Sorting Signals That Mediate Traffic of Chitin Synthase III between the TGN/Endosomes and to the Plasma Membrane in Yeast

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Abstract
Traffic of the integral yeast membrane protein chitin synthase III (Chs3p) from the trans-Golgi network (TGN) to the cell surface and to and from the early endosomes (EE) requires active protein sorting decoded by a number of protein coats. Here we define overlapping signals on Chs3p responsible for sorting in both exocytic and intracellular pathways by the coats exomer and AP-1, respectively. Residues 19DEESLL24, near the N-terminal cytoplasmically-exposed domain, comprise both an exocytic di-acidic signal and an intracellular di-leucine signal. Additionally we show that the AP-3 complex is required for the intracellular retention of Chs3p. Finally, residues R374 and W391, comprise another signal responsible for an exomer-independent alternative pathway that conveys Chs3p to the cell surface. These results establish a role for active protein sorting at the trans-Golgi on route to the plasma membrane (PM) and suggest a possible mechanism to regulate protein trafficking.

Introduction
The selective packaging of transmembrane cargo proteins into transport vesicles requires specific recognition of signals within the cytosolic part of the cargo by coat protein complexes. Such a mechanism has been described for almost all intracellular vesicular transport events. Selective export of membrane cargo proteins from the endoplasmic reticulum (ER) is mediated by interaction of the Sec24 subunit of the COPII coat with diverse di-acidic or hydrophobic motifs, and retrograde transport from the cis-Golgi is driven by the recognition of C-terminal di-lysine motifs by the COP1 coat complex [1,2]. Later in the secretory pathway, the adaptor protein complexes (APs), Golgi-localized gamma ear-containing ARF-binding proteins (GGAs), and epsin-related proteins sort transmembrane proteins between the TGN, endosomes, and lysosomes [3–5]. These transport events are usually mediated by tyrosine-based sorting signals or di-leucine-based signals (for review [6]).

In addition to the conserved GGA adaptors, which transport proteins from the TGN to late endosomes, there are four AP complexes in mammalian cells and three in Saccharomyces cerevisiae (S. cerevisiae). All are composed of four subunits: one small subunit (σ1–4), one medium subunit (μ1–4), and two large subunits (β1–4 and y1, y2, δ3, or ε4) [7]. The AP-1 complex mediates trafficking between the TGN and endosomes [3], AP-2 mediates endocytosis [8], AP-3 directs proteins toward lysosomes in mammalian cells and the vacuole in yeast [9,10], and AP-4 may be involved in lysosomal and/or basolateral protein sorting [11,12] and in the selective transport of cargo from the TGN to the endosome [13].

The types of signals to which AP complexes and GGAs bind is best characterized in mammalian cells. The μ subunits of the APs bind to YXXΦ sorting signals (where Φ represents bulky hydrophobic amino acids) and a combination of the σ and γ (AP-1), δ (AP-2), or γ (AP-3) subunits bind to [D/E]XXXL[L/I/V] signals [14–16]. The GGA proteins bind to a smaller di-leucine signal, DXXL, in which there are two residues between the critical acidic and di-leucine residues instead of three [6]. Yeast adaptors recognize sorting determinants that are conserved and non-conserved with respect to their mammalian counterparts. In yeast and mammalian cells, AP-3 acts on [D/E]XXXL[L/I/V] signals [17,18]. Conversely, the yeast GGA proteins lack the binding pocket required to interact with DXXL signals and instead use ubiquitin as a signal [19,20]. Finally, at least two AP-1-dependent signals have been described thus far in yeast. The first is found in the protein DPAPA, encoded by STE13, and is of the non-canonical sequence, MSASTHISHKRRKN [21]. The second is found in the vacuolar membrane protein Snα2p and is of the sequence YSHL [22].

Protein transport from the TGN to the PM is mediated by a less well-understood process, as there are limited examples of specific

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recognition of cytosolic signals within cargos by coat protein complexes. In some cases export signals may not exist and traffic through this route may be by default or governed by the length of the transmembrane (TM) span, and in turn dictated by differences in the lipid composition of the TGN and PM [23]. Increasing or decreasing the length of the TMs of single transmembrane domain proteins, or the use of synthetic sequences of different lengths fused to soluble reporters showed that long TM domains direct PM localization in yeast and mammalian cells [24,25]. In other cases, however, signals are necessary for efficient transport from the TGN to the PM.

In mammalian cells, transport of Kir2.1, a member of the superfamily of inward rectifier potassium channels, constitutes a key example of selective export at the TGN for the surface expression of a native protein. In this case, two independent signals in the primary structure of the protein each contribute to cell-surface transport: basic residues in the cytosolic N terminus and a tyrosine motif within the cytosolic C terminus are both necessary for delivery to the PM. The coat proteins that recognize these motifs have not been identified [26]. Recently a non-linear signal in the quaternary structure of Kir2.1 has been shown to be involved in trafficking proteins from the TGN to the PM [27].

The exomer of S. cerevisiae is a novel coat protein complex involved in trafficking proteins from the TGN to the PM. This complex is composed of the proteins Chs3p and four paralogous ChAps (Chs3p-etr1p binding proteins), and is required for trafficking the type I cell-cell fusion protein Fus1p to the PM during the mating response [28]. Characterization of exomer-mediated trafficking of Fus1p revealed the presence of a non-linear signal in the cytosolic portion of the protein which is of the sequence IXTPK and is required for interaction of Fus1p with exomer and its trafficking to the PM during the mating response [28].

The exomer complex has also been shown to be required for transport of the major chitin synthase Chs3p from the TGN to the PM in a cell cycle regulated manner [29–31]. At steady state, Chs3p is localized at the bud neck but is also maintained in an intracellular reservoir by continuously cycling between the TGN and the EE [32,33]. Cells harboring individual deletions of CHS3 or the ChAP CHS6 or double mutations in the ChAP BCH1 and BUD7 fail to traffic Chs3p to the cell surface [34,35]. Additionally, Chs3p has been shown to physically interact with the exomer complex using both in vivo crosslinking experiments and in vitro pull down assays [30,31].

The intracellular pool of Chs3p is maintained by its cycling between the TGN and endosomes, dependent on the activity of the clathrin adaptors AP-1, Gga1p/Gga2p, and the epsin related proteins Ent3p/Ent5p [33,36]. In cells carrying mutations in these adaptors, Chs3p reaches the plasma membrane from the TGN or the EE by at least one alternative exocytic pathway that bypasses the requirement for exomer [35]. The sequences within Chs3p required for intracellular trafficking, exomer-dependent trafficking, and alternative exocytic transport remain to be identified. Here, we demonstrate that Chs3p contains specific information that is necessary for its transport through the exomer pathway, intracellular AP-1-dependent pathway, and the alternative exocytic pathway. Additionally, we show that yet another AP complex, AP-3, is involved in the intracellular retention of Chs3p.

