for DNA transformation into yeast have been described (Ito et al., 1983). The *NCE2* cDNA was removed from the library plasmid pACYL17 as a 1.2-kb EcoRI to Bam HI fragment that was subcloned into the *E. coli* vector pBluescript (Stratagene, La Jolla, CA) to generate the plasmid pACE5. The *nce2* disruption was constructed by subcloning a 1.5-kb SspI fragment encoding the *HIS3* gene from the plasmid pRS303 (Sikorski and Hieter, 1989) into the unique SnaB1 site of pACE5 within the *NCE2* insert yielding plasmid pACE6. The disruption allele was linearized from the pBluescript vector with an EcoRI–BamHI digest of pACE6 that was used to transform yeast. His^+ transformants were assayed by Southern analyses for the presence of the *nce2* disruption allele. Southerns were performed using the Genius system from Boehringer Mannheim (Indianapolis, IN). The probe for the southern analyses was a 600-bp KpnI fragment from pACE5 containing 50 bp of the pBluescript vector and bases −181 to 340 of the *NCE2* cDNA.

Library Screen

The yeast strain WKK7 was cotransformed with the tag-galectin-1 expression vector pACY6 and a yeast cDNA library (McKnight and McConaughy, 1983). Ura^+, Trp^+ transformants were selected at room temperature. Transformants that appeared to exhibit wild-type growth were picked and then screened by Western blotting for high levels of tag-galectin-1 expression. Positive clones were recovered and reintroduced into yeast expressing galectin-1. Clones that seemed to increase galectin-1 export from strain WKK7 by a semiquantitative Western assay were examined further by the labeling export assay.

Radiolabeling and High pH Extraction of Yeast Cell Wall Proteins

Yeast were grown at 25°C or 30°C in selective minimal media for plasmid maintainence. 2.5 A_{600} units of cells (cultures at A_{600} ~2.0 for late log experiments or at A_{600} ~0.5–0.9 for early log experiments) were concentrated into 0.5 ml of media + protease inhibitors (1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml chymostatin final) and radiolabeled with 250 μCi Tran^{35}S-label (ICN Radiochemicals, Irvine, CA) for 10 or 20 min. For experiments with the *sec^{ts}* strains, cells were left at 25°C or shifted to 37°C for 30 min before radiolabeling to impose the temperature-sensitive block. Incorporation of radiolabel was stopped by the addition of 5 mM methionine, 1 mM cysteine (final concentration). After a 30-min chase, the cells were pelleted, rinsed once in 10 mM NaN_3, and immediately resuspended in extraction buffer (0.1 M Tris, pH 9.4, 10 mM DTT) and incubated for 15 min at 37°C. The cells were separated from the released proteins by centrifugation at 10,000 rpm for 5 s. To insure that no whole cells remained in the suspension, the supernatant was centrifuged through a 0.22-μm SPIN-X filter (Costar Corp., Cambridge, MA) that had been prerinsed with 0.1% BSA to prevent sticking to the filter. The released proteins were precipitated with TCA. For immunoprecipitations, after high pH extraction, cells and TCA-precipitated protein were resuspended in 50 μl 50 mM Tris, pH 7.5, 1.0% SDS. The cells were lysed by vortexing for 1 min with glass beads and then both cell-associated and extracted protein fractions were boiled for 3 min. 1 ml IP buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.1 mM EDTA, pH 8.0, 0.5% Tween 20) was added and the extracts were clarified by centrifugation at 14,000 rpm for 5 min. Total cpm in the cell-associated vs. the released protein fractions were determined in a scintillation counter. Rabbit anti-33 kD cell wall protein (CWP; obtained from R. Sentandreu, University of Valencia, Valencia, Spain) and rabbit anti-SEC14p (obtained from V. Bankaitis) antibodies were used at a 1:1000 dilution. Affinity-purified rabbit anti–galectin-1 and rabbit polyclonal anti-carboxypeptidase Y (obtained from T. Stevens, University of Oregon) were used at a 1:500 dilution. Rabbit anti-phospho-glycerate kinase serum (obtained from J. Thorner, University of California, Berkeley) was used at a 1:50,000 dilution. Immune complexes were recovered using protein G–Sepharose, and the beads were washed twice with IP buffer, twice with urea wash buffer (200 mM NaCl, 100 mM Tris, pH 7.5, 2.0 M urea, 0.5% Tween 20), and once with 0.1% SDS. Cells and released protein fractions were converted to SDS-PAGE samples and were resolved on 15% acrylamide gels. The gels were exposed to Amersham hyperfilm, or ^{35}S-labeled proteins were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Western blots were performed by standard methods.

Results

Exported Proteins Can Be Extracted from Yeast Using High pH

Many secreted proteins in yeast remain associated with the cell wall. We have found that secreted proteins can be effectively released from yeast by perturbing the cell wall with an extraction buffer that contains reducing agents (Orlean et al., 1986) and has a high pH (Reid, 1983). Although enzymatic digestion of the cell wall can also release cell wall–associated proteins, lyticase treatment resulted in too much variability in cell lysis for studying nonclassical export. After 15 min at 37°C in extraction buffer (0.1 M Tris, pH 9.4, 10 mM DTT), there was almost undetectable lysis of yeast cells. The percentage of total radiolabeled

Table I. Yeast Strains

Genotype	Source
W3031A (*MATa ura3-1 leu2-3,112 his3-11,15 trpl-1 ade2-1 can1-100*)	Rod Rothstein, Columbia University
WKK7 (*MATa ura3-1 leu2-3,112 his3-11,15 trp1-1 ade 2-1 can1-100 ste6Δ::HIS3*)	Kuchler et al., 1989
CTY182 (*MATa ura3-52 Δhis3-200 lys2-801$_{am}$*)	Bankaitis et al., 1989
CTY270 (*MATα ura3-52 sec 18-1^{ts}*)	NY432 from Peter Novick, Yale University
ACYD1 (*MATa ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*) (*MATα ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*)	JN173 from Jodi Nunnari, University of California, Davis
ACYD2 *nce2:: HIS3* derivative of ACYD1	This study
ACY1 *nce2::HIS3* derivative of W3031A	This study
ACY3 *nce2::URA3* derivative of CTY270	This study
CTY78 (*MATα ade2-101 ura3-52 Δhis3-200 lys2-801$_{am}$ Δtrp1 sec14-1^{ts}*)	Vytas Bankaitis, University of Alabama, Birmingham, AL

protein that was extracted under these conditions was 0.15% (n = 11, SE = 0.01%) for strains CTY182 (wild-type) and CTY270 (*sec18^{ts}*), and 0.34% (n = 17, SE = 0.03%) for strains W3031A (wild-type) and WKK7 (*Δste6*). The extraction of a reproducible and relatively low percentage of total labeled protein supported the hypothesis that the treatment was not causing lysis of the yeast, but rather, was removing a consistent fraction of the cell wall proteins. Furthermore, this procedure did not cause lysis because when cell-associated and extracted proteins were compared by SDS-PAGE for wild-type and *sec18^{ts}* yeast (Fig. 1 *A*), total cell-associated (lanes *1–4*) vs. extracted proteins (lanes *5–8*) were not identical.

