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MCD4 Encodes a Conserved Endoplasmic Reticulum Membrane Protein Essential for Glycosylphosphatidylinositol Anchor Synthesis in Yeast

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Glycosylphosphatidylinositol (GPI)-anchored proteins are cell surface-localized proteins that serve many important cellular functions. The pathway mediating synthesis and attachment of the GPI anchor to these proteins in eukaryotic cells is complex, highly conserved, and plays a critical role in the proper targeting, transport, and function of all GPI-anchored protein family members. In this article, we demonstrate that *MCD4*, an essential gene that was initially identified in a genetic screen to isolate *Saccharomyces cerevisiae* mutants defective for bud emergence, encodes a previously unidentified component of the GPI anchor synthesis pathway. Mcd4p is a multimembrane-spanning protein that localizes to the endoplasmic reticulum (ER) and contains a large NH_2-terminal ER lumenal domain. We have also cloned the human *MCD4* gene and found that Mcd4p is both highly conserved throughout eukaryotes and has two yeast homologues. Mcd4p's lumenal domain contains three conserved motifs found in mammalian phosphodiesterases and nucleotide pyrophosphases; notably, the temperature-conditional *MCD4* allele used for our studies (*mcd4–174*) harbors a single amino acid change in motif 2. The *mcd4–174* mutant (1) is defective in ER-to-Golgi transport of GPI-anchored proteins (i.e., Gas1p) while other proteins (i.e., CPY) are unaffected; (2) secretes and releases (potentially up-regulated cell wall) proteins into the medium, suggesting a defect in cell wall integrity; and (3) exhibits marked morphological defects, most notably the accumulation of distorted, ER- and vesicle-like membranes. *mcd4–174* cells synthesize all classes of inositolphosphoceramides, indicating that the GPI protein transport block is not due to deficient ceramide synthesis. However, *mcd4–174* cells have a severe defect in incorporation of [^{3}H]inositol into proteins and accumulate several previously uncharacterized [^{3}H]inositol-labeled lipids whose properties are consistent with their being GPI precursors. Together, these studies demonstrate that *MCD4* encodes a new, conserved component of the GPI anchor synthesis pathway and highlight the intimate connections between GPI anchoring, bud emergence, cell wall function, and feedback mechanisms likely to be involved in regulating each of these essential processes. A putative role for Mcd4p as participating in the modification of GPI anchors with side chain phosphoethanolamine is also discussed.

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INTRODUCTION

Protein transport through the secretory pathway involves multiple steps that are conserved throughout eukaryotes (Palade, 1975; Schekman, 1985; Rothman, 1994; Schekman and Orci, 1996). Secreted or cell surface proteins are first translocated into the endoplasmic reticulum (ER) and then packaged into COPII-coated vesicle intermediates for transport to the Golgi complex. Upon reaching the trans-Golgi, these proteins undergo another vesicle packaging event and enter secretory vesicles that ultimately dock and fuse with the plasma membrane. A striking and readily observable example of the secretory process is that of polarized secretion in the budding yeast *Saccharomyces cerevisiae*, where efficient targeting of secretory vesicles to newly emerging buds ensures that nascent daughter cells receive important protein, lipid, and cell wall components required both for bud emergence and for survival of the daughter cell after cytokinesis (Herskowitz, 1988; Preuss *et al.*, 1992; Cid *et al.*, 1995; Drubin and Nelson, 1996).

Glycosylphosphatidylinositol (GPI)-anchored proteins comprise an important and well-characterized family of proteins found at the cell surface of all eukaryotic cells (Lisanti *et al.*, 1990; McConville and Ferguson, 1993; Klis, 1994; Udenfriend and Kodukula, 1995). These proteins serve a variety of functions; in yeast, for example, GPI-anchored proteins have been identified as cell wall proteins (although most GPI-anchored proteins reside in the plasma membrane), flocculation proteins, aspartyl proteases, proteins required for glucan synthesis, and proteins involved in inositol metabolism (Klis, 1994; Udenfriend and Kodukula, 1995; Orlean, 1997). Many additional proteins likely to be GPI anchored can be found in the yeast genome and still await characterization (Caro *et al.*, 1997). Regardless of their function, GPI-anchored proteins must both acquire a GPI anchor and traffic from their site of synthesis (the ER) to their ultimate final destination (the cell surface) to execute their roles.

Proteins destined to be GPI anchored (i.e., Gas1p [Conzelmann *et al.*, 1988; Nuoffer *et al.*, 1991; Vai *et al.*, 1991]) are initially synthesized with an N-terminal signal sequence, which is cleaved shortly after translocation into the ER, and a C-terminal hydrophobic region, which anchors the protein in the ER membrane and serves as part of the signal for GPI anchor attachment (McConville and Ferguson, 1993; Udenfriend and Kodukula, 1995). Shortly after protein synthesis, the C-terminal transmembrane region is replaced with the preformed GPI anchor (Englund, 1993), a reaction thought to be mediated by a transamidase complex that simultaneously cleaves the hydrophobic domain and replaces it with the preformed GPI anchor (Maxwell *et al.*, 1995). The GPI anchor is synthesized in the ER. The core GPI anchor consists of a lipid group (serving as the membrane anchor), myo-inositol, GlcN, several mannose groups, and a phosphoethanolamine group, which ultimately connects the GPI anchor to the protein via an amide linkage (McConville and Ferguson, 1993; Udenfriend and Kodukula, 1995; Orlean, 1997). GPI anchors also contain side chain molecules that vary by organism; for example, a side chain found in mammalian and (very recently) yeast cells but that is absent from lower eukaryotes such as trypanosomes is a phosphoethanolamine (EtN-P) residue attached to the first mannose of the anchor (McConville and Ferguson, 1993; Canivenc-Gansel *et al.*, 1998; Sütterlin *et al.*, 1998). Yeast mutants defective for either GPI anchor synthesis or GPI anchor attachment all exhibit specific defects in ER-to-Golgi transport of GPI-anchored proteins such as Gas1p but not of other proteins (Benghezal *et al.*, 1995, 1996; Hamburger *et al.*, 1995; Schönbächler *et al.*, 1995), unless the mutation is in a gene affecting more than just the GPI synthesis pathway (i.e., *SEC53* [Kepes and Schekman, 1988; Conzelmann *et al.*, 1990]). This transport defect is most likely due to a failure of nonanchored GPI proteins to be packaged into ER-derived COPII vesicles (Doering and Schekman, 1996); thus, the GPI anchor may also function as a packaging signal.

Consistent with this idea, another specific requirement for ER-to-Golgi transport of GPI-anchored proteins is sphingolipid/ceramide synthesis. Ceramides are a major, essential component of the plasma membrane (Smith and Lester, 1974), and drugs that inhibit ceramide synthesis (Horvath *et al.*, 1994) as well as a yeast mutant defective for the first step in the ceramide synthesis pathway (Skrzypek *et al.*, 1997; Sütterlin *et al.*, 1997) impair ER-to-Golgi transport of GPI-anchored (but not other cargo) proteins. It is currently thought that ceramides and GPI-anchored proteins are copackaged into COPII vesicles (Horvath *et al.*, 1994; Doering and Schekman, 1996; Sütterlin *et al.*, 1997), with ceramides perhaps acting to cluster GPI-anchored proteins into membrane microdomains (Brown and Rose, 1992; Fiedler *et al.*, 1993), which in turn encourages the packaging event. Some GPI-anchored proteins also undergo remodeling reactions in the ER and in the Golgi, where ceramide is introduced into the GPI anchor after its attachment to the protein (Conzelmann *et al.*, 1992; Reggiori *et al.*, 1997; Sipos *et al.*, 1997); whether this influences transport is currently unclear.