**Materials and Methods**

**Growth Conditions**

Yeast cultures were grown in YPD (1% yeast extract, 2% peptone, 2% glucose), or synthetic complete (SC) dropout media (0.67% nitrogen base, 2% glucose, complete drop-out supplements (Q-biogene, Carlsbad, CA)). Resistance to calcofluor (CF) was assessed by growth on SC-Ura agar plates supplemented with 50 or 100 µg/ml Fluorescent Brightener 28 (calcofluor) (Sigma Chemical Co., St. Louis, MO). To prevent precipitation of CF in the SC-Ura+ CF agar plates, we adjusted the minimal medium near pH 7.0 by the addition of 0.7 M KH2PO4, pH 7.0 to a final concentration of 10%. Growth on 1/2 YPD agar medium (0.5% yeast extract, 1% peptone, 1% glucose, 1% agar) was used in the genetic selection that identified the DEESLL signal.

**Yeast Strain Construction**

Strains (Table 1) were constructed either by tetrads dissection of sporulated diploid strains or by integration of disruption cassettes that were generated from plasmid templates or pre-existing chromosomal deletions [37,38]. All allelic replacements were confirmed by PCR.

**Plasmid Construction**

Point mutations and deletions within CHS3 were introduced by QuickChange Mutagenesis (Stratagene, La Jolla, CA) using primers containing the desired changes and plasmid pJC345 as a template. Plasmid pJC345 contains a copy of CHS3 under the control of its own promoter inserted into the EcoR1/SalI sites of pRS316 [All plasmids used in this study are listed in Table 2].

**Quantitative Chitin Assays**

The protocol used for quantitative chitin assays was largely adopted from Bulik et al. [39]. Yeast cultures were grown in YPD to saturation, after which 35-50 mg of cells were collected for analysis. Yeast cells were lysed in 500 ul of 6% KOH at 95°C for 90 min. Cell wall material was sedimented for 10 min at top speed in a microcentrifuge. Pellets were rinsed with 1 ml 1× PBS, then 500 ul McIlvaine’s buffer (63% 0.2 M Na2HPO4, 37% 0.1 M citric acid), pH 6.0, and then resuspended in 100 ul McIlvaine’s buffer, pH 6.0, by sonication with a microtip sonicator. Chitin in the cell wall material was digested by addition of 8 ul 7 mg/ml chitinase from Trichoderma viride (T. viride) (Sigma Chemical Co., St. Louis, MO #C8241) at 37°C for 18 h. After incubation, the remaining cell wall was sedimented at top speed for 10 min. Quantification of the liberated GlcNAc was achieved with a colorimetric assay. Aliquots (10 ul) of sample were mixed with 10 ul of 0.27 M sodium borate, pH 9.0, and heated at 99°C for 10 min and then cooled on ice for 5 min. Immediately before use, DMAB solution (1 g p-dimethylaminobenzaldehyde in 1.25 ml concentrated HCL and 8.75 ml glacial acetic acid) was diluted 1:10 into glacial acetic acid and then 100 ul added to each tube. Tubes were incubated at 37°C for 20 min to allow development of color. Tubes were briefly chilled on ice and 90 ul of each reaction were aliquoted to a microtiter plate. The intensity of the reaction was measured at OD570 in a Dynatech MR3000 microtiter plate reader. Experimental samples were compared against a standard curve using reactions with pure GlcNAc solutions (0 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM, and 2 mM), and calculated as nmol of GlcNAc/mg cells. For each experiment, two to three replicates were conducted for each strain and used to determine the average amount of GlcNAc released per strain. Averages were normalized against wt values and graphed.
Sucrose Gradient Fractionation

The analysis of organelles by sucrose gradient fractionation was performed as described [40]. In brief, 10 OD600 units of mid-log cells were harvested by centrifugation and washed with ice-cold 20 mM Na_2HPO_4/20 mM KF. Cells were digested with lyticase and the resulting spheroplasts were lysed by osmotic shock with 0.35 ml of lysis buffer (5% sucrose in 20 mM triethanolamine, pH 7.2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Unlysed cells were removed by centrifugation (500 g, 20 min). Soluble cell lysates (15 ml) were incubated with 2 ml glutathione beads and incubated with 12 ml HKSG buffer containing 10 mM reduced glutathione. A total of 10 fractions were collected and protein concentration was measured by the Bradford assay. For all proteins purified for this experiment, more than 2 mg of GST-tagged protein were recovered with at least 90% purity.

Microscopy

Yeast cultures were grown to mid-log phase in SC-Ura and then fixed by addition of 37% formaldehyde to a final concentration of 4%, followed by incubation on ice for 30 min. Fixed cells were washed twice with 1 ml dH_2O and resuspended in 250 ul of a 50 ug/ml calcofluor solution for 30 min on ice. Cells were washed two times with 1 ml dH_2O and visualized with a DAPI filter on a Nikon epifluorescence microscope. Images were captured with a CCD camera and processed with Adobe Photoshop.

Chs3p/Chs5p Binding Experiments

Escherichia coli (E. coli) BL21(DE3)pLysS cells (Stratagene, La Jolla, CA) harboring plasmids containing GST-Chs3 fragments were grown in 500 ml cultures to an OD600 of 0.5–1.0, at which time IPTG was added to a final concentration of 250 mM. Soluble cell lysates (15 ml) were incubated with 2 ml glutathione agarose at 4°C for 3 h. Beads were washed three times with 10 ml PBSG buffer (phosphate buffer, pH 7.3, 140 mM NaCl, 2.7 mM KCl, 10% glycerol) followed by one additional wash in 10 ml HKSG (50 mM HEPES, pH 7.4, 50 mM KOAc, 200 mM sorbitol, 10% glycerol). Bound proteins were eluted with 12 ml HKSG buffer containing 10 mM reduced glutathione.