Yeast possess at least one nonclassical export pathway, the peptide **a** factor transporter Ste6p. If nonclassical export pathways for proteins exist in yeast, there should be unique proteins secreted, even when the classical secretory pathway is blocked. Sec18p is the yeast homologue of *N*-ethylmaleimide–sensitive factor (NSF) (Wilson et al., 1989) and is required for the transport of proteins through the secretory pathway in vivo (Novick et al., 1980). In a *sec18^{ts}* strain, classical vesicular protein traffic can be blocked by shifting the growing cells from 25°C to 37°C (Novick et al., 1980). Fig. 1 *A*, lanes *4* and *8*, show the total and extractable proteins from a *sec18^{ts}* strain that had been shifted to the nonpermissive temperature before radiola-

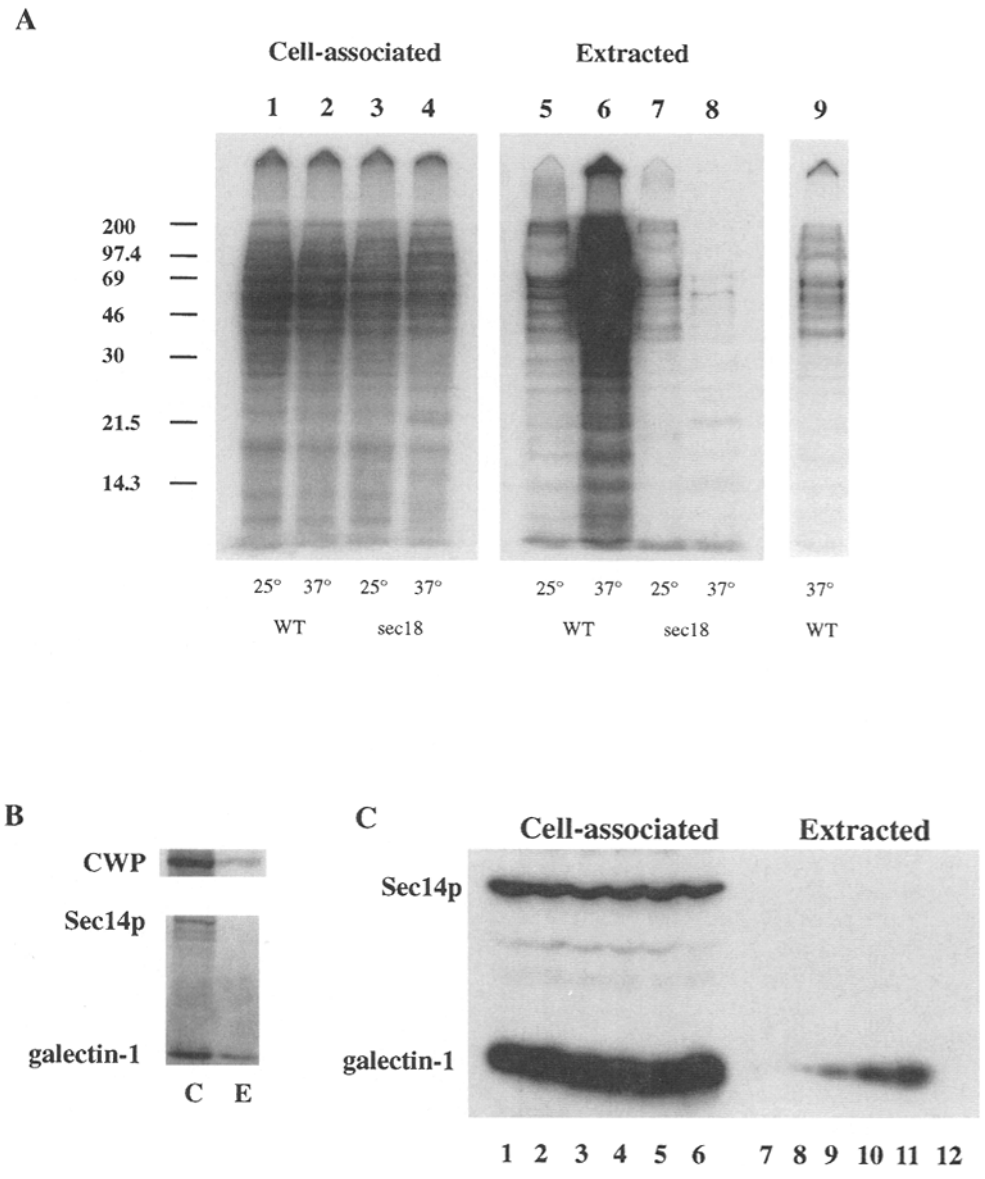

Figure 1. High pH extraction of yeast cell wall proteins. (*A*) Yeast strains CTY182 (*WT*) and CTY270 (*sec18^{ts}*) were shifted to 25°C (lanes *1*, *3*, *5*, and *7*) or 37°C (lanes *2*, *4*, *6*, and *8*), radiolabeled, and treated with high pH extraction buffer (0.1 M Tris, pH 9.4, 10 mM DTT), as described in Materials and Methods. The cell-associated proteins (lanes *1–4*) and extracted proteins (lanes *5–8*) from the wt and *sec18^{ts}* strains are shown. Note that the extracted protein profile is distinct from that of the cell-associated proteins, and that the extracted profile for the *sec18^{ts}* strain at 37°C is distinct from that at 25°C and the wild-type extracted profiles. The gels containing the cell samples and the extracted proteins were exposed to Amersham hyperfilm for 20 min (lanes *1–4*) and 5 h (lanes *5–8*), respectively. Lane *9* is a 20-min exposure of lane *6*. (*B*) A yeast cell wall protein and galectin-1 are high pH-extractable. Strain CTY182 expressing rat galectin-1 was radiolabeled and treated with high pH extraction buffer. Cells and released proteins were converted to immunoprecipitation samples. Galectin-1, SEC14p, and the 33-kD CWP were immunoprecipitated from the cell-associated (lane *1*) and the extracted protein (lane *2*) fractions. ^{35}S-labeled proteins were quantitated using a PhosphorImager. (*C*) Western blot showing extraction of galectin-1 from cells at steady state. Increasing amounts of galectin-1 were released from cells with time in extraction buffer (lanes *7–11*), but not in neutral pH buffer (lane *12*). CTY182 expressing galectin-1 was grown to $A_{600} \sim 2.0$. Cells were pelleted from growth media and immediately resuspended in extraction buffer at room temperature. Aliquots were removed at 0 (lanes *1* and *7*), 5 (lanes *2* and *8*), 10 (lanes *3* and *9*), 15 (lanes *4* and *10*), and 30 (lanes *5* and *11*) min, and cells (lanes *1–6*) were separated from released proteins (lanes *7–12*). As a control, one aliquot of cells was resuspended in 0.1 M Tris, pH 7.5, for 30 min and separated into cell-associated (lane *6*) and released protein (lane *12*) fractions. No detectable amount of the abundant cytosolic protein Sec14p was released by this treatment.

beling. A distinct profile of extractable proteins externalized in the absence of classical secretory pathway function suggests that several exported proteins in yeast do not require the classical secretory pathway for export. (This profile is also shown in Figs. 6 *F* and 7 *B*.) The increase in the total extractable protein in the wild-type strain at 37°C may reflect a faster secretion rate in yeast at the higher temperature. A shorter exposure of this sample (Fig. 1 *A*, lane *9*) reveals a similar profile of total extractable protein in the wild-type strain (independent of temperature) to that secreted by the *sec18*^{ts} strain at 25°C. These results show that secreted proteins are different from cell-associated proteins in yeast, and that the proteins released when the conventional pathway is blocked are different from those that are secreted when it is intact.