This article describes an essential *S. cerevisiae* protein that is likely to function in the GPI anchor synthesis pathways of multiple eukaryotic systems. This protein, Mcd4p, was identified in a screen to isolate mutants defective for bud emergence and polarized growth independent of the requirement for the actin cytoskeleton in those processes (Mondésert and Reed, 1996; Mondésert *et al.*, 1997).

The mutants isolated in this screen were called "morphogenesis checkpoint dependent" (*mcd*), since cells defective for polarized growth cannot survive without the morphogenesis checkpoint, a cell cycle machinery-driven regulatory process that normally ensures that mitosis is delayed until new buds emerge and thereby prevents the formation of multinucleated cells (Lew and Reed, 1995a,b; Mondésert and Reed, 1996). Somewhat surprisingly, of the seven complementation groups of *mcd* mutants isolated, six corresponded to previously identified genes that fell into two distinct classes. The first class consisted of genes whose products are required for ER- or Golgi-specific glycosylation processes: Mnn10p/Bed1p (Mcd1p), a Golgi-localized membrane protein required for elongating α1,6 mannosyltransferase activity (Dean and Poster, 1996); Anp1p/Gem3p (Mcd2p), a component of a multisubunit complex (Hashimoto and Yoda, 1997) exhibiting in vitro α1,6-mannosyltransferase activity (Jungmann and Munro, 1998); Gog5p/Vrg4p/Van2p (Mcd3p), the Golgi GDP-mannose transporter (Dean *et al.*, 1997); and Sec53p/Alg4p (Mcd5p), the ER-localized phosphomannomutase that provides the ER with GDP-mannose for N- and O-linked glycosylation and GPI anchor synthesis (Kepes and Schekman, 1988). Proteins encoded by the second class of *MCD* genes, Sec3p/Psl1p (Mcd6p) and Sec5p (Mcd7p), consisted of two subunits of the multisubunit exocyst complex; this complex defines the localized site for docking/fusion of secretory vesicles at bud tips (TerBush *et al.*, 1996). These results clearly established important roles for glycosylation and exocyst function in polarized secretion.

Unlike most of the *mcd* mutants described above, *mcd4* mutants did not affect glycosylation of at least one secretory pathway protein and also did not correspond to any known gene (Mondésert *et al.*, 1997). We therefore reasoned that Mcd4p might affect polarized growth and secretion by a mechanism distinct from either N-linked glycosylation or exocyst function. In an effort to understand the role this protein might play in bud emergence and perhaps other secretory pathway functions as well, we cloned *MCD4*, characterized the subcellular location and topology of the Mcd4 protein, and initiated an extensive analysis of *mcd4* mutant phenotypes. Interestingly and somewhat unexpectedly, these studies indicated that Mcd4p is a previously unidentified yet highly conserved component of the GPI anchor synthesis pathway, potentially participating in modification of GPI anchors with side chain phosphoethanolamine. The implications of this finding on how GPI anchoring, bud emergence, and cell wall integrity/remodeling are connected are also discussed.

MATERIALS AND METHODS

Media, Plasmids, Strains, Yeast Genetic Techniques

Yeast cells were grown in YPD, YNB (yeast nitrogen base), or SD media supplemented as necessary (Sherman *et al.*, 1979). Plasmids used in this study were YEpMCD4 (*MCD4*, 2μ, *URA3*), pRS425-MKC7HA (*MKC7-HA*, 2μ, *LEU2*), YCp50-mcd4–174 (*mcd4–174*, *CEN*, *URA3*), YCp50-MCD4 (*MCD4*, *CEN*, *URA3*). Yeast strains used in this study were SEY6210 (*MAT*α *ura3 leu2 his3 trp1 lys2 suc2*Δ*9*; [Robinson *et al.*, 1988]), SEY6210a/α (*MAT***a**/*MAT*α *ura3 leu2 his3 trp1 lys2 suc2*Δ*9*; Emr lab strain), BF264–15D (*MAT*α *ade1 his2 leu2 trp1*; [Reed *et al.*, 1985]), GY1450 (SEY6210; *mcd4*::*KAN*^{R}, YCp50-mcd4–174; this study); GY1446 (SEY6210; *mcd4*::*KAN*^{R}, YCp50-MCD4; this study), SEY5188 (*MAT*α *sec18–1 ura3 leu2 his3 suc2*Δ*9*; [Graham and Emr, 1991]), EGY111–2 (*MAT*α *sec1–1 ura3 leu2 his3 suc2*Δ*9*; [Gaynor and Emr, 1997]), YMW2 (*ade3 ade3 his3 leu2 trp1 MAT***a** [P. Orlean]), YMW2 *gpi1*Δ*::TRP1* [P. Orlean], gpi2–5a (*gpi2*^{ts} ura3 his3 *MAT***a** [P. Orlean]), gpi3–6a (*gpi3*^{ts} ade2 ura3 his3 *MAT***a** [P. Orlean]). The cDNA clones containing *hMCD4* (clones 627105 and 612824, GenBank accession numbers aa191163 and aa181731) were from the I.M.A.G.E. Consortium (Lennon *et al.*, 1996). Standard yeast genetic techniques were used throughout (Sherman *et al.*, 1979). Construction of GY1450 and GY1446 was as follows. One chromosomal copy of *MCD4* was disrupted in SEY6210a/α using the *KAN*^{R} gene as previously described (Wach *et al.*, 1994). The resulting strain was transformed with YCp50-mcd4–174 or YCp50-MCD4 and sporulated, and Kan^R, Ura$^+$ spores were isolated. GY1450 was also tested for temperature-conditional growth.

^{35}S-Methionine Labeling, Immunoprecipitation, Glycosylation Analyses, Subcellular Fractionation, Electron Microscopy, Bud Emergence Assays

Labeling of cells with ^{35}S-methionine and processing for immunoprecipitation were performed essentially as previously described (Gaynor and Emr, 1997). For the experiments shown in Figures 4 and 5 (CPY, HSP150, media proteins, CWP33), pulse-labeled and chased cells were centrifuged at 5000 $\times$ *g* for 5 min and separated into cell and media fractions before immunoprecipitation or processing for SDS-PAGE. Gas1p was also immunoprecipitated from media fractions. For the experiments shown in Figure 6, pulse-labeled and chased whole cells were prepared for immunoprecipitation as described for CPY (Gaynor and Emr, 1997). Endo H treatment and subcellular fractionation were performed as previously described (Gaynor *et al.*, 1994; Gaynor and Emr, 1997). Electron microscopy was performed as previously described (Rieder *et al.*, 1996). Tunicamycin treatment was performed by treating exponentially growing cells with 10 μg/ml tunicamycin (Sigma Chemical, St. Louis, MO) for 30 min at 30°C. Bud emergence assays (i.e., propidium iodide staining, fluorescence-activated cell sorter analysis, microscopy) were performed as previously described (Mondésert and Reed, 1996; Mondésert *et al.*, 1997).

Antisera

Antiserum against Mcd4p was prepared as follows. A *Bgl*II–*Hin*dIII fragment from the N-terminal region of *MCD4* was cloned into pQE-9 (Qiagen, Chatsworth, CA) to produce an N-terminal–6-HIS fusion protein in *Escherichia coli*. The almost entirely insoluble fusion protein was gel purified and used to immunize New Zealand white rabbits as previously described (Grandin and Reed, 1993). The resulting antiserum was affinity purified by standard procedures (Pringle *et al.*, 1991). The affinity-purified antiserum was used at a 1:150 dilution for immunoblots. Antisera to CPY (Klionsky *et al.*, 1988), Gas1p (Doering and Schekman, 1996), HSP150 (Russo *et al.*, 1992), CWP33 (Toyn *et al.*, 1988; Sanz *et al.*, 1989), Vam3p (Darsow

et al., 1997), α1,6-mannose (Franzusoff and Schekman, 1989), Yap3p (Ash *et al.*, 1995), and the 12CA5 monoclonal antibody (Boeringer Mannheim, Indianapolis, IN) were described previously.