Tandem Affinity Purification (TAP) Tag co-purification of AP-1 and Chs3p

Two liters of each strain were grown overnight in SC-Ura medium to an OD 600 of ~1.0. Cells (1500 OD_600) were centrifuged, washed with 50 ml dH_2O, and resuspended in 40 ml of spheroplasting pre-treatment buffer (100 mM Tris, pH 9.4, 40 mM β-mercaptoethanol) and incubated at 30°C for 10 min. Treated cells were resuspended in 40 ml of spheroplasting buffer (0.7 M sorbitol, 0.75×YPD, 20 mM Hepes, pH 7.5, and 4 mM β-mercaptoethanol) and cells were converted to spheroplasts during a 45 min incubation at 30°C. Spheroplasts were washed in 40 ml cross-linking buffer (0.7 M sorbitol, 20 mM Hepes pH 7.5, and 125 mM KOAc) and then resuspended in 30 ml fresh cross-linking buffer. A 100 ml stock of DSP was prepared in DMSO immediately before use. DSP was added to the spheroplast suspension at a final concentration of 5 mM and incubated at room temperature for 30 min. To quench any

### Table 1. Strains used in this study.

| Name    | Genotype                                                                 | Reference |
|---------|--------------------------------------------------------------------------|-----------|
| YPH499  | MATa ade2-101oc his3-1200 leu2-11 lys2-801am trp1-ΔΔ3 ura3-52             | [52]      |
| JCY306  | MATa ade2 his3 leu2 lys2 trp1 ura3 chs3::LEU2                             | [50]      |
| SPY06   | MATa ade2 his3 leu2 lys2 trp1 ura3 chs3::LEU2 apl4::ΔTRP                  | This study|
| SPY21   | MATa ade2 his3 leu2 lys2 trp1 ura3 chs3::LEU2 apl4::ΔTRP                 | This study|
| SPY10   | MATa ade2 his3 leu2 trp1 ura3 chs3::LEU2 Chs5::KANMX                     | This study|
| TSY49   | MATa ade2-101oc his3-1200 leu2-11 lys2-801am trp1-ΔΔ3 ura3-52 chs6::His5  | This study|
| TSY131  | MATa prb1-1122 pep4-3 pcr1-407 gal2 trp1 ura3-52 AP51::S-Tag-Tev-ΔΔ3::KANMX | This study|
| TSY178  | MATa ade2-101oc his3-1200 leu2-11 lys2-801am trp1-ΔΔ3 ura3-52 chs6::His5  | This study|
| TSY194  | MATa ade2-101oc his3-1200 leu2-11 lys2-801am trp1-ΔΔ3 ura3-52 chs3::LEU2  | This study|
| TSY269  | MATa ade2-101oc his3-1200 leu2-11 lys2-801am trp1-ΔΔ3 ura3-52 chs6::His5  | This study|
| TSY300  | MATa ade2-101oc his3-1200 leu2-11 lys2-801am trp1-ΔΔ3 ura3-52 chs6::His5  | This study|
| RSY3393 | MAT7 ade2-101oc his3-1200 leu2-11 lys2-801am trp1-ΔΔ3 ura3-52 chs6::His5  | [36]      |

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remaining DSP, 2 M Tris, pH7.5, was added to a final concentration of 100 mM and the mixture was incubated for 15 min at room temperature. Treated spheroplasts were resuspended in 15 ml of lysis buffer (50 mM Hepes, pH 7.5, 1% Triton X-100, 0.4 M NaCl and protease inhibitors) and lysis was achieved with 15 strokes of a dounce homogenizer. Lysates were incubated on ice for 30 min to allow extraction of membrane proteins. Unbroken cells and large debris were removed from the lysates by centrifugation at 500 g for 10 min. The supernatant was further clarified by centrifugation at 13,000 g for 20 min. Proteins that bind nonspecifically to agarose beads were removed by incubation of the lysates with 2 ml CL-6B agarose at 4°C for 30 min. The resulting lysates were incubated with a 100 ul bed volume of IgG Sepharose for 3 h at 4°C. Beads were washed with 10 ml of lysis buffer three times. AP-1-TAP was eluted from the beads by incubation with TEV protease overnight at 4°C. To achieve the second round of purification, we incubated the elution sample with a 30 ul bed volume of S-protein agarose for 3 h at 4°C. Beads were washed with 1 ml of lysis buffer 5 times. Proteins were eluted with 30 ul of 2× sample buffer containing 100 mM DTT at 55°C for 10 minutes. Proteins were resolved on 10% polyacrylamide gels and total protein was visualized by staining with sypro-red, prior to transfer to PVDF membranes. Immunoblot analysis to determine the amount of co-purified Chs3 was carried out using anti-Chs3 antibodies.

Table 2. Plasmids used in this study.

| Name                  | Description                                      | Source                |
|-----------------------|--------------------------------------------------|-----------------------|
| pJC345                | EcoRI-SalI genomic fragment containing CHS3 cloned in pRS316 | [50]                  |
| pCHS3QC1              | W391R (Chs3W391R) mutation in pJC345             | This study            |
| pCHS3QC2              | W391L (Chs3W391L) mutation in pJC345             | This study            |
| pCHS3QC3              | W391F (Chs3W391F) mutation in pJC345             | This study            |
| pCHS3QC4              | W391S (Chs3W391S) mutation in pJC345             | This study            |
| pCHS3QC5              | W391A (Chs3W391A) mutation in pJC345             | This study            |
| pCHS3QC6              | W391T (Chs3R374T) mutation in pJC345             | This study            |
| pCHS3QC7              | W391A (Chs3R374A) mutation in pJC345             | This study            |
| pCHS3QC103            | N17AQ18A (Chs3N17AQ18A) mutation in pJC345       | This study            |
| pCHS3QC133            | D19A (Chs3D19A) mutation in pJC345               | This study            |
| pCHS3QC135            | E20A (Chs3E20A) mutation in pJC345               | This study            |
| pCHS3QC137            | E21A (Chs3E21A) mutation in pJC345               | This study            |
| pCHS3QC131            | S22A (Chs3S22A) mutation in pJC345               | This study            |
| pCHS3QC106            | L23A (Chs3L23A) mutation in pJC345               | This study            |
| pCHS3QC107            | L24A (Chs3L24A) mutation in pJC345               | This study            |
| pCHS3QC139            | R25A (Chs3R25A) mutation in pJC345               | This study            |
| pCHS3QC115            | N17AQ18A (Chs3N17AQ18A) mutation in pCHS3QC1     | This study            |
| pCHS3QC134            | D19A (Chs3D19A) mutation in pCHS3QC1             | This study            |
| pCHS3QC136            | E20A (Chs3E20A) mutation in pCHS3QC1             | This study            |
| pCHS3QC138            | E21A (Chs3E21A) mutation in pCHS3QC1             | This study            |
| pCHS3QC132            | S22A (Chs3S22A) mutation in pCHS3QC1             | This study            |
| pCHS3QC118            | L23A (Chs3L23A) mutation in pCHS3QC1             | This study            |
| pCHS3QC119            | L24A (Chs3L24A) mutation in pCHS3QC1             | This study            |
| pCHS3QC144            | R25A (Chs3R25A) mutation in pCHS3QC1             | This study            |
| pD15                  | chs3p deleted of N-terminal 15 AA (Δ15CHS3) in p416MET25 | This study            |
| pD15WR                | W391R (Δ15CHS3(W391R)) mutation in pD15          | This study            |
| pD25                  | chs3p deleted of N-terminal 25 AA (Δ25CHS3) in p416MET25 | This study            |
| pD25WR                | W391R (Δ25CHS3(W391R)) mutation in pD25          | This study            |
| pJC322                | N-terminal 171AA of Chs3p GST-tagged (Chs3(1–170)) in pGEX-2T | [50]                  |
| pCHS3QC164            | D19AE21A (Chs3(1–170)D19AE21A) mutation in pJC322 | This study            |
| pCHS3-2GST            | Second cytosolic loop of Chs3p GST-tagged (Chs3(224–451)) in pGEX-2T | This study            |
| pTS11                 | L24H (Chs3L24H) mutation in pJC345               | This study            |
| pTS13                 | L24F (Chs3L24F) mutation in pJC345               | This study            |
| pTS15                 | L24P (Chs3L24P) mutation in pJC345               | This study            |
| pTS18                 | D19N (Chs3D19N) mutation in pJC345               | This study            |
| pTS166                | DEESLLLΔ (Chs3-DEESLLLΔ) mutation in pJC345      | This study            |