Galectin-1 Is Exported from Yeast

The evidence for residual protein secretion when the classical secretory pathway is blocked suggested that yeast might have an alternate protein export pathway. Data in support of this hypothesis were obtained by testing for galectin-1 export from yeast carrying a galectin-1 expression vector. Cell-associated and extracted protein fractions were assayed by immunoprecipitation, and the amount of a given protein in each fraction was quantitated by PhosphorImager analysis. Under late log phase growth conditions, significant amounts of labeled galectin-1 could be released from intact yeast with high pH. To estimate the efficiency of the high pH extraction, cell-associated (Fig. 1 *B*, lane *C*) and extracted protein fractions (Fig. 1 *B*, lane *E*) were also tested for the presence of an endogenous 33-kD CWP (Sanz et al., 1987). Quantitation indicated that 14.8% (n = 3, SE = 2.7%) of the CWP was removed from the cells by this method (Fig. 1 *B*, lane *E*). This was not caused by cell lysis since <1% of an abundant cytosolic protein, Sec14p (Bankaitis et al., 1989), was released (Fig. 1 *B*, lane *E*). In contrast, 12.5% (n = 4, SE = 0.6%) of galectin-1 was extracted (Fig. 1 *B*, lane *E*), demonstrating that about the same fraction of galectin-1 was extractable as an endogenous cell wall protein. The amount of galectin-1 extracted from CTY182 cells was dependent on the time of extraction, measured by Western blotting (Fig. 1 *C*). Increasing amounts of galectin-1 were released with incubation time in the high pH buffer, whereas the SEC14p was not released (Fig. 1 *C*, lanes *7–11*). 30 min of incubation in 0.1 M Tris, pH 7.5, did not release galectin-1 from yeast (Fig. 1 *C*, lane *12*). The amount of extractable galectin reached a plateau at 30 min at room temperature and 15 min at 37°C. The validity of the alkanization process was confirmed by using two other measures of externalization. Similar levels of galectin-1 could also be released from yeast by digestion of the cell wall with lyticase (data not shown), although lysis was occasionally observed. Secondly, galectin-1 could be labeled in whole cells by membrane-impermeable biotinylation reagents, confirming that galectin-1 was indeed exported across the plasma membrane, but was mostly trapped by the yeast cell wall (data not shown).

Galectin-1 Export Does Not Require the Classical Secretory Pathway

If galectin-1 is secreted by an alternative pathway in yeast, its secretion should not be markedly inhibited in a *sec18*^{ts} strain at 37°C (Novick et al., 1980; Fig. 2). As an internal control for *sec18p*^{ts} function, the maturation of the vacuolar protease CPY was monitored (Stevens et al., 1982). In the *sec18*^{ts} strain at 37°C, all of the CPY was recovered in the ER form p1 at 37°C (Fig. 2 *A*, lane *7*). In the *sec18*^{ts} cells at 25°C, 16.6% (n = 2, ± 2.4%) of galectin-1 was extractable (Fig. 2 *A*, lane *4*), similar to what was observed in wild-type cells (Figs. 1 *B* and 2 *A*, lane *2*). In the *sec18*^{ts} cells at the nonpermissive temperature of 37°C, externalization was not inhibited, since 43.4% (n = 2, ± 2.0%) was extracted (Figs. 2 *A*, lane *8*). This result indicated that the export of galectin-1 did not occur through the classical secretory pathway that uses the *SEC18* gene product.

Galectin-1 Export Does Not Require the Peptide Transporter Ste6p

A candidate for a molecule involved in nonclassical export of galectin-1 from yeast is the Ste6 protein. This member of the ATP-binding cassette superfamily of transporters, which includes the multidrug resistance protein, is an integral membrane protein that contains 12 α-helical membrane–spanning domains, 2 ATP-binding domains, and is known to transport the 12–amino acid lipopeptide **a** factor directly from the cytosol to the periplasm (Kuchler et al., 1989). Thus, it was reasonable to test the involvement of the Ste6p in nonclassical export of galectin-1 from yeast. We therefore expressed galectin-1 in *ste6* null yeast and an isogenic wild-type strain. The *ste6* mutation caused a reduction of galectin-1 secretion, but did not eliminate it (Fig. 2 *B*): 30.2% (n = 5, SE = 4.5%) and 20.2% (n = 5, SE = 1.6%) of galectin-1 was high pH extractable from wild-type (W3031A) and *Δste6* (WKK7) yeast, respectively. Galectin-1 was also externalized in α yeast strains (Fig. 2 *A*, lane *8*), cells that do not express Ste6p (an **a** cell-specific gene product). These data demonstrated that Ste6p is not essential for galectin-1 export.

An Epitope-tagged Rat Galectin-1 Is Exported from Yeast

To determine if an amino-terminally modified form of galectin-1 could be secreted by yeast, tag-galectin-1 was expressed in yeast. The tag consisted of 13 amino acids of the T7 phage gene 10 peptide at the extreme amino terminus plus 5 new amino acids from the construction followed by the entire 135–amino acid rat galectin-1. Western blot analyses revealed that the expression levels of the two forms of galectin-1 were significantly different in the wild-type strain W3031A that was used earlier (Fig. 3). Thus, CTY182 was used to determine the export efficiency of tag-galectin-1, since this strain expressed similar levels of the two forms of galectin-1. PhosphorImager analysis indicated that in a 20-min pulse and 30-min chase, about two times as much tag-galectin-1 was made as wild-type galectin-1 in strain CTY182. In a 15-min extraction at 37°C, 6.9% (n = 2, ± 2.9%) of tag-galectin was released from yeast compared to 12.5% for wild-type galectin-1. Since tag-galectin-1 was secreted, the authentic amino terminus of galectin-1 can be preceded by novel amino acids without preventing galectin-1 export from yeast.