Labeling of Proteins with myo-[2-[^{3}H]]Inositol

Labeling and processing of cells was done essentially as previously described (Horvath *et al.*, 1994; Schönbächler *et al.*, 1995; Sütterlin *et al.*, 1997); a description of the procedure is as follows. Cells were grown in SDYE (SD + 0.2% yeast extract) overnight to midlog phase, washed twice in SD–inositol medium, and 5 OD-eq were resuspended in 1 ml SD–inositol. Cells were incubated at 24° or 38°C for 15 min, and then labeled for 45 min with 80 μCi myo-[2-[^{3}H]]Inositol (Amersham, Arlington Heights, IL). Transport was stopped by the addition of NaN_3 and NaF to a 10 mM final concentration each after which the cells were placed on ice. Cells were collected by centrifugation, washed once in 10 mM NaN_3/NaF, and resuspended in 250 μl TEPI (50 mM Tris pH 7.5, 5 mM EDTA, 2 mM PMSF, 30 μg/ml each leupeptin, antipain, and pepstatin). Glass beads (0.2 g) were added to the suspension, and cells were lysed by vortexing four times for 30 s each, placing the cells on ice between each vortexing cycle. The glass beads were allowed to settle, and the lysate was collected and transferred to a new tube. The glass beads were washed with another 250 μl TEPI. The lysates were pooled, and proteins were precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10%. Proteins were acetone washed twice and dried in a speed-vac. Proteins were solubilized by sonication in 50 μl Laemmli sample buffer containing 2.5% β-mercaptoethanol, boiled for 5 min, and spun hard in a microcentrifuge for 10 min. Ten microliters (~1 OD-eq) were run out on a 10% SDS gel, which was then fixed, processed for fluorography with 1 M sodium salicylate, and exposed to film at −70°C.

Lipid Analyses

Lipids were labeled, extracted, and analyzed essentially as previously described (Puoti *et al.*, 1991; Horvath *et al.*, 1994); a description of the procedure is as follows. Cells were grown in YPD overnight to midlog phase and washed twice in SD medium lacking inositol (SD − inositol), after which 3 OD-eq were resuspended in 600 μl SD − inositol (5 OD/ml). Cells were incubated at 24 or 38°C for 15 min, labeled for 20 min with 20 μCi myo-[2-[^{3}H]]inositol (Amersham), and then chased for 60 min by the addition of an equal volume of YPD + 80 μg/ml cold myo-inositol. Transport was stopped by the addition of NaN_3 and NaF to a 10 mM final concentration each and the cells were placed on ice. Cells were collected by centrifugation, washed once in 10 mM NaN_3/NaF, and resuspended in 500 μl CMW extraction solvent ($CHCl_3$/CH_3OH/H_2O, 10:10:3 [vol/vol]). Glass beads (0.5 g) were added to the suspension, and cells were lysed by vortexing four times for 30 s each. The lysate was spun at top speed for 5 min in a microcentrifuge, and the organic phase was collected and transferred to a new tube. An additional 300 μl CMW were added to the remaining aqueous phase/glass beads, vortexed briefly, and spun, and the resulting organic phase was collected and pooled with the first organic phase. The pooled lipids were dried in a speed-vac. For desalting, the dried lipids were resuspended in 150 μl H_2O-saturated butanol and extracted with 75 μl H_2O. The organic phase was collected, and the aqueous phase was back extracted with an additional 75 μl H_2O-saturated butanol. The pooled organic phases were dried in a speed-vac and resuspended in 25 μl CMW. Five microliters (~0.5 OD-eq) were loaded onto TLC plates (Kieselgel 60, Merck, West Point, PA), developed using either solvent 1 ($CHCl_3$/CH_3OH/0.25% KCl, 55:45:10 [vol/vol]), solvent 2 ($CHCl_3$/CH_3OH/H_2O, 10:10:2.5 [vol/vol]), or solvent 3 ($CHCl_3$/CH_3OH/13 M NH_4OH/1 M NH_4OAc/H_2O, 180:140:9:9:23 [vol/vol]), and exposed to x-ray film. Treatment of lipids with methanolic NH_3 was performed as previously described (Costello and Orlean, 1992).

RESULTS

Mcd4p Is a Multimembrane-spanning ER Protein with a Large, Lumenal N-Terminal Domain

Mcd4 Protein Features

MCD4 corresponds to the *S. cerevisiae* open reading frame YKL165c and was cloned by complementation of the lethality that results when *mcd* mutations are combined with Clb2 overexpression (Mondésert and Reed, 1996; Mondésert *et al.*, 1997). The *MCD4* gene predicts a 919-amino acid protein. Kyte-Doolittle hydropathy analysis (Kyte and Doolittle, 1982) and PredictProtein programs (Rost *et al.*, 1995) were used to predict the presence and orientation of transmembrane domains (TMDs); both analyses predicted 14 TMDs in Mcd4p. The first putative TMD occurs near the N terminus (amino acids [aa] 14–31) and is likely to function as a stop-transfer sequence (i.e., like a signal sequence, it directs the protein's translocation into the ER; however, it is not cleaved after translocation). The remaining N-terminal half (aa 31–459) of Mcd4p is highly hydrophilic, with the next TMD predicted to begin at aa 459. The last 13 TMDs all occur in the C-terminal half of Mcd4p and are relatively evenly spaced within this region. The C terminus of the protein ends with the amino acids "KKTQ" and is likely to extrude into the cytoplasm; thus, Mcd4p also contains the well-characterized "KKXX" ER retrieval motif (Jackson *et al.*, 1993; Gaynor *et al.*, 1994) found at the cytoplasmic C terminus of many membrane proteins known either to reside in or cycle through the ER. Finally, Mcd4p also contains eight potential N-linked glycosylation sites: one preceding the first TMD, six within the large N-terminal hydrophilic region, and one immediately following the fourth predicted TMD.

Mcd4p Localizes to the ER

Antiserum to the N-terminal hydrophilic portion of the protein was generated in rabbits and affinity purified (see MATERIALS AND METHODS). The affinity-purified anti-Mcd4p antiserum recognized a distinct, ~100-kDa band by immunoblot of lysates from wild-type cells (Figure 1, B–D). Consistent with this band corresponding to the Mcd4 protein, its intensity increased dramatically in immunoblots using lysates where Mcd4p was overproduced from a 2μ vector. The antiserum also recognized an ~100-kDa band by immunoprecipitation of pulse-labeled cell lysates overexpressing Mcd4p (Figure 1C); however, endogenous levels of Mcd4p were nearly impossible to detect by immunoprecipitation using either crude or affinity-purified antiserum. Therefore, unless otherwise stated, most of our Mcd4p studies were performed by immunoblot analysis of Mcd4p from wild-type cells, which allowed easy detection of chromosomally expressed levels of the protein.