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Results
Identification of a Sorting Signal Required for the Intracellular Retention of Chs3p

We have previously reported that a number of clathrin adaptor proteins are involved in transporting Chs3p between the TGN and endosomes [33,36]. To better understand how these intracellular transport pathways are achieved we conducted a genetic selection to identify the sorting signals in Chs3p that mediate these events. We relied on the fact that mutations that inhibit Chs3p intracellular trafficking cause Chs3p to engage in an exomer-independent alternative exocytic pathway [33]. Deletion of the exomer component CHS6 blocks Chs3p localization to the PM, which in turn results in greatly reduced levels of chitin in the cell wall. Phenotypically, chs6Δ strains are resistant to the toxic effects of the chitin binding drug calcofluor and are sensitive to growth on ½ YPD, a hypo-osmotic medium. Restoration of Chs3p to the PM of a chs6Δ strain, via the alternative exocytic pathway, results in cells that are sensitive to calcofluor and resistant to growth on ½ YPD [33]. We reasoned that mutations in the sorting signal required for intracellular retention of Chs3p would allow Chs3p to localize to the PM of chs6Δ cells, conferring calcofluor-sensitive and ½ YPD-resistant phenotypes.

To carry out the selection, we randomly mutagenized a low copy plasmid that carried wt CHS3, under the control of its own promoter, by propagating the plasmid in XL1-Red E. coli cells (Stratagene, La Jolla, CA), a strain of E. coli that is deficient in DNA repair pathways. Mutagenized plasmid pools were transformed into an chs6Δ strain, and transformants were replicated onto ½ YPD. Colonies resistant to this medium were further tested for sensitivity to medium containing calcofluor. Using this method, four alleles of CHS3 that conferred resistance to ½ YPD and sensitivity to calcofluor in the chs6Δ chs6Δ strain were isolated (Figure 1A). The mutations coded for the following amino acid substitutions: D19N, L24H, L24F, and L24P. These were isolated (Figure 1A). The mutations coded for the following amino acid substitutions: D19N, L24H, L24F, and L24P. These were isolated (Figure 1A).

To confirm that the mutant variants of Chs3p were able to localize to the PM of chs6Δ chs6Δ cells, we assayed chitin in the cell wall by staining cells with calcofluor (Figure 1B). During the cell cycle Chs3p synthesizes a ring of chitin around the mother-bud neck, marking sites of current and previous cell division. In cells expressing Chs3p at the PM, staining with calcofluor reveals numerous bright chitin rings, whereas in cells that lack PM-localized Chs3p, such as chs6Δ cells expressing wt Chs3p, chitin rings are absent. Figure 1B shows that, as expected, chitin was absent from the cell walls of chs6Δ chs6Δ cells harboring a plasmid expressing wt CHS3, but present in cells expressing the Chs3-2L4P point mutant, confirming that the DEESLL signal is required for the intracellular retention of Chs3p.

In signals of this type the most critical residues are the first acidic residue and the di-leucine residues [6]. We tested if this was the case for the DEESLL signal by conducting alanine-scanning mutagenesis of nucleotides that correspond to amino acid residues 17 through 25 of Chs3p, and assayed for the ability of these mutants to confer a calcofluor sensitive phenotype to chs6Δ chs6Δ cells (Figure 1C). Of all the alanine substitutions tested, only L23A and L24A resulted in calcofluor sensitivity when introduced in chs6Δ chs6Δ cells (Figure 1B). Interestingly, the D19A mutation had no obvious effect, even though the substitution D19N was isolated in our ½ YPD selection (Figure 1A). These results indicate that the aspartic acid residue plays only a minor role in the intracellular trafficking of Chs3p, whereas the leucine residues are essential.

The DEESLL Signal is Required for a Physical Interaction between AP-1 and Chs3p

The DEESLL signal matches the consensus motif for AP binding, and we have previously shown that AP-1 and Chs3p physically interact [6,36]. Thus, it seemed likely that AP-1 might interact with this sequence to facilitate intracellular transport of Chs3p. To test this hypothesis, we utilized a strain in which CHS3 was deleted and the smallest subunit of AP-1 (Apl1p) was fused to a tandem affinity purification tag [36]. Into this strain we transformed a plasmid expressing either wt Chs3p or Chs3-DEESLLA. We then added the crosslinking agent DSP to living cells to stabilize the interaction between AP-1 and the Chs3p variants and subsequently purified AP-1 from yeast cells. As demonstrated previously [36], wt Chs3p co-purified with AP-1 (Figure 2). In contrast to the result with wt Chs3p, Chs3-DEESLLA failed to co-purify with AP-1, demonstrating that the DEESLL signal is required for the physical interaction of Chs3p and AP-1 (Figure 2).