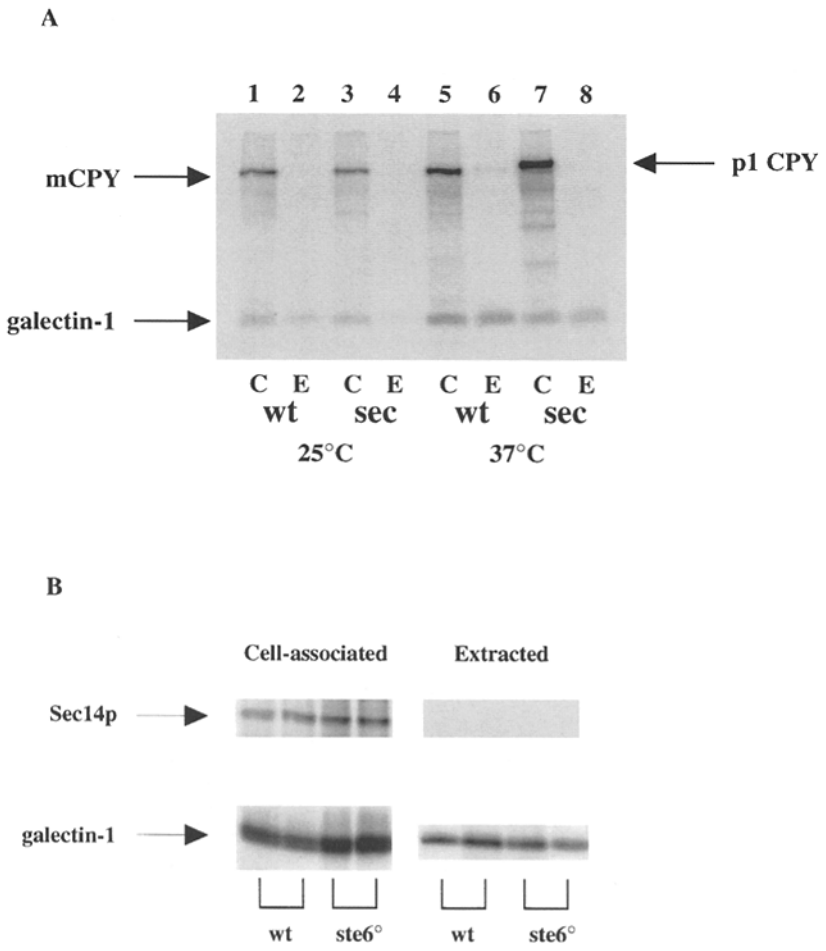

Figure 2. Galectin-1 is exported from yeast by a novel mechanism. (*A*) Galectin-1 export does not require the classical secretory pathway. Wild type (*wt*) (lanes *1*, *2*, *5*, and *6*) vs. *sec18*^{ts} (*sec*) (lanes *3*, *4*, *7*, and *8*) yeast at 25°C (lanes *1–4*) and 37°C (lanes *5–8*). Cell-associated (*C*; lanes *1*, *3*, *5*, and *7*) and extracted protein (*E*; lanes *2*, *4*, *6*, and *8*) fractions were prepared. Cells were radiolabeled and extracted as described in Materials and Methods. Cells and released protein fractions were assayed by immunoprecipitation for the presence of galectin-1, SEC14p (not shown), and CPY. The shift in mobility for p1 vs. mCPY appears small because a 15% acrylamide gel was used. The apparent increase in galectin-1 export at 37°C from wild-type cells was not consistently observed. (*B*) Ste6p is not required for galectin-1 export. Strains W3031A (*wt*), and WKK7 (*Δste6*) were radiolabeled and extracted as described in Materials and Methods. Cell-associated and released protein fractions were assayed by immunoprecipitation for the presence of SEC14p and galectin-1. Duplicate trials are shown for each strain. Less than 1.0% of the total SEC14p was released in all trials.

Screen for Gene Products Involved in Nonclassical Export

A screen for components of the nonclassical export machinery took advantage of a serendipitous observation. We observed that expression of tag-galectin-1 in wild-type strain W3031A gave very high levels of expression (Fig. 3), whereas expression of tag-galectin-1 in the isogenic *Δste6* strain WKK7 resulted in poor growth. Since Ste6p is known to be the transporter for the nonclassical export of peptide **a** factor, we hypothesized that poor growth in the *Δste6* strain was in some way related to nonclasssical protein export. If poor growth was caused by a defect in the export machinery in the *Δste6* strain, then normal growth might be restored by overexpressing gene products that are involved in nonclassical protein secretion. In that light, the *Δste6* strain WKK7 was cotransformed with the tag-galectin-1 expression vector pACY6 and a multicopy yeast cDNA library. The library was made from an α strain, which should lack the *STE6* cDNA (McKnight and McConaughy, 1983). Colonies that exhibited normal growth at 25°C were picked and the library plasmids were recovered. Plasmids that conferred normal growth upon reintroduction into *Δste6* strains that expressed high levels of tag-galectin-1 were examined further.

To determine if the library clones affected galectin-1 secretion, the recovered library plasmids were introduced into strains that expressed galectin-1. Using late log phase cultures ($A_{600} \sim 2.0$) for strains W3031A and WKK7, no significant difference was seen in the fraction of the total labeled galectin-1 released with high pH (20–30%) in the presence of the clones. Stimulation could be observed, however, in early log phase cultures that exhibited submaximal secretion. In early log cultures ($A_{600} \sim 0.5$–0.9), almost no detectable galectin-1 could be alkali extracted from the wild-type strain W3031A (0.67%, $n = 3$, SE = 0.04%) or the isogenic *Δste6* strain WKK7 (0.5%, $n = 5$, SE = 0.2%). The significant difference in the export efficiency of galectin-1 between early and later phases of growth could reflect a growth phase–dependent regulation of nonclassical export. Using early log cultures, the effect on nonclassical export of two of the library clones was revealed. The presence of either library plasmid pACYL07 or pACYL17 caused a 10-fold increase in galectin-1 export; high pH-extractable galectin-1 in WKK7 was increased from 0.5% to 6.8% for pACYL07 ($n = 5$, SE = 1.7%) and to 5.6% for pACYL17 ($n = 2$, ± 0.4) (Fig. 4). Thus, these clones appear to be affecting the nonclassical protein secretion machinery for galectin-1.

Novel Gene Products Involved in Nonclassical Export

We have named the clone07 and clone17 genes *NCE1* and *NCE2* (nonclassical export), respectively. The DNA and amino acid sequences of the *NCE1* and *NCE2* cDNAs are shown in Fig. 5. The *NCE1* cDNA encodes a 53–amino

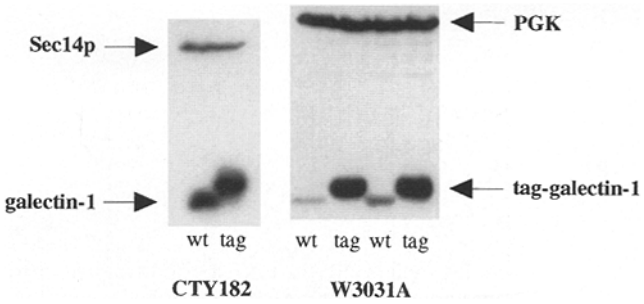

Figure 3. Expression of galectin-1 and tag-galectin-1 in yeast. The steady-state expression levels of normal and epitope-tagged galectin-1 are similar in CTY182, but very different in W3031A, the wild-type yeast strains used in this study. The W3031A data is shown in duplicate. The Western blots were performed by standard methods. SEC14p and PGK were probed to normalize the data.

acid protein. This was one of four novel proteins smaller than 100 amino acids identified in this screen (Cleves, A.E., and R.B. Kelly, manuscript in preparation). The *NCE1* sequence was confirmed by homology to the genomic sequence SCPRP21 in the database. SCPRP21 is a 36-kb genomic sequence on the left arm of yeast chromosome X (Purnelle et al., 1994). The sequence for *NCE1* lies between two hypothetical proteins, J0316 and J0318, but was not marked as an open reading frame. Interestingly, the genomic sequence of *NCE1* showed a 143-bp intron between nucleotides 32 and 33 of our cDNA sequence (i.e., between the second and third nucleotide of the Lys11 codon). The small size of the *NCE1* open reading frame and the intron are probably the reasons the open reading frame was not designated in the database sequence.