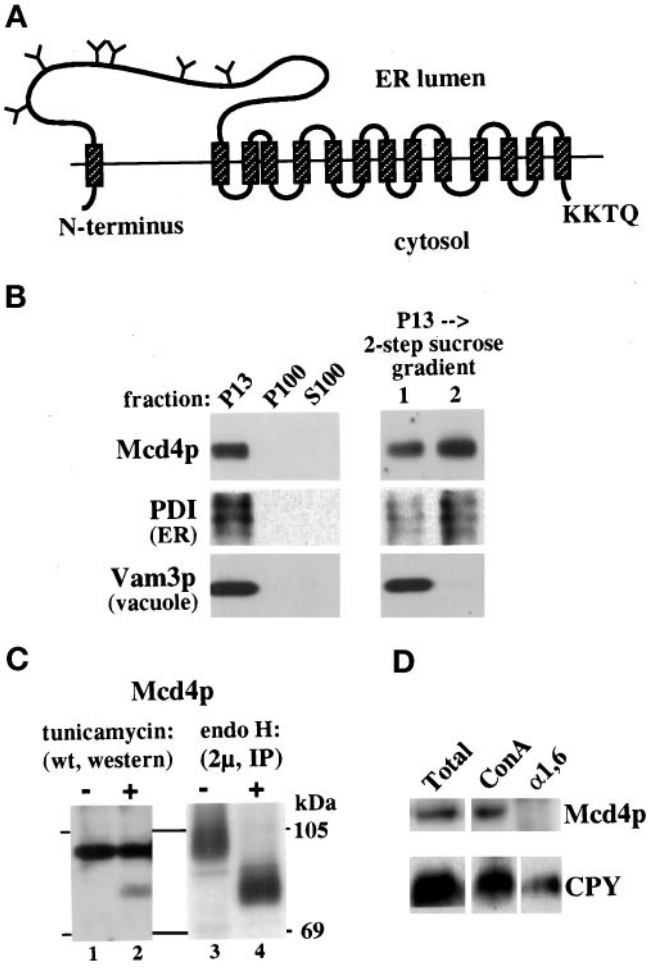

Figure 1. Mcd4p is an ER-localized, multimembrane-spanning protein with a large, lumenal N-terminal domain. (A) Predicted topology of Mcd4p in the ER membrane, based on data shown in panels B, C, and D. Predicted TMDs (hatched boxes), N-linked glycosylation sites likely to be utilized (Y), and the C-terminal KKXX motif are shown. (B) Subcellular localization of Mcd4p. Wild-type (SEY6210) cells were spheroplasted and subjected to differential centrifugation. The 13,000 × *g* pellet (P13) was further resolved on a two-step sucrose gradient. Shown are the immunoblots of the P13, P100, and S100 fractions for Mcd4p (1:150), PDI (1:320), and Vam3p (1:2500). (C) N-linked glycosylation of Mcd4p. Lanes 1 and 2: wild-type (SEY6210) cells were either treated with tunicamycin (+) or mock-treated (−) for 30 min, lysed, and resolved by SDS-PAGE. Mcd4p was visualized by immunoblotting. Lanes 3 and 4: wild-type cells overexpressing Mcd4p (SEY6210 harboring yEp-MCD4) were pulse labeled, chased, and lysed. Mcd4p was recovered by immunoprecipitation and either treated with endo H (+) or mock-treated (−). The positions of molecular mass markers are shown to the right. (D) Post-ER modification of Mcd4p. Lysates were generated from wild-type (SEY6210) cells; 5 OD-eq of cells were prepared for immunoprecipitation with anti–α1,6-mannose antiserum (2 μl/OD) or precipitation with ConA-Sepharose; 1 OD-eq of total lysate (Total), and 2.5 OD-eq of ConA-Sepharose (ConA) and α1,6-mannose antiserum precipitates (α1,6) were resolved by SDS-PAGE, and Mcd4p (1:150) and CPY (1:5000) were visualized by immunoblotting.

The KKXX motif found at the C terminus of Mcd4p suggested, but did not demonstrate, ER localization of the protein. To determine the intracellular location of Mcd4p, we performed subcellular fractionation and sucrose density gradient experiments. In brief, spheroplasts generated from exponentially growing wild-type cells were gently lysed and subjected to sequential differential centrifugation at 300 × *g*, 13,000 × *g*, and 100,000 × *g*. The pellet and supernatant fractions from each step were harvested and precipitated, and the proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for Mcd4p. For simplicity, only the 13,000 × *g* pellet, 100,000 × *g* pellet, and 100,000 × *g* supernatant fractions are shown. Mcd4p was found exclusively in the low-speed (13,000 × *g*) pellet fraction (Figure 1B). This fraction typically contains large, dense membranes such as the ER, vacuole, and plasma membrane but is relatively free of less dense membranes such as Golgi and transport vesicles, each of which are primarily found in the P100 (Gaynor *et al.*, 1994; Marcusson *et al.*, 1994).

This analysis suggested that Mcd4p is most likely to reside either in the ER, plasma membrane, or the vacuole. To differentiate among these possibilities, the P13 was further resolved on a two-step sucrose gradient. This gradient yields two distinct membrane bands (Gaynor *et al.*, 1994): a less dense membrane band migrating within the upper sucrose layer (fraction 1), and a sharp, dense membrane band that migrates at the interface of the two sucrose steps (fraction 2). Approximately 60% of Mcd4p was found in the dense membrane band (fraction 2), with the remainder found in fraction 1 (Figure 1B). This distribution was nearly identical to that observed for the ER resident protein disulfide isomerase (PDI; Figure 1B) as well as to that of other well-characterized ER resident proteins; i.e., Wbp1p (Gaynor *et al.*, 1994) and Gpi8p (Benghezal *et al.*, 1996). In contrast, the vacuolar membrane protein Vam3p (Darsow *et al.*, 1997) was found exclusively in the less dense membrane band (fraction 1; Figure 1B). To address the possibility that Mcd4p resides at the plasma membrane, similar fractionation studies were carried out using a *sec1–1* mutant incubated for 120 min at a nonpermissive temperature. Sec1p is required for docking/fusion of secretory vesicles at the plasma membrane (Novick and Schekman, 1979), and *sec1–1* mutants rapidly accumulate secretory vesicles at a nonpermissive temperature (Novick *et al.*, 1981); thus, if Mcd4p resides at the plasma membrane, newly synthesized protein would be expected to accumulate in the P100 at a nonpermissive temperature, as has been observed for other plasma membrane-localized proteins (Ash *et al.*, 1995). However, the distribution of Mcd4p in *sec1–1* was indistinguishable from that in wild-type cells (Gaynor and Emr, unpublished observations). Together with 1) the

fact that Mcd4p does contain a KKXX motif, 2) glycosylation studies of Mcd4p, and 3) characterization of *mcd4* mutant defects (see below), these data are most consistent with Mcd4p residing in the ER.

Mcd4p's Hydrophilic N-Terminal Domain Extends into the ER Lumen

The predicted topology of Mcd4p based on sequence analysis and the above localization data suggested that the large, hydrophilic N-terminal domain extends into the lumen of the ER (Figure 1A). If so, then the six potential N-linked glycosylation sites contained within this region (i.e., between TMDs 1 and 2) would be predicted to receive N-linked carbohydrate modification; however, the N-linked sites preceding TMD 1 and immediately following TMD 4 would not, as they would face the cytosol. N-linked modification begins in the ER, where core oligosaccharides are added to appropriate asparagine residues immediately after protein translocation (Herscovics and Orlean, 1993). Tunicamycin is a drug that inhibits core N-linked modification. Wild-type cells were treated with tunicamycin for 30 min and prepared for immunoblot analysis with antiserum to Mcd4p. Tunicamycin-treated cells accumulated a form of Mcd4p that was ~12–14 kDa smaller than normal (Figure 1C, lanes 1 and 2). As a single-core N-glycan is typically ~2 kDa in mass, this result is consistent with newly synthesized Mcd4p in the treated cells failing to acquire core oligosaccharides on the six N-linked glycosylation sites between TMDs 1 and 2. As further support for these N-linked sites being utilized, cells overexpressing Mcd4p from a 2μ vector were pulse-labeled for 10 min with ^{35}S-methionine and chased with cold methionine and cysteine for 30 min, and Mcd4p was recovered by immunoprecipitation. Half of the immunoprecipitate was treated with endo H, which removes N-linked carbohydrates. All of the endo H-treated protein exhibited the ~12- to 14-kDa mobility shift (Figure 1C, lanes 3 and 4), indicating that all of the Mcd4p synthesized during the pulse labeling had been modified with at least core N-linked sugars. This analysis is thus consistent with the predicted protein topology shown in Figure 1A.