The AP-3 Complex Is Required for the Intracellular Retention of Chs3p

We previously reported that AP-2 and AP-3 do not have roles in transporting Chs3p [33]. However, during the course of our studies we re-examined this issue. We introduced mutations in the β2 subunit of AP-2 (apl2Δ) and the β3 subunit of AP-3 (apl3Δ) into a chs6Δ strain and tested the ability of these mutations to confer calcofluor sensitivity (Figure 3A). When grown on rich YPD medium containing 50 μg/ml calcofluor, strains harboring the (AP-2Δ) and (AP-3Δ) mutations exhibited unaltered calcofluor-resistance. However, on synthetic complete (SC) minimal medium the chs6Δ AP-3Δ cells were partially calcofluor-sensitive.

To resolve the discrepancy between the YPD and SC media, we conducted qualitative chitin assays to determine the extent to which chitin was restored to the cell walls of these strains. To conduct these assays, we lysed cells in an alkaline solution from
which cell wall material was recovered in an insoluble fraction. This material was digested with chitinases to yield N-acetyl-
glucosamine (GlcNAc), which was quantified using a colorimetric
assay. As expected, a chs6Δ mutant, where Chs3p is not targeted to
the PM, had low levels of chitin in the cell wall, compared to wt
(Figure 3B). The chs6Δ AP-2Δ strain had low levels of chitin
equivalent to the parental chs6Δ strain, confirming that AP-2 does
not play a significant role in trafficking Chs3p. Conversely, the
chs6Δ AP-1Δ strain had a level of chitin that was much greater
than that of the chs6Δ single mutant, and interestingly, the chs6Δ
AP-3Δ strain contained a level of chitin that was intermediate
between that of the chs6Δ single mutant and the chs6Δ AP-1Δ
mutant. This result indicated that AP-3 is important for the
intracellular transport of Chs3p, but plays a less significant role
than AP-1. The lower level of chitin in chs6Δ AP-3Δ cells may
explain why this strain did not show obvious calcofluor
sensitivity on YPD. Our general experience with chitin assays is that growth in SC medium leads to higher levels of chitin in the cell wall compared to growth in YPD. The reason for this difference is unknown, but may explain why the chs6Δ AP-3Δ strain showed partial calcofluor sensitivity on SC medium but not YPD.

We also wished to determine if AP-1 and AP-3 function in the same or distinct pathways of Chs3p transport. We reasoned that if these adaptors function in the same pathway, the amount of chitin restored to the cell wall of chs6Δ AP-1Δ AP-3Δ cells would not exceed that of the chs6Δ AP-1Δ mutant. Conversely, if these adaptors function in distinct pathways of Chs3p transport, the effects of the APA mutations should be additive, resulting in more chitin in the cell walls of the chs6Δ AP-1Δ AP-3Δ mutant than the chs6Δ AP-1Δ or the chs6Δ AP-3Δ mutant. Figure 3 shows that, when averaged from five different experiments, we did not observe a significant enhancement of chitin levels in the triple mutant compared to the double mutant. Based on these findings

we conclude that AP-1 and AP-3 may serve overlapping functions in transport of Chs3p.

Finally, it is important to note that at a higher calcofluor
concentration (100 µg/ml) the chs6Δ AP-2Δ strain was sensitive to
growth on YPD + calcofluor. The quantitative chitin assay in
Figure 3 demonstrated that this strain had a low level of chitin,
therefore, Chs3p does not traffic to the PM of this strain. We
presume that the sensitivity of this strain to high calcofluor
concentrations may be caused by other defects imparted by the
ap1Δ1 mutation. Taken together we conclude that AP-2 plays no
significant role in trafficking Chs3p, whereas AP-3 plays an
important, although less significant role than AP-1 in Chs3p
transport.

Mutations in Residues R374 and W391 Impair the Transport of Chs3p to the PM through the Alternative
Exocytic Pathway

In this and previous studies we relied on the transit of Chs3p
through the alternative exocytic pathway to characterize the role
of coat protein adaptors and the DEESLL signal in the
intracellular trafficking of Chs3p [33,36]. To better understand
the nature of the alternative exocytic pathway, we conducted a
genetic screen to isolate chs3Δ alleles specifically unable to access
this route. As before, this selection was based on the calcofluor
phenotype. As noted previously, chs6Δ AP-1Δ cells are sensitive to
calcofluor. We reasoned that a mutation in the signal required for
transit of Chs3p through the alternative exocytic pathway would
confer calcofluor-resistance to chs6Δ AP-1Δ cells. A library of
randomly mutagenized chs3Δ was created by error-prone PCR and
introduced into chs3Δchs6Δ AP-1Δ (ap1Δ47) cells on a low copy
plasmid, under the control of its own promoter. Clones from this
library, producing Chs3p unable to reach the PM through the
alternative exocytic pathway, were selected for their calcofluor-
resistant phenotype. Plasmids from calcofluor-resistant transfor-

Figure 2. The DEESLL signal is required for a physical interaction between AP-1 and Chs3p. Cells of the genotype chs3Δ pep4Δ pth1Δ prc1Δ1 AP3-TAP (TSY131), expressing either wt Chs3p or Chs3-DEESLLA were grown overnight, converted to spheroplasts, and treated with the crosslinking agent DSP prior to lysis and purification of the AP-1 complex via a TAP tag. Samples were separated on a 10% polyacrylamide gel, which was visualized with sypro red stain prior to transfer to a PVDF membrane for evaluation by immunoblot. Shown is a western for Chs3p and a total protein stain for the AP-1 purification. Note that Chs3-DEESLLA does not copurify with AP-1.
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Figure 3. AP-3 is required for the intracellular retention of
Chs3p. (A) Ten-fold serial dilutions of wt (YPH499), chs6Δ (TSY49),
chs6Δ ap1Δ1 (RSY3393), chs6Δ ap1Δ4 (TSY300) and chs6Δ ap1Δ1
(TSY269) cells on YPD, YPD + 50 µg/ml calcofluor (YPD + CF), synthetic complete medium (SC), and synthetic complete medium +50 µg/ml calcofluor (SC + CF). (B) An average of 5 quantitative chitin assays. Cultures were grown to saturation and cell walls were isolated by lysing 35–50 mg of cells in 6% KOH. Chitin was digested with chitinase from T. viride and the resulting GlnAc was quantified. For each assay the results of 2–3 replicates of each strain were averaged and normalized against the wt level of chitin. Five separate normalized experiments were then averaged together.
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randomly mutagenized chs3Δ was created by error-prone PCR and
introduced into chs3Δchs6Δ AP-1Δ (ap1Δ47) cells on a low copy
plasmid, under the control of its own promoter. Clones from this
library, producing Chs3p unable to reach the PM through the
alternative exocytic pathway, were selected for their calcofluor-
resistant phenotype. Plasmids from calcofluor-resistant transfor-
mants were recovered and introduced into a \textit{chs3\_A} strain. We selected for further characterization only those clones that were able to complement the calcofluor-resistant phenotype of \textit{chs3\_A}. The ability to rescue the \textit{chs3\_A} phenotype indicated that the mutated copy of \textit{CHS3} encoded a stable and active Chs3p protein that was able to exit the ER and, more importantly, traffic to the PM through the normal exomer-dependent pathway. Each plasmid recovered in the initial screen contained multiple mutations in the \textit{CHS3} sequence, but after each mutation was individually reproduced by site-directed mutagenesis, a single mutation, W391R, conferred the expected phenotype: calcofluor resistance in \textit{chs3\_A chs6\_A AP\_1\_A} cells and calcofluor sensitivity in \textit{chs3\_A} cells (Figure 4A).