The *NCE2* cDNA contains a single open reading frame that encodes a 173–amino acid protein. The presence of in-frame stop codons upstream of the initiator Met indicated that the clone was a complete cDNA. In vitro translation of the NCE2 gene product resulted in a protein with an apparent molecular mass of ~21 kD on SDS-PAGE (Fig. 6 *A*), which was in good agreement with the predicted molecular mass of 19 kD. Examination of the primary sequence and hydropathy plot for the NCE2 protein (Fig. 6 *B*) revealed four potential membrane-spanning domains. A diagram of the NCE2 protein indicating these potential transmembrane domains is shown in Fig. 6 *C*. Database searches revealed no significant homologies to the NCE2 gene product.

To test the involvement of the NCE2 gene product in nonclassical export, a null allele was generated by inserting a 1.5-kb fragment encoding the *HIS3* gene into the unique SnaB1 site that cuts between bases 28 and 29 of the *NCE2* sequence within the 10th codon of the *NCE2* open reading frame. Diploid and haploid yeast were transformed to His$^+$ with linear DNA encoding the *nce2* null allele, and the presence of the disrupted allele in the genome was confirmed by Southern analyses (Fig. 6 *D*). The ability

A

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-19  ACACAAGATC ATCGATAACA TGGTTCAGTA CGCTCCCTTT CTATTAGGAA AATTTTCTGA   41
                        M  V  Q  Y  A  P  F  L  L  G  K  F  S  D    14

 42  TCCGTTATTG GCAATTATGG TAGGCTGTCT CAGCTACTAT GTGTACGAAA GGAAAATGGG  101
 15   P  L  L  A  I  M  V  G  C  L  S  Y  Y  V  Y  E  R  K  M  G     34

102  GCGTCCACAA GGTCATCATT TACACGAGCT CATAAAGAAG CGATGGGACG ATCGCAAATA  161
 35   R  P  Q  G  H  H  L  H  E  L  I  K  K  R  W  D  D  R  K  *     53

162  ATAATCCGGC GTGAGGGGAG TATATATAAG TTTCTGGCGA TATGTAAATA CCATAATAAT  221
      *

222  TAATCCTAGG ATCTACCTTT ATACGCATTT ATCCTATAAA AACCCAATCT TTCAGATCCA  281

282  CAGTATTTTT ATATGTGTGA TTTCCTTGAT TGTCTCA  318
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B

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-181  TTTGTTCGCA ATTGATCAAG AAAAAATACA ATTGAAAAGG TTTTACATTT TTAATTTTTC  -122

-121  TGCTCATCGC GCTTTTTTAA AAGGATAAAT AAACATTTCT TTAAAAAACA TCTTCAATAA   -62

 -61  GAAAAATCGG TTAAAAAAAC TTTTCTTCTC AAAGCATACC TAATAACAAT ATAATCCCAT    -2

  -1  AATGCTAGCC CTAGCTGATA ACATTCTACG TATAATAAAT TTCCTATTTT TGGTTATTTC    59
   1   M  L  A  L  A  D  N  I  L  R  I  I  N  F  L  F  L  V  I  S      20

  60  CATCGGTTTA ATCAGTTCGT TGTTAAACAC CCAACATAGG CACAGCTCCA GAGTAAACTA   119
  21   I  G  L  I  S  S  L  L  N  T  Q  H  R  H  S  S  R  V  N  Y      40

 120  CTGTATGTTT GCTTGTGCAT ATGGTATATT CACCGATTCA TTGTACGGTG TCTTTGCCAA   179
  41   C  M  F  A  C  A  Y  G  I  F  T  D  S  L  Y  G  V  F  A  N      60

 180  CTTCATTGAA CCATTGGCAT GGCCACTAGT TTTGTTCACA CTGGACTTTT TGAACTTTGT   239
  61   F  I  E  P  L  A  W  P  L  V  L  F  T  L  D  F  L  N  F  V      80

 240  GTTCACTTTC ACTGCCGGTA CAGTGTTGGC CGTTGGTATC AGAGCTCACT CATGTAACAA   299
  81   F  T  F  T  A  G  T  V  L  A  V  G  I  R  A  H  S  C  N  N     100

 300  CAGCTCATAC GTTGACAGTA ACAAGATTAC TCAAGGTTCC GGTACCAGAT GTAGACAAGC   359
 101   S  S  Y  V  D  S  N  K  I  T  Q  G  S  G  T  R  C  R  Q  A     120

 360  TCAAGCCGCT GTTGCATTCC TCTACTTCTC TTGTGCCATC TTTTTGGCTA AGACCCTGAT   419
 121   Q  A  A  V  A  F  L  Y  F  S  C  A  I  F  L  A  K  T  L  M     140

 420  GTCTGTTTTC AACATGATCT CCAATGGTGC CTTTGGTTCT GGTTCTTTCT CCAAGAGAAG   479
 141   S  V  F  N  M  I  S  N  G  A  F  G  S  G  S  F  S  K  R  R     160

 480  AAGAACTGGC CAAGTCGGTG TTCCAACCAT TTCCCAAGTC TAAATTTTAC GCCA  533
 161   R  T  G  Q  V  G  V  P  T  I  S  Q  V  *                  173
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Figure 5. Sequence of *NCE1* and *NCE2* cDNAs. Shown are the DNA and deduced amino acid sequences for the (*A*) *NCE1* and (*B*) *NCE2* cDNAs. Base 1 is the first base of the initiator codon. Stop codons are indicated with asterisks.

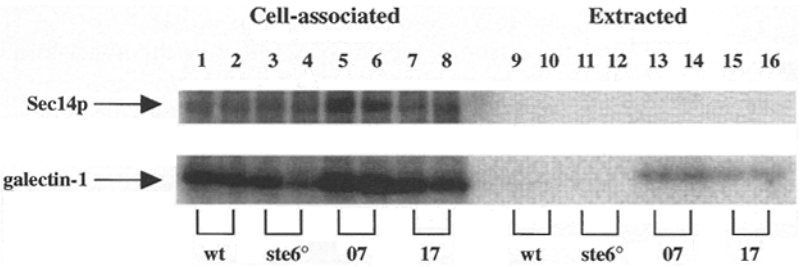

Figure 4. Gene products involved in nonclassical export. Galectin-1–expressing strains of W3031A (*MATa*, *wt*), WKK7 (*MATa*, *Δste6*), WKK7 bearing pACYL07, and WKK7 bearing pACYL17 were radiolabeled in the early log phase (A_{600} 0.5) as described in Materials and Methods, and samples were separated into cell-associated and extracted fractions. Fractions were probed with anti–galectin-1 and anti-Sec14p antibodies. Immunoprecipitates were resolved on 15% polyacrylamide gels and quantitated using a PhosphorImager. Samples are shown in duplicate.