Mcd4p Is Unlikely to Cycle through the Golgi

Finally, although Mcd4p is likely to reside in the ER, the C-terminal KKXX ER retrieval motif suggested that the protein might also cycle through the Golgi complex (Jackson *et al.*, 1993; Gaynor *et al.*, 1994; Schröder *et al.*, 1995; Stamnes *et al.*, 1995). In yeast, the first known post-ER secretory pathway compartment is defined by the initiating α1,6-mannosyltransferase Och1p (Gaynor *et al.*, 1994), which catalyzes the first Golgi-specific N-linked glycosylation step (Nakayama *et al.*, 1992; Nakanishi-Shindo *et al.*, 1993). If Mcd4p cycles through the Golgi, then its core N-linked sugars should acquire initial α1,6-mannose modification, an event that is readily detectable using α1,6-mannose-specific antiserum (Franzusoff and Schekman, 1989; Graham and Emr, 1991; Gaynor *et al.*, 1994). To determine whether Mcd4p is α1,6-mannose-modified exponentially growing wild-type cells were harvested and prepared for immunoprecipitation. Lysates were precipitated with either Concanavalin A (ConA)-Sepharose (ConA is a lectin that recognizes all mannose moieties) or with α1,6-mannose–specific antiserum and protein A–Sepharose. 2.5 OD-eq from each precipitation or 1 OD-eq of total lysate (as a control) were run on an SDS gel, transferred to nitrocellulose, and immunoblotted for Mcd4p (Figure 1D). Mcd4p was readily detected in the ConA–Sepharose precipitation, consistent with it receiving at least core N-linked modification. However, no Mcd4p was detected from the α1,6-mannose–specific immunoprecipitate. As a positive control, the α1,6-mannose immunoprecipitate was also immunoblotted for the vacuolar hydrolase carboxypeptidase Y (CPY), and, as expected, signal was observed (Figure 1D). Consistent with the immunoblot data, when cells overexpressing Mcd4p were pulse-labeled and chased, Mcd4p immunoprecipitated, and the protein subjected to reimmunoprecipitation with α1,6-mannose–specific antiserum, Mcd4p was also not detected with α1,6-mannose antiserum (Gaynor and Emr, unpublished observations). Furthermore, haploid cells deleted for wild-type Mcd4p but harboring a mutant Mcd4p in which the KKXX motif was destroyed (by site-directed mutagenesis of KKTQ to KSTQ or SSTQ) were viable and grew like wild-type cells. This indicated that the KKXX motif is not required for normal function and thus, presumably, normal localization of Mcd4p. Together, these analyses suggest that Mcd4p does not traffic or cycle through the Och1p compartment and may instead, like many other ER resident proteins (i.e., Wbp1p [te Heesen *et al.*, 1992; Gaynor *et al.*, 1994]), simply be retained in the ER.

Mcd4p Has Human and Schizosaccharomyces pombe *Homologues, Is Conserved throughout Eukaryotes, and Contains Three Motifs Found in Phosphodiesterases and Pyrophosphatases; the mcd4–174 Mutation Is in Motif 2*

To investigate whether *S. cerevisiae* contains related Mcd4-like proteins and/or if Mcd4-like proteins exist in systems other than *S. cerevisiae*, BLAST (basic local alignment search tool [Altschul *et al.*, 1990]) analysis was performed using the NCBI BLAST program to search for homologues within the GenBank nonredundant, dbest, and At (*Arabidopsis thaliana*) databases.

This analysis first revealed that Mcd4p is highly conserved in multiple eukaryotic organisms, including humans. We cloned and sequenced the human

MCD4 gene (*hMCD4*) using cDNAs initially identified as ESTs zp86f05.s1 (locus AA190634) and zp49g05.s1 (locus AA1811731). The complete *hMCD4* sequence can be found as GenBank accession no. AF109219. The *S. pombe MCD4* gene (*spMCD4*) was sequenced by the *S. pombe* sequencing project (SPBC24E9.08c; locus 2879870). Alignment of the complete *S. cerevisiae* (S.c.), *S. pombe* (S.p.), and human (H.s.) Mcd4 protein sequences (Figure 2A) was performed using the CLUSTALW Multiple Sequence Alignment program. These Mcd4 proteins are all roughly the same length (S.c. = 919 aa; S.p. = 935 aa; H.s. = 931 aa) and exhibit remarkable homology to each other both in sequence identity and predicted structure. Over the entire length of the proteins, percent identities are 39% between S.c. and S.p., 35% between S.c. and H.s., and 34% between S.p. and H.s. (with similarities ranging from 50 to 60%). The highest degree of homology occurs in Mcd4p's N-terminal lumenal domain: within the first 350 aa, percent identities increase to 57% between S.c. and S.p., 51% between S.c. and H.s., and 48% between S.p. and H.s. Kyte-Doolittle hydropathy analysis also suggests a high degree of structural similarity with respect to location and number of the predicted TMDs (Figure 2A, boxes). EST sequence comparisons indicate that *MCD4* homologues also exist in *Caenorhabditis elegans* (CELK021H3F; locus D35559), mouse (mz94all.r1; locus AA267602), and *A. thaliana* (F28J24TFB; locus B25867).

This analysis also identified two putative *S. cerevisiae* Mcd4p homologues, encoded by open reading frames YJL062w and YLL031c, that exhibit both sequence and structural (by hydropathy analysis) similarity to Mcd4p. Because Mcd4p is essential, these homologues obviously cannot substitute for Mcd4p; rather, Mcd4p and its *S. cerevisiae* homologues may comprise a family of proteins with similar but nonoverlapping functions. Consistent with this idea, YJL062w and YLL031c, as with Mcd4p, also have strikingly similar *S. pombe* counterparts.

Interestingly, PSI-BLAST analysis using Mcd4p's N-terminal lumenal domain also revealed homologies to several known mammalian phosphodiesterases, nucleotide pyrophosphatases, and certain sulfatases; for example, OryzaNucPdeAse (a nucleotide pyrophosphatase/nucleotide phosphodiesterase), HsPdeAse (human phosphodiesterase Iα), AlkPDE (mouse alkaline phosphodiesterase I/nucleotide pyrophosphatase), and sterolsulfatase. Motif searches using the MEME (Multiple Expectation Maximization for Motif Elucidation) and MAST (Motif Alignment and Search Tool) programs (Bailey and Gribskov, 1998) also defined three well-conserved motifs present in the known phosphodiesterases, pyrophosphatases, Mcd4p, and its homologues (Figure 2B; Figure 2A, thick lines), with the DHGM sequence at the end of Motif 3 being particularly well conserved. Notably, in all of these proteins, Motifs 1, 2, and 3 are found in the same order with very similar spacing between the motifs (89–107 aa between nos. 1 and 2; 30–35 aa between nos. 2 and 3; see "#AAs" in Figure 2B).

Finally, we wanted to determine the precise site of the mutation in *mcd4–174*, the allele used for the mutant characterization studies described in the following sections. Restriction digest analyses first indicated that the mutation lay within the protein's N-terminal lumenal domain. Sequencing this region of *mcd4–174* revealed that glycine 227 was mutated to glutamic acid (Figure 2, A and B, arrowhead). Glycine 227 is in the middle of motif 2 and, even more strikingly, is conserved in all of the proteins described above (Mcd4p, its human and *S. pombe* counterparts, its yeast homologues, and the known phosphodiesterases and pyrophosphatases; see Figure 2B). The fact that the *mcd4–174* mutation maps to a highly conserved amino acid in motif 2, together with the nearly identical spacing between the motifs in all of these proteins, strongly suggests that these motifs are significant to the function of Mcd4p. The relevance of these homologies, motifs, and the G227E mutation in *mcd4–174* to a proposed activity for Mcd4p is addressed in DISCUSSION.

mcd4–174 Cells Exhibit Marked Morphological Defects

Prior to the *mcd4* mutant studies described in the following sections, we crossed the two *mcd4* alleles identified in the *mcd* screen into the SEY6210 strain background, a strain whose morphology and protein transport characteristics have been extensively characterized. Although both alleles (*mcd4–154* and *mcd4–174*) behaved similarly in preliminary protein transport studies in the original strain background used for the *mcd* selection, the *mcd4–154* allele was nearly lethal in SEY6210. Therefore, all data shown in this and subsequent sections are from analysis of the *mcd4–174* allele in the SEY6210 strain background.