The W391R mutation represents a dramatic substitution, which could induce a conformational change that would impair the accessibility of a signal situated nearby. To discriminate between these two possibilities, we tested the effect of less drastic substitutions at W391. Whereas the most conservative substitution, W391F, conferred an intermediate phenotype, W391A, W391L, and W391S, when expressed in the \textit{chs3\_A chs6\_A AP\_1\_A} strain, presented a calcofluor-resistant phenotype comparable to that of W391R (Figure 4A).

The amino acid residues D(387)LLD(390), found immediately upstream of W391, resemble a canonical di-leucine signal. The potential involvement of L389 could not be assessed because L389A and L389S mutations destabilized the Chs3p protein. However, mutation of any of the other residues in this sequence

![Figure 4. Mutations in residues R374 and W391 specifically impair access to the alternative exocytic pathway.](image-url)

(A) Ten-fold serial dilutions of \textit{chs3\_A} (RSY1699) or \textit{chs3\_A chs6\_A AP\_1\_A} (SPY10) cells expressing alanine-substitution mutants of \textit{CHS3} were spotted onto synthetic complete medium -Ura + 100 \textmu g/ml calcofluor -Ura + CF. (B) Subcellular fractionation of wt Chs3, Chs3-W391R and Chs3-R374A expressed in \textit{chs3\_A} and \textit{chs3\_A chs6\_A AP\_1\_A} cells on step sucrose/EDTA gradients. Total membranes from spheroplasts were separated on a step sucrose/EDTA gradient. The protein composition of fractions obtained from differential centrifugations and sucrose gradients were analyzed by SDS/PAGE and immunoblotting (PM marker: Pma1p; Golgi/EE markers: Tlg1p).

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resistance when introduced in the tions for W391, both R374A and R374T showed calcofluor therefore, examined the role of R374 in promoting access of residue is located 17 residues upstream of the critical W391. We, Although Chs3p does not contain a canonical EH domain, an Arg upstream from the Trp also plays a critical role in NPF binding. motifs, where a conserved tryptophan residue within the pocket is absolutely necessary for NPF binding [44]. In addition to this critical Trp, another conserved residue (R/K) located 16 residues upstream from the Trp also plays a critical role in NPF binding. Although Chs3p does not contain a canonical EH domain, an Arg residue is located 17 residues upstream of the critical W391. We, therefore, examined the role of R374 in promoting access of Chs3p to the alternative exocytic pathway. As with the substitutions for W391, both R374A and R374T showed calcofluor resistance when introduced in the chs3Δ chs6Δ AP-1A strain and a calcofluor-sensitive phenotype in the chs3Δ strain (Figure 4A), suggesting that R374 and W391 were part of the same signal and that this signal was specific to the alternative exocytic pathway.

To test the Chs3p traffic defect of these mutants, we performed membrane fractionation on sucrose/TEA gradients under conditions where intracellular endosomal/Golgi membranes were separated from the PM (Figure 4B). For these experiments we compared the localization of either wt Chs3p, Chs3W391R or Chs3R374A in chs3Δ or chs3Δ chs6Δ AP-1A cells. In chs3Δ cells ~40% of wt or the mutant variants of Chs3p co-fractionated with the PM marker Pma1p, which confirmed the observation that the mutants were able to access the normal exomer-dependent pathway. Conversely, in the chs3Δ chs6Δ AP-1A strain, ~50% of wild type Chs3p was present in the PM fractions, whereas Chs3W391R was not detected in those fractions and only ~15% of the Chs3R374A mutant protein co-fractionated with Pma1p. These localization results supported the phenotypic observation that Chs3W391R and Chs3R374A mutant proteins were deficient in traversing the alternative exocytic route.

Identification of a Signal Required for the Exomer-Dependent Transport of Chs3p

Having identified a signal that mediates transport of Chs3p via the alternative exocytic pathway, we applied a similar approach in an attempt to isolate CHS3 alleles impaired for transport to the PM through the exomer pathway. However, we were unable to isolate point mutant alleles by this approach, and therefore chose truncation analysis as an alternative. Deletion of a region between amino acids 15 and 25 identified a segment essential for normal sorting of Chs3p.

We examined which exocytic pathway, the exomer pathway or the alternative exocytic pathway, N-terminally truncated forms of Chs3p use for their transport to the PM. Deletion of the N-terminal 15 or 25 amino acids produced stable and active proteins, capable of leaving the ER and of being transported to the PM, as shown by the calcofluor-sensitive phenotype they conferred on chs3Δ cells (Figure 5A). It was unclear, however, if these proteins arrived at the PM strictly via the exomer pathway, the alternative exocytic pathway, or both. As previously demonstrated, the DEESLL signal resides at residues 19–24, and variants of Chs3p carrying mutations in this signal engage in the alternative exocytic pathway. Thus, if the signal required for exomer-dependent traffic resided within this region any trafficking defect imparted by deleting it may have been masked by the transit of Chs3p through the alternative exocytic pathway. To test this we expressed Δ15Chs3 and Δ25Chs3 in chs3Δ chs6Δ cells and assayed the calcofluor phenotype. Cells expressing either wt Chs3p or Δ15Chs3 in the chs3Δ chs6Δ strain were resistant to calcofluor (not shown). This demonstrated that the first 15 amino acids of Chs3p contained no information required for intracellular retention of the protein and thus Δ15Chs3 arrives at the PM solely by the exomer-dependent pathway. In contrast to this, the absence of the DEESLL signal in Δ25Chs3 allowed this protein to access the PM via the alternative exocytic pathway, as confirmed by the calcofluor-sensitive phenotype (not shown). This complicated our ability to test the Δ25Chs3 N-terminal truncation mutant for dependence on exomer. To obviate this problem and determine if Δ25Chs3 relied on both the exomer and alternative exocytic pathways or only one, the W391R mutation, which blocks access to the alternate route, was introduced into the truncation constructs. In chs3Δ cells, expression of Δ15Chs3-W391R conferred a calcofluor-sensitive phenotype, confirming that access of this variant to the PM was independent of the alternative exocytic pathway, and thus reliant on the exomer pathway (Figure 5A). In contrast, cells expressing Δ25Chs3-W391R in a chs3Δ strain were resistant to calcofluor, demonstrating that Chs3p lacking the first 25 residues was transported to the PM strictly through the alternative pathway and, due to the loss of residues 16–25, had lost its capacity to traffic via the exomer route (Figure 5A). This result indicated that residues between 16 and 25 of Chs3p were those required for exomer-dependent transport.