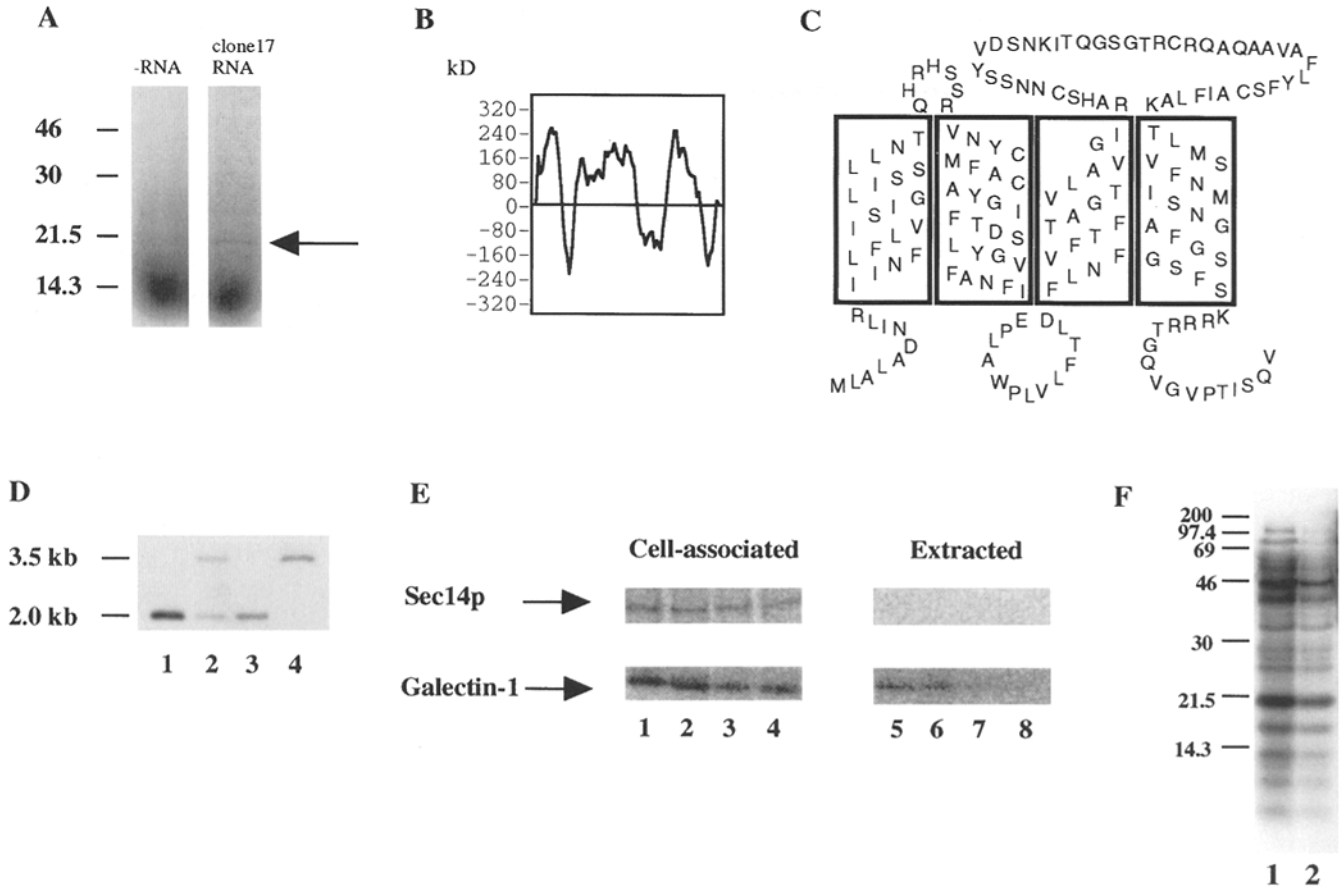

Figure 6. Model of the NCE2 gene product. (*A*) In vitro translation of the *NCE2* cDNA. The *NCE2* RNA was generated in vitro from the T7 promoter in the *E. coli* vector pBluescript (Stratagene) using the RiboMAX system (Promega). RNA was translated in vitro using wheat germ extract (Promega) and Tran^{35}S-label. An aliquot of each translation reaction was diluted in sample buffer and resolved on a 15% acrylamide gel. (*B*) Hydropathy analysis of the NCE2 protein. The inferred NCE2p amino acid sequence was used to generate a hydropathy profile by the method of Kyte and Doolittle (1982). (*C*) Model of the NCE2 protein with boxes indicating transmembrane domains. The four transmembrane domains correspond to the four peaks of hydrophobicity in the hydropathy plot. (*D*) Southern analyses of the *NCE2* gene. Genomic DNA was prepared from diploid strains ACYD1 (lane *1*) and ACYD2 (lane *2*), as well as haploid strains W3031A (lane *3*) and ACY1 (lane *4*). DNA was digested with EcoRI and BamHI, resolved in a 0.8% agarose gel, transferred to a nylon membrane, and probed with a fragment of the *NCE2* cDNA. The wild-type diploid (lane *1*) and haploid (lane *3*) strains reveal a 2.0-kb fragment representing the wild-type *NCE2* allele. The diploid bearing the *nce2* disruption shows the 2.0-kb wild-type allele and the 3.5-kb disruption allele containing the 1.5-kb HIS3 insertion (lane *2*). The haploid bearing the disuption allele shows only the 3.5-kb fragment (lane *4*). (*E*) Galectin-1 export in the absence of the NCE2 gene product. The galectin-1 export assay was performed on strain ACY1 carrying pACYL17 (lanes *1*, *2* and *5*, *6*) and the *nce2* disruption strain ACY1 (lanes *3*, *4* and *7*, *8*). Duplicate samples are shown. (*F*) Exported proteins in a *sec18^{ts}*, *nce2* null double mutant. *sec18^{ts}* (CTY270, lane *1*) and *sec18^{ts}*, *Δnce2* (ACY3, lane *2*) yeast were shifted to the nonpermissive temperature for 30 min and then radiolabeled and extracted as described in Materials and Methods. Samples were resolved on a 17% acrylamide gel.

to generate haploid yeast strain ACY1 with the *nce2* null allele as the sole copy of this gene demonstrated that the *NCE2* gene is not essential for yeast viability. Furthermore, the yeast strain ACY1 grew on solid media as well as the isogenic wild-type strain W3031A at 14°, 30°, and 37°C. To test the effects of the *nce2* disruption on nonclassical export, the strain ACY1 was transformed with the galectin-1 expression vector pACY1 and the galectin-1 export assay was performed on this strain. Using late log cultures, the strain ACY1 exhibited 8.2% (n = 10, SE = 1.0%) high pH extractable galectin-1 (Fig. 6 *E*, lanes *7* and *8*) compared to 30.2% for the wild-type strain W3031A (Fig. 2 *B*). Furthermore, the introduction of the *NCE2* expression vector pACYL17 into the *nce2* disruption strain restored secretion of galectin-1 to wild-type levels (28.0%, n = 2, ±3.5%) (Fig. 6 *E*, lanes *5* and *6*). The significant reduction in galectin-1 export observed in strain ACY1 suggests that the NCE2 gene product is involved in nonclassical protein export in yeast. Moreover, tag-galectin expression results in lethality in *nce2* null yeast, reminiscent of the growth defect observed in *ste6* null yeast, further establishing a link between *NCE2* and nonclassical export.