First, electron microscopy was used to examine the morphology of the *mcd4–174* mutant at the ultrastructural level. Cells were grown at 24°C to early log phase, incubated for 3 h at either 24 or 38°C, and prepared for electron microscopy. For the most part, *mcd4–174* cells incubated at 24°C (Figure 3B) appeared quite similar to wild-type cells incubated at either 24 or 38°C (Figure 3A). The nucleus, vacuole, and mitochondria were clearly apparent, buds were round and well formed, and the cells were relatively free of other membrane structures. In contrast, *mcd4–174* cells incubated at 38°C exhibited several striking morphological aberrancies (Figure 3, C–F). For example, significant accumulation of abnormal membranes was observed. Some of these membranes were continuous with the nuclear membrane and most likely correspond to ER (Figure 3D; arrows). Other accumulated

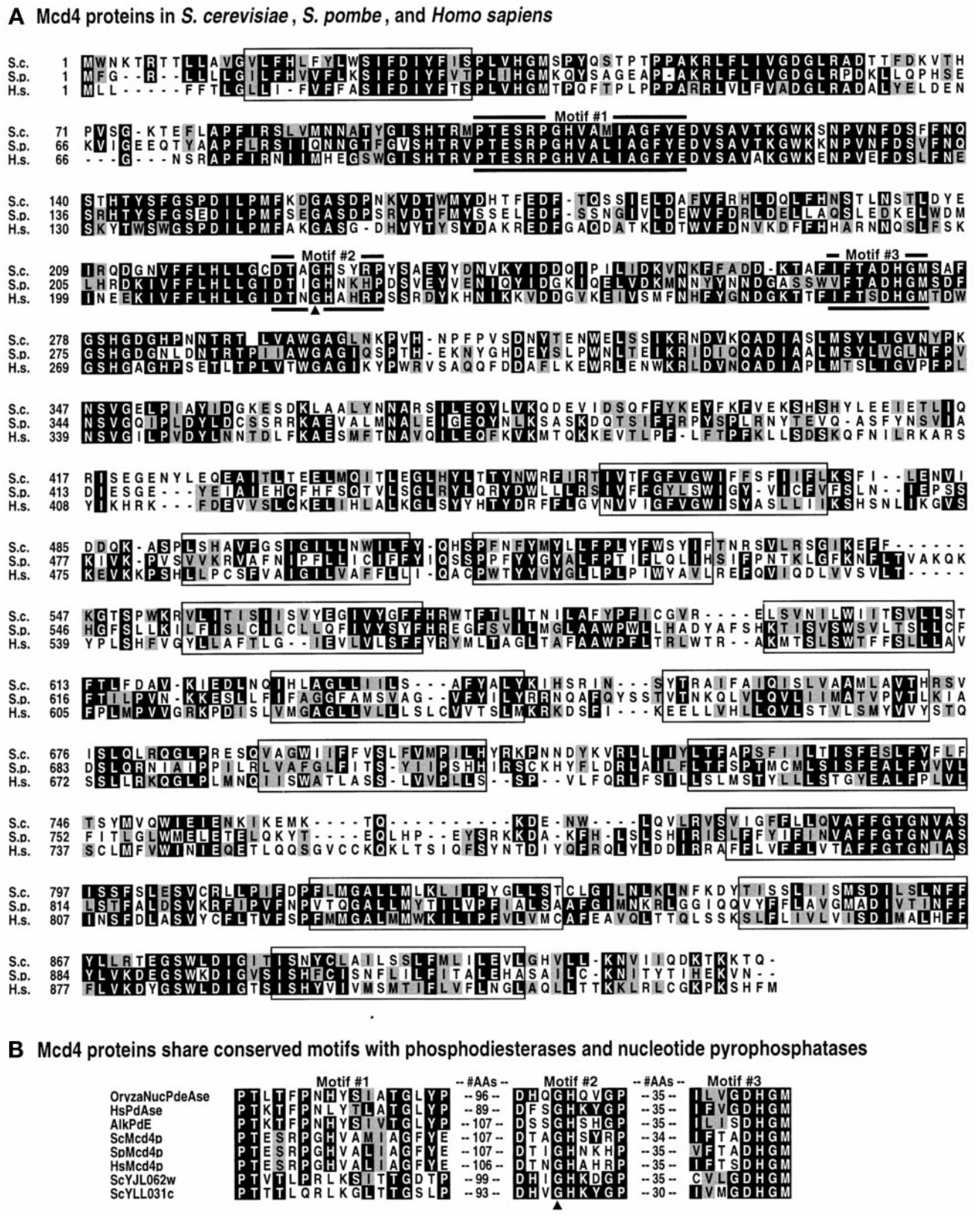
A Mcd4 proteins in S. cerevisiae, S. pombe, and Homo sapiens
Motif #1
Motif #2
Motif #3
B Mcd4 proteins share conserved motifs with phosphodiesterases and nucleotide pyrophosphatases
Motif #1
-- #AAs --
Motif #2
-- #AAs --
Motif #3
OryzaNucPdeAse
HsPdAse
AlkPdE
ScMcd4p
SpMcd4p
HsMcd4p
ScYJL062w
ScYLL031c

Figure 2.

membranes were more clustered and looped (Figure 3, C, E, and F; also see arrowheads) and may be derived from either ER or possibly Golgi membranes. A higher magnification photograph of these membranes is shown in Figure 3G. Some of the cells also accumulated secretory vesicles and, unexpectedly, the vacuoles appeared somewhat fragmented. Most (>90%) of the cells did not form buds; however, when buds were observed (Figure 3F), they were very small, misshapen, and contained many strange membrane structures, including the above mentioned clustered, looped membranes (which often appeared to accumulate near the bud necks). The *mcd4–174* mutation thus clearly causes marked morphological defects that are apparent at both the light microscope and ultrastructural levels.

Transport and Modification of Two Secretory Pathway Proteins, CPY and HSP150, Are Normal in mcd4–174

Given our morphological observations, the ER localization of Mcd4p, and the fact that the other *mcd* mutants affect some aspect of secretory pathway transport or protein modification, we reasoned that *mcd4* mutants might also exhibit defects in protein transport or modification within the secretory pathway, in particular at the level of the ER and Golgi. The only secretory protein analysis previously done with *mcd4* mutants was to examine invertase glycosylation (Mondésert *et al.*, 1997). As different secretory cargo proteins have distinctly different requirements for efficient secretory transport (Horvath *et al.*, 1994; Hamburger *et al.*, 1995; Schimmöller *et al.*, 1995; Gaynor and Emr, 1997; Sütterlin *et al.*, 1997), we decided to assay transport of additional proteins in *mcd4–174* cells. One differential requirement for secretory transport is that of COPI. COPI vesicles mediate Golgi-to-ER protein retrieval; however, some anterograde cargo proteins are also indirectly affected (Gaynor and Emr, 1997; Sütterlin *et al.*, 1997). As Mcd4p contains the C-terminal KKXX motif that signals COPI-mediated ER retrieval, we decided initially to test the *mcd4–174* mutant for its ability to transport at least one COPI-dependent cargo protein (CPY) and one additional COPI-independent cargo protein other than invertase (HSP150).