To further define the exomer signal within this region, we mutated individual residues to alanine in the context of Chs3-W391R and tested the phenotype of these mutants in a chs3Δ AP-1A background. The choice of this strain was motivated by our desire to ensure that the alternative pathway would be fully accessed by the different mutants. The expression of Chs3-W391R in chs3Δ AP-1A cells resulted in calcofluor-sensitivity because Chs3p employed the exomer pathway (Figure 5B, top row). However, when a chs6Δ mutation was introduced, cells became calcofluor-resistant because neither the exomer pathway nor the alternative pathway was accessed (Figure 5B, second row). This was the phenotype that we wished to phenocopy by specifically mutating the exomer sorting signal in Chs3-W391R. Of the individual point mutants, only Chs3-D19A/W391R and Chs3-E21A/W391R showed calcofluor-resistance when expressed in the chs3Δ AP-1A strain (Figure 5B), suggesting that these residues comprised the exomer sorting signal.

We confirmed that the calcofluor phenotype shown by the Δ15Chs3-W391R, Δ25Chs3-W391R and Chs3-D19A/E21A/W391R mutants correlated with a defect of transport to the PM by membrane fractionation on sucrose/EDTA gradients. Whereas the Δ15Chs3-W391R protein co-fractionated with the PM marker Pma1p, Δ25Chs3-W391R and Chs3-D19A/E21A/W391R failed to populate these fractions (Figure 5C). Finally, we observed that targeted deletions of amino acid residues 50–60 and 60–170, comprising the remainder of the N-terminal cytosolic domain of Chs3p, failed to reveal additional information necessary for exomer-dependent traffic. Deletion of residues 41–50 destabilized Chs3p, preventing us from testing this region (not shown).

D19 and E21 Define a DXE Motif Necessary for the Interaction of Chs3p with Chs5p

We hypothesized that residues D19 and E21 were required for the transport of Chs3p through the exomer pathway and thus would promote the recognition of Chs3p by the exomer complex [30,31]. To test this, we initially attempted experiments utilizing the same strategy we employed to confirm a role of the DEESLL
signal in interaction between Chs3p and the AP-1 complex: in vivo crosslinking followed by purification of TAP-tagged Chs5p. Using this method, we previously showed that Chs3p co-purifies with Chs5-TAP [30], however, experiments testing for reduced co-purification of a Chs3-D19AE21A mutant with Chs5-TAP yielded inconsistent results. We therefore, employed an alternative strategy in which we examined the binding of different regions of Chs3p, purified as soluble GST fusions, to purified His-tagged Chs5p or the entire His-tagged exomer complex immobilized on Ni-NTA beads. Whereas the construct corresponding to the second cytosolic domain of Chs3p (Chs3 (224–451)) bound poorly to the exomer complex, Chs3p (1–170), corresponding to the full N-terminal cytoplasmic tail of Chs3p, showed a robust interaction. Mutation of the key di-acidic motif in the N-terminus of Chs3p (1–170D19AE21A) reproducibly reduced the binding of this domain to the immobilized exomer components. In the case of experiments using only His-tagged Chs5p as bait the reduction ranged from 42–67% of the level of binding of the wt fragment (not shown). Accordingly, in the case of similar experiments in which the complete exomer complex, including His-Chs5, was used the level of Chs3-D19AE21A binding was reduced to 62–63% of wt. (Figure 6A and 6B).

Figure 5. Mutations of D19 and E21 result in intracellular retention of Chs3p. (A) Ten-fold serial dilutions of chs3Δ (RSY1699) or chs3Δ AP-1Δ (SPY21) cells expressing N-terminal deletion mutants of CHS3, with and without the W391R substitution, were spotted onto synthetic complete medium -Ura plates +100 ug/ml calcofluor (-Ura + CF). (B) Ten-fold serial dilutions of chs3Δ AP-1Δ cells expressing alanine substitution mutants from residue 16 to residue 25 of Chs3-W391R were spotted onto - Ura + CF. (C) Subcellular fractionation of Δ15Chs3-W391R, Δ25Chs3-W391R and Chs3-D19AE21AW391R expressed in chs3Δ AP-1Δ cells on step sucrose/EDTA gradients. Total membranes from spheroplasts were separated on a step sucrose/EDTA gradient. The protein composition of fractions obtained from the sucrose gradients was analyzed by SDS/PAGE and immunoblotting (PM marker: Pma1p; Golgi/EE markers: Tlg1p).

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Discussion

The current model for trafficking of Chs3p within the late secretory pathway proposes a key sorting decision at the TGN: transport to the PM through the exomer pathway or transport to and from the EE to maintain an intracellular reservoir. Mutations in AP-1, AP-3, GGA1/2, Ent3/5 or the DEESLL signal cause Chs3p to be rerouted to the PM via a poorly understood alternative pathway, independent of exomer. Here we showed that distinct events in the post-Golgi traffic of Chs3p are controlled by multiple sorting determinants.

In our studies of the intracellular transport of Chs3p, we employed a genetic selection for an intracellular sorting signal and identified four alleles of CHS3 that define the DEESLL signal in the N-terminus of Chs3p. This signal matches the consensus sequence for AP-dependent signals in mammalian and yeast cells [6]. Importantly, mutation of this signal exerts the same effect as mutation of the AP-1 complex: Chs3p is able to access the alternative exocytic pathway and reach the PM in chs6Δ cells.