To test if any major endogenous yeast nonclassical export substrates require the NCE2 gene product for export, a *sec18^{ts}*, *nce2* null double mutant was constructed. The profile of total exported proteins for the double mutant was not significantly different than the profile from the *sec18^{ts}* strain (Fig. 6 *F*). This result, along with the fact that the *NCE2* gene is not essential for viability, suggests that redundant mechanisms for nonclassical export exist in

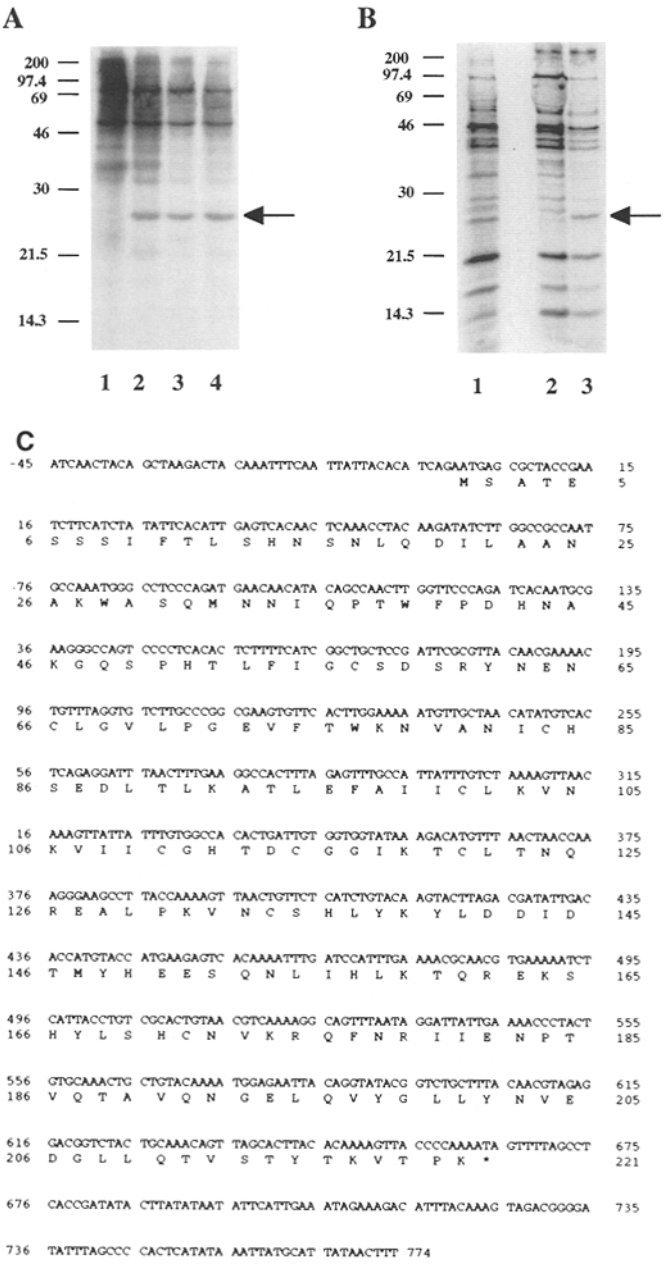

Figure 7. An endogenous yeast nonclassical export substrate. (*A*) Yeast strains bearing pACYL04 exhibit a novel 25-kD band (indicated by the arrow) in the profile of exported proteins. Shown are the high pH extractable protein profiles from wild type (W3031A) + library vector (lane *1*), wild type (W3031A) + pACYL04 (lane *2*), *Δste6* (WKK7) + pACYL04 (lane *3*), and *Δnce2* (ACY1) + pACYL04 (lane *4*). Samples were resolved on a 15% acrylamide gel. (*B*) The novel 25-kD band is a nonclassical export substrate. *sec18*^{ts} (lane *1*), *sec14*^{ts} + library vector (lane *2*), and *sec14*^{ts} + pACYL04 (lane *3*) yeast were shifted to 37°C for 30 min and then labeled and extracted as described in Materials and Methods. The arrow indicates the 25-kD band seen in the strain bearing pACYL04. Samples were resolved on a 15% acrylamide gel. Note: The novel 25-kD band is not seen in the *sec18*^{ts} profile (lane *1*). The three bands between 21.5 and 30 kD are seen in the *sec18*^{ts} (lane *1*) and *sec14*^{ts} (lane *2*) profiles, albeit at different intensities. The novel band indicated by the arrow migrates between the two lower bands of the triplet between 21.5 and 30 kD. (*C*) Shown are the DNA and deduced amino acid sequences for the *NCE3* cDNA. Base 1 is the first base of the initiator codon. The stop codon is indicated with an asterisk.

yeast. Furthermore, Fig. 7 *B* shows that the profile of nonclassical exported proteins from yeast is the same regardless of the particular *sec*^{ts} mutant tested (Fig. 7 *B*, lanes *1* and *2*). Also, the nonclassical export of proteins observed in the *sec*^{ts} strains cannot be attributed to Ste6p because both *sec*^{ts} mutants are α strains.

Identification of an Endogenous Yeast Nonclassical Export Substrate

One function of nonclassical protein export could be to rid the cytosol of proteins that are normally required or tolerated at low expression levels, but become toxic when present at high levels. In the library screen, galectin-induced toxicity could be relieved by overexpression of the nonclassical export machinery. In addition, overexpression of certain yeast proteins may indirectly upregulate the nonclassical export machinery. Thus, our screen might also have identified endogenous yeast cytosolic proteins whose overexpression is toxic.

If an overexpressed cytosolic protein is indeed removed from the cell by nonclassical export, then that protein should appear as a novel band in the profile of exported proteins. Such a novel protein band was detected among exported proteins from yeast carrying another cDNA isolated in our screen. Clone04 encodes a hydrophilic protein with a predicted molecular mass of 25 kD (Fig. 7 *C*). Wild-type strain W3031A carrying plasmid pACYL04 exhibits a novel band at ~25 kD in the profile of exported proteins (Fig. 7 *A*, lane *2*) compared to the profile from the same strain carrying the library vector only (Fig. 7 *A*, lane *1*). The novel 25-kD band is also exported in *Δste6* (Fig. 7 *A*, lane *3*) and *Δnce2* (Fig. 7 *A*, lane *4*) strains. The export of this protein in both the *Δste6* and *Δnce2* strains again suggests that there are redundant nonclassical export mechanisms in yeast and that deleting only one transporter will not result in a global defect in nonclassical export.