CPY is a vacuolar hydrolase that is synthesized as a precursor form. It is glycosylated first in the ER, generating the p1 form, and then in the Golgi, generating the p2 form. In the late Golgi, CPY is sorted and transported to the vacuole, where it is cleaved to generate the mature (m), active hydrolase (Stevens *et al.*, 1982). Wild-type and *mcd4–174* cells were incubated at permissive (24°C) or nonpermissive (38°C) temperature for 15 min and pulse-labeled with ^{35}S-methionine for 10 min. After 0 and 30 min of chase, cells were TCA precipitated, and lysates were prepared for immunoprecipitation with CPY antiserum. At both 24 and 38°C, CPY processing and maturation were normal in *mcd4–174* (Figure 4A; Gaynor and Emr, unpublished observations), indicating that Mcd4p is not likely to participate in trafficking or modification of CPY. HSP150 is a heavily O-glycosylated protein that is secreted into the growth medium (Russo *et al.*, 1992). Pulse-labeled and chased samples from the experiment described above were also separated into cell (internal; C) and media (external; M) fractions, and each fraction was TCA precipitated and processed for immunoprecipitation with HSP150 antiserum. For simplicity, only the 30-min, 38°C chase point is shown. Like CPY, HSP150 was processed and transported normally in *mcd4–174* under nonpermissive growth conditions, as evidenced by its appropriate migration in SDS gels and presence in the media (Figure 4B). Thus, at least one COPI-dependent and two COPI-independent cargo proteins do not appear to require Mcd4p for secretory transport. Together with previous work (Mondésert *et al.*, 1997), this analysis also indicated that Mcd4p does not participate in either the N-linked or O-linked glycosylation pathways.

Figure 2 (facing page). Mcd4 protein sequence alignments and motifs. (A) Alignment of Mcd4 proteins from *S. cerevisiae*, *S. pombe*, and *Homo sapiens*, using the CLUSTALW Multiple Sequence Alignment Program. Regions of amino acid identity are shaded black. Regions of amino acid identity are shaded gray. TMDs are in boxes. Motifs 1, 2, and 3 (labeled; also see part B) are denoted by thick lines. (B) Three conserved motifs found in phosphodiesterases, pyrophosphatases, Mcd4p, and Mcd4p homologues. Motifs were identified using MEME and MAST analyses. Shading is used to highlight identities and similarities between the known enzymes and Mcd4p and/or its homologues: within a given amino acid position, the most prevalent residue in Mcd4p or its homologues that is identical to an amino acid in the same position in a known enzyme are shaded black, while those that are similar are shaded gray. Amino acids that are identical or similar only between Mcd4p and its homologues are not shaded. "AAs" signifies the number of amino acids separating the motifs. In both panels A and B, the arrowhead points to glycine 227, which is mutated to glutamic acid in *S. cerevisiae mcd4–174*.

The **mcd4–174** *Mutant Exhibits a Marked Increase in Secretion of Proteins into the Medium*

Rather than simply screening through all known secretory cargo proteins, we next decided to take a more general approach to assay for secretory pathway defects in *mcd4–174*. This approach was previously successful in revealing secretory pathway abnormalities in COPI mutants and involves simply assaying for proteins secreted into the growth medium during pulse-chase analysis (Gaynor and Emr, 1997).

Samples of the entire labeled media fractions from the experiment described in Figure 4 were TCA precipitated, and the proteins were resolved by SDS-

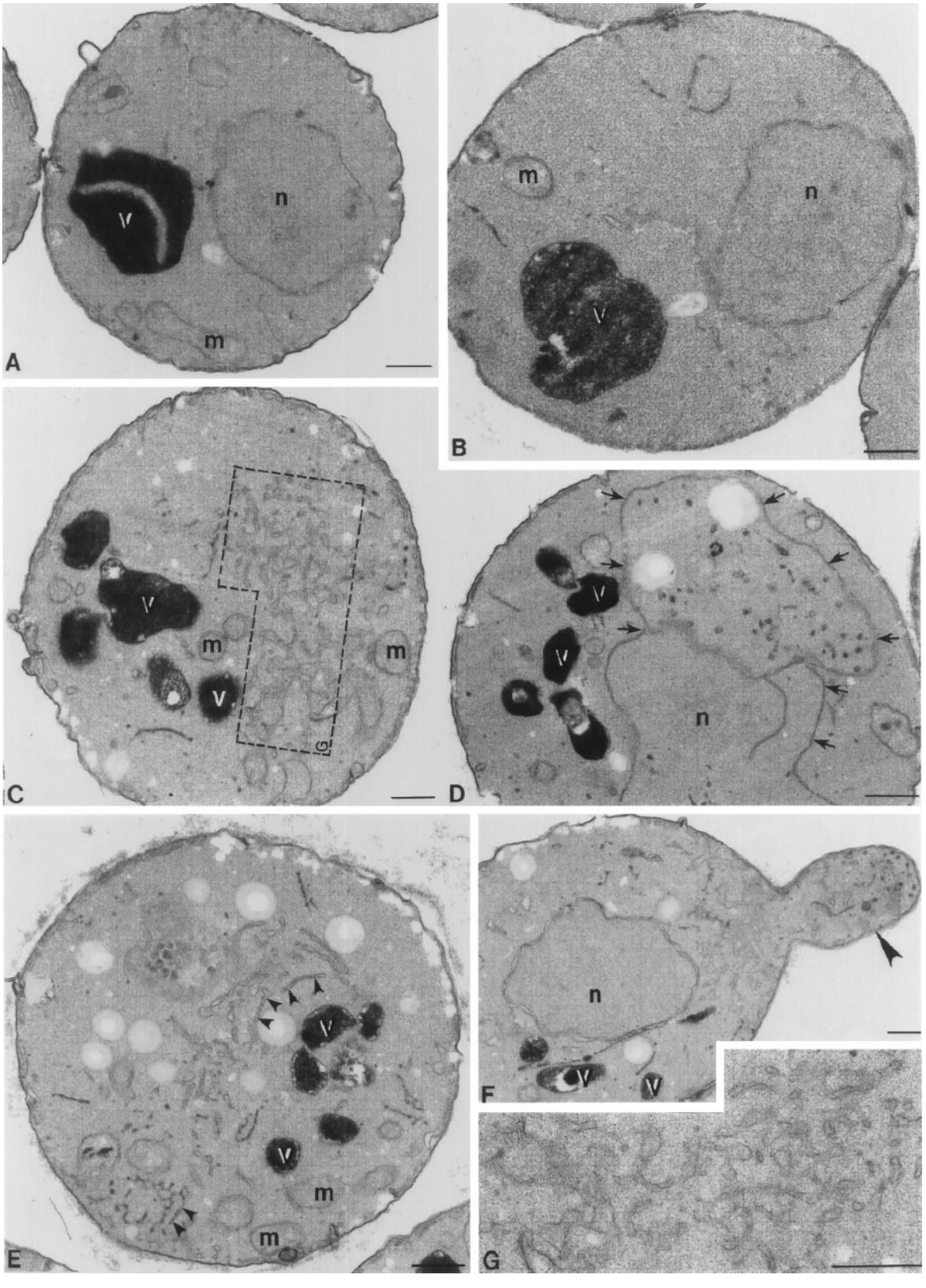

Figure 3.