Additionally, biochemical experiments confirmed that AP-1 physically interacts with Chs3p via the DEESLL signal (Figure 2). Thus the DEESLL signal constitutes the first [D/E][XXX][L/V/I] signal found to be dependent on AP-1 in yeast cells. Furthermore, the DEESLL signal is remarkable in that it also harbors a DXE motif used by the exomer complex for delivery of Chs3p to the PM. Whereas residues D19 and E21 are most important for the exomer-dependent exocytic pathway, residues L23 and L24 are most critical for the AP-1-dependent intracellular pathway.

The involvement of AP-3 in trafficking Chs3p was surprising given our previous conclusion to the contrary [33]. However, the difference may be due to strain background differences, which may alter the sensitivity of cells to calciofluor and ½ YPD media. We previously used a strain from the SEY6210 background to test the involvement of AP-3 in Chs3p trafficking [33], but have used strains from the YPH499 background in this study. The involvement of AP-3 in Chs3p trafficking was also surprising because AP-3 is implicated in delivering the proteins ALP and Vam3p from the TGN directly to the vacuole, bypassing the endosomal system [45,46], whereas Chs3p does not obviously localize to the vacuole. We were unable to trap Chs3p in the vacuole in a vac7Δ mutant, which is impaired in a vacuolar retrieval pathway, even when CHS6 was also deleted to restrict Chs3p to intracellular compartments (not shown). Thus our findings raise the possibility that AP-3 may transport Chs3p between the TGN and endosomes instead of to the vacuole. The mechanism by which AP-3 transports Chs3p is unknown, but AP-3 also acts on [D/E][XXX][L/V/I] signals [6], and thus may also have some interaction with the DEESLL signal. This possibility remains to be tested. Finally it is important to note that AP-3 is also required for the intracellular retention of the other known exomer-dependent PM protein, Fus1p (personal communication, Robyn Barfield).

In our studies of the alternative exocytic pathway we showed by two independent methods (calciofluor phenotype and sucrose gradient fractionation) that mutations in residues R374 and W391 block access of Chs3p to the route responsible for targeting Chs3p to the PM independently of the exomer complex. The exact route of this pathway remains to be determined. Depending on the directionality of the trafficking event mediated by AP-1, the W391 signal may control transport of Chs3p from the TGN to the EE, from the EE to the PM, or from the TGN to the PM.
where the APs function in Chs3p transport has been difficult, thus
we have not yet been able to distinguish these possibilities.

Chs3p and its partners present many features consistent with a
function as coat proteins for the transport of transmembrane
proteins from the TGN to the PM: (1) In chs3Δ mutants, Chs3p
and Fus1p are not transported to the PM and are retained within
the cell [47,48], (2) Chs3p, Chs6p and three Chs6p paralogs
assemble into the ~1 MDa exomer complex that binds Chs3p in
vivo and forms a spiky coat on synthetic liposomes in vitro [30,31]
and (3) Chs3p and the exomer components Bch1p and Bi67p are
required for the exocytic trafficking of Fus1p to the PM [28]. We
show here that the interaction of exomer with Chs3 in vitro is
dependent on the residues D19 and E21. Furthermore, these
residues are essential for the transport of Chs3p from the TGN to
the PM. Together these results suggest that the di-acidic motif D19
and E21 of Chs3p define a cytosolic sorting determinant decoded
by exomer for transport from the TGN to PM. It is worth noting
that the D19A E21A mutation, although conferring strong
calcofluor resistance in our genetic analysis of the exomer motif
(Figure 5B), impairs only by ~40% the direct binding to exomer
when tested with recombinant proteins (Figure 6B), illustrating
that the effect of this mutation on the binding to exomer is more
stringent in vivo in the context of the full-length chs3p than in vitro.

It is also worth noting that DXE motifs can also serve as ER-
export signals [49], and therefore the possibility of residues
19DEE21 acting in ER exit of Chs3p must be considered. Analysis
of Chs3-GFP lacking the DESSLL signal, and thus lacking
residues 19DEE21, does not reveal obvious entrapment of Chs3-
GFP in the ER, indicating that the elimination of the DXE signal
does not confer a major ER export defect (not shown). We have
not made measurements of the kinetics of Chs3p exit from the ER,
and therefore are unable to formally rule out the possibility that
the D19A E21A mutation causes a slight ER exit defect. However,
based on the lack of obvious ER accumulation in combination
with the strong trafficking defects revealed by our calcofluor and
fractionation studies, we believe the bulk of the defect is in
traficking from the Golgi to the PM. Finally, the observation of
the proximity within the N-terminal domain of Chs3p of the
motifs for the exomer pathway and the AP-1 recycling pathway
raises the interesting possibility of regulation of Chs3p traffic
through competitive interactions between these two signals and
their respective coat proteins. Such regulation would be consistent
with the known trafficking events that mediate specific subcellular
delivery of Chs3p within the late secretory pathway. Chitin ring
formation early in the cell cycle is controlled by the timed
transport of Chs3p from the TGN to the PM by the exomer
complex [50]. Moreover, cell stress, such as high temperature,
causes similar relocation of the internal pool of Chs3p to the PM
[40]. This environmental regulation is dependent on the action of
protein kinase C, Pkc1p, which, among many other functions,
is necessary for the phosphorylation of Chs3p [40]. In mammalian
cells, the phosphorylation of a serine or threonine is known to
influence the traffic of membrane proteins through the TGN/
endosomal system by interfering with the activity of a nearby
sorting signal [3,51]. At least three serines (S22, S26, and S29) are
located in the vicinity of the DESSLL signal, and the phosphor-
ylation state of these residues may influence the affinity of this
region for different coat proteins. However, preliminary character-
ization of the DEESLL signal suggests that modification of these
residues is not a positive determinant to engage Chs3p in either
the exomer pathway or the AP-1 pathway, as the single substitutions
S22A, S26A and S29A did not block access to either of these
transport pathways (Figure 5B, 5C). Nevertheless, the additive
contribution of these mutations, or a negative influence caused by
the phosphorylation of these residues, or the contribution of more
distal serine or threonine residues cannot be excluded. Further
studies of the traffic of Chs3p should continue to present
opportunities to investigate the regulation of vesicular transport
in the late secretory pathway of eukaryotic cells.

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Author Contributions

Conceived and designed the experiments: TLS SP CWW RS. Performed
the experiments: TLS SP CWW. Analyzed the data: TLS SP CWW RS.
Contributed reagents/materials/analysis tools: TLS SP CWW. Wrote the
paper: TLS SP RS.

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