To verify that the novel 25-kD protein was exported by a nonclassical mechanism, plasmid pACYL04 was introduced into a *sec*^{ts} strain. As seen in Fig. 7 *B*, the profile of exported proteins from a *sec14*^{ts} strain at the nonpermissive temperature shows the novel band at 25 kD (lane *3*) compared to the profile from the same strain carrying the library vector only (lane *2*). This suggests that the 25-kD band is an endogenous substrate for yeast nonclassical export.

The cDNA encoded on plasmid pACYL04 has been named *NCE3*. The DNA and deduced amino acid sequences of *NCE3* are shown in Fig. 7 *C*.

Discussion

This work has shown that yeast have the machinery for nonclassical protein secretion in addition to the **a** factor transporter, Ste6p. To observe nonclassical secretion, it was necessary to use high pH treatment that reliably extracted the same percentage of total labeled protein without causing yeast cell lysis. A known substrate for nonclassical export, rat galectin-1, was stably expressed in and exported from yeast. Export assays performed in mutant backgrounds provide genetic evidence that galectin-1 export requires neither the classical secretory pathway nor Ste6p, a member of the multidrug resistance protein fam-

ily; therefore, a novel nonclassical protein export pathway is involved. The observation that mammalian galectin-1 is selectively exported from yeast suggests that the molecular machinery that is involved in nonclassical export of proteins may be evolutionarily conserved, similar to the homologies found in the classical secretory pathway (reviewed by Salama and Schekman, 1995; Bennett, 1995). It is possible, however, that the mechanisms of galectin-1 export from myoblasts and yeast are different.

Typical labeling conditions for yeast in which cultures were labeled in the early logarithmic phase (A_{600} 0.5–0.9) did not result in significant amounts of high pH-extractable galectin-1. One explanation of the growth phase dependence of high pH-extractable galectin-1 is that nonclassical export of proteins in yeast is a regulated pathway such as a stress response. The alternate explanation, that the extractability of galectin-1 varies with growth conditions, would require that both library clones *NCE1* and *NCE2* alter properties of the yeast cell wall rather than affect nonclassical export directly. This is unlikely, given that tag-galectin-1 expression is lethal in a *nce2* null strain.

An unexpected result was that blocking the classical secretory pathway enhanced nonclassical secretion in yeast. However, similar observations have been made in mammalian cells. Several unrelated reagents such as monensin, brefeldin A, dinitrophenol, carbonyl cyanide chlorophenylhydrazone, cycloheximide, and cytochalasin D cause an increase in IL-1β and thioredoxin export (Rubartelli et al., 1990, 1992). Rubartelli and Sitia (1991) suggested that the enhancement of nonclassical export might be caused by cellular stress, which results in increased heat shock protein (hsp) expression. It is reasonable that hsps may be involved in nonclassical export. hsp70 is a chaperone protein that is upregulated in response to heat shock and is believed to prevent aggregation of proteins (reviewed by Becker and Craig, 1994). Heat shock has been reported to increase the export of proteins through a nonclassical mechanism (Jackson et al., 1992; Rubartelli et al., 1990), and hsp70 has been demonstrated to be involved in the translocation of proteins across many membranes, including the ER, mitochondria, lysosome, nucleus, and peroxisome (Chirico et al., 1988; Deshaies et al., 1988; Chiang et al., 1989; Shi and Thomas, 1992; Walton et al., 1994).

A possible physiological function for nonclassical export is to remove toxic proteins from the cytoplasm. Ste6p is known to be involved in **a** factor secretion, and our data suggest that it may also participate in detoxification mechanisms. These two putative functions for Ste6p, peptide transport and detoxification, are not as unrelated as might first appear. The Ste6p is a member of the ABC family of transporters that function both as peptide transporters and in multidrug resistance (Higgins, 1992). A similar link between endogenous substrate transport and detoxification has been noted in the nervous system. The catecholamine transporter responsible for proton-driven biogenic amine uptake into chromaffin granules is capable of protecting cells from death by *N*-methyl-4-phenylpyridinium (MPP^+) poisoning (Liu et al., 1992). Furthermore, the sequence of the catecholamine transporter shows highest homology to transporters that confer tetracycline resistance to bacteria. Perhaps detoxification mechanisms and endogenous substrate transport are evolutionarily related.

Overexpression of tag-galectin results in a growth defect in cells that lack *NCE2* or *STE6*, a gene already implicated in nonclassical peptide secretion. Furthermore, overexpressing the *NCE2* gene suppresses the growth defect caused by the *ste6* deletion. Thus, the NCE2 gene product is implicated genetically in nonclassical protein secretion. Since it is a null mutation in *ste6* that is suppressed, it is likely that the NCE2 gene product participates in a parallel nonclassical secretion pathway. Redundant nonclassical export mechanisms would explain why (*a*) deletion of the *NCE2* gene has no detectable effect on the secretion of major labeled proteins in a $sec18^{ts}$ mutant at the nonpermissive temperature (Fig. 7 *A*); and (*b*) Ste6p is not required for the export of proteins when the classical secretory pathway is blocked (both sec^{ts} strains used in these experiments were α strains and thus do not express Ste6p). Whatever the explanation, the effect of tag-galectin-1 overexpression in the *ste6* null background allowed a genetic screen for genes that affect this hitherto unknown aspect of yeast cell biology. Three genes have already been identified. NCE2p is a candidate for a component of the export machinery, and NCE3p appears to encode an endogenous yeast nonclassical export substrate. The small *NCE1* gene product may be part of the export machinery, or, along with the other low molecular weight proteins identified in the screen, may also be a substrate for nonclassical export.

It is possible that the 173–amino acid NCE2 protein is a component of the nonclassical export machinery in yeast. There is precedent for the existence of small membrane proteins that function as transporters. "miniTEXANs" (mini toxin-extruding antiporters) are a family of bacterial proteins that are ~100 amino acids in length, contain four putative transmembrane domains, and confer antibiotic resistance most likely by active extrusion (Lewis, 1994). One of these proteins, EmrE, although only 110 amino acids in length, has been demonstrated to be an antiporter that exchanges protons with toxic cations such as ethidium and methyl viologen (Yerushalmi et al., 1995). Our model for the structure of the NCE2 gene product resembles models of the miniTEXANs. The acquisition of antibodies to the NCE2 gene product will allow us to test the hypothesis that the NCE2 protein is a multimembrane-spanning domain protein involved in nonclassical protein export. It should be noted that NCE2p may be an accessory subunit to an essential core component of the nonclassical export machinery or, alternatively, the role of NCE2p in nonclassical export may be indirect.

The development of this system is an important step in the study of nonclassical export mechanisms. The expression of galectin-1 in yeast made it possible to show genetically that the classical secretory pathway is not required for all protein secretion. A specific ABC transporter has been shown not to be required for galectin-1 export, and at least three gene products involved in nonclassical export, one a potential transporter and one a nonclassical export substrate, have been identified. The yeast system can now be used to screen for mammalian gene products involved in nonclassical export.

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