PAGE. At 24°C, media protein secretion profiles were nearly identical for both wild-type and *mcd4–174* cells. Somewhat surprisingly, at 38°C the *mcd4–174* mutant secreted significantly more proteins into the media than wild-type cells (Figure 5A). Some of these secreted proteins appeared to represent an increase in proteins normally observed in the media from wild-type cells (arrows), while some of these proteins were not apparent in the wild-type media (●). This result was not due simply to increased ^{35}S-methionine incorporation into proteins by *mcd4–174*, as there was no difference in CPY or HSP150 signal strength in wild-type versus mutant cells (Figure 4). This result was also not likely to be due to cell lysis, as neither CPY nor the cytosolic protein glucose-6-phosphate dehydrogenase were observed in the *mcd4–174* media (Gaynor and Emr, unpublished observations). Furthermore, HSP150 synthesis and secretion were identical in *mcd4–174* and wild-type cells (Figure 4B), indicating that at least some normally secreted media proteins were unaffected by the *mcd4* mutation. Two proteins were also apparently secreted by wild-type but not *mcd4–174* cells (Figure 5A, open arrowheads); these proteins may require GPI anchoring and/or GPI protein transport for their normal secretion (see later sections).

One of the abundant proteins secreted from *mcd4–174* cells (Figure 5A, large arrow) migrated at a molecular mass which suggested that it might correspond to the 33-kDa cell wall protein CWP33 (Toyn *et al.*, 1988; Sanz *et al.*, 1989; Herman *et al.*, 1991); immunoprecipitation of cell and media fractions from the 38°, 30-min chase point with antiserum to CWP33 confirmed that this was the case (Figure 5B). Similar amounts of CWP33 were found in both the wild-type and *mcd4–174* cell (intracellular, C) fractions. No CWP33 was immunoprecipitated from wild-type media (Figure 5B, M), although some CWP33 (or a similarly migrating protein) was observed as a "media fraction protein" in wild-type cells (Figure 5A). In contrast, CWP33 was quite abundant in the media from *mcd4–174* cells (Figure 5B, M). Therefore, not only was CWP33 secreted into the media in *mcd4–174*, suggestive of a cell wall defect, but CWP33 synthesis was also markedly increased in the mutant.

Figure 3 (facing page). mcd4–174 Cells exhibit morphological aberrancies at nonpermissive temperature. Wild-type (SEY6210) and *mcd4–174* (GY1450) cells were incubated at 24 or 38°C for 3 h and prepared for electron microscopy. Wild-type cells at 38°C are shown in panel A. *mcd4–174* Cells at 24°C are shown in panel B. *mcd4–174* Cells at 38°C are shown in panels C–F. The nucleus (N), vacuole (v), and mitochondria (m) are indicated. Arrows point to accumulated membranes likely to correspond to ER. Arrowheads point to other accumulated membranes, which may also represent ER. Panel G is a higher magnification photograph of the accumulated membranes observed in the cell shown in panel C. Bar, 0.5 μm.

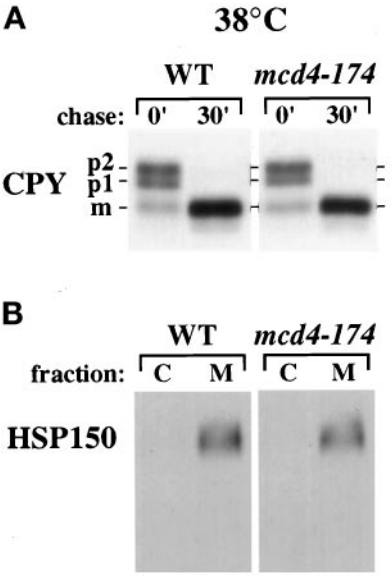

Figure 4. mcd4–174 Is not defective for transport or modification of CPY and HSP150. Wild-type (WT; GY1446) and *mcd4–174* (GY1450) cells were incubated at 38°C for 15 min, pulse-labeled with ^{35}S-methionine for 10 min, chased for 30 min, separated into intracellular (C) and extracellular/media (M) fractions, and processed for immunoprecipitation with antisera to CPY (A) or HSP150 (B). In panel A, the positions of p1 (ER), p2 (Golgi), and m (mature/vacuolar) CPY are indicated, and only the intracellular fraction is shown (no CPY was found in the extracellular [M] fraction).

The mcd4–174 Mutant Is Defective for ER-to-Golgi Transport of Multiple GPI-anchored Proteins

The aberrant secretion/release of at least one (and probably more) cell wall proteins into the media in *mcd4–174* mutant cells prompted us to look more closely at additional secretory pathway proteins. As GPI-anchored proteins have been implicated in maintenance of normal cell wall composition and function (Benghezal *et al.*, 1995; Ram *et al.*, 1995; Vossen *et al.*, 1995; Leidich and Orlean, 1996; Orlean, 1997), and as many cell wall proteins are GPI anchored (Schreuder *et al.*, 1993; Van der Vaart *et al.*, 1996; Skrzypek *et al.*, 1997), we next decided to look at transport of the well-characterized GPI-anchored protein Gas1p.

Gas1p is a plasma membrane-localized protein that is both N and O glycosylated (Conzelmann *et al.*, 1988; Fankhauser and Conzelmann, 1991; Nuoffer *et al.*, 1991). ER-to-Golgi transport of Gas1p results in a characteristic shift in the protein's mobility in SDS gels from a 105-kDa, ER-glycosylated form to a 125-kDa, Golgi-modified form. To test whether Mcd4p participates in ER-to-Golgi transport of Gas1p, cells were incubated either at 24 or 38°C for 15 min, pulse-labeled with ^{35}S-methionine, chased, and processed for immunoprecipitation with antiserum to Gas1p (Figure 6A). Wild-type cells matured Gas1p normally at all temperatures tested (for

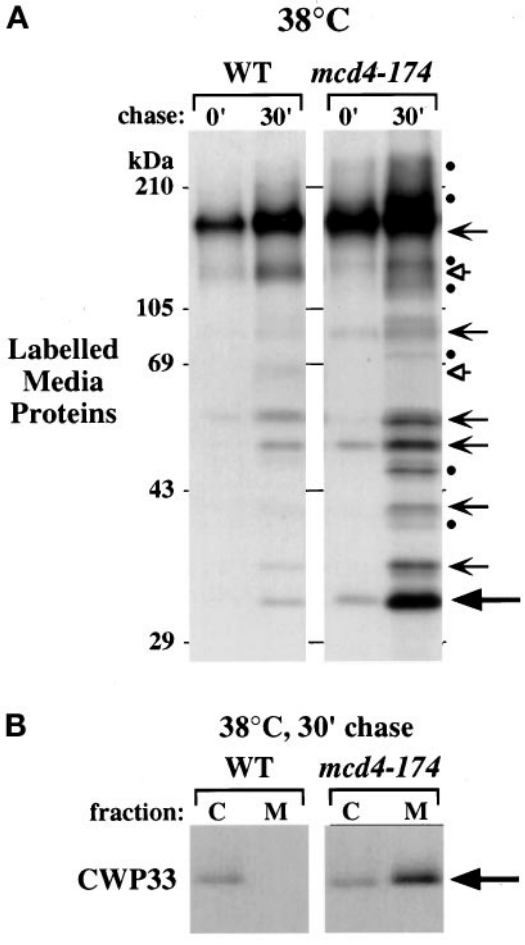

Figure 5. mcd4–174 Cells secrete/release multiple additional proteins into the media, at least one of which is a known and apparently up-regulated cell wall protein (CWP33). (A) Media proteins. Total labeled media fraction proteins from the experiment described in Figure 3 were resolved by SDS-PAGE. The positions of molecular mass markers are shown to the left. To the right, arrows (←) indicate proteins that appear in both the wild-type (WT) and *mcd4–174* media but that are more abundant in the media from *mcd4–174* cells, bullets (●) indicate proteins secreted from *mcd4–174* cells that are not apparent in the wild-type media lane, and open arrowheads indicate proteins secreted from wild-type but not *mcd4–174* cells. The thick arrow denotes the position of an abundant, ~33-kDa protein shown to be CWP33 (cell wall protein 33). (B) CWP33. Pulse-labeled and chased intracellular (C) and extracellular/media (M) fractions were immunoprecipitated for CWP33 (thick arrow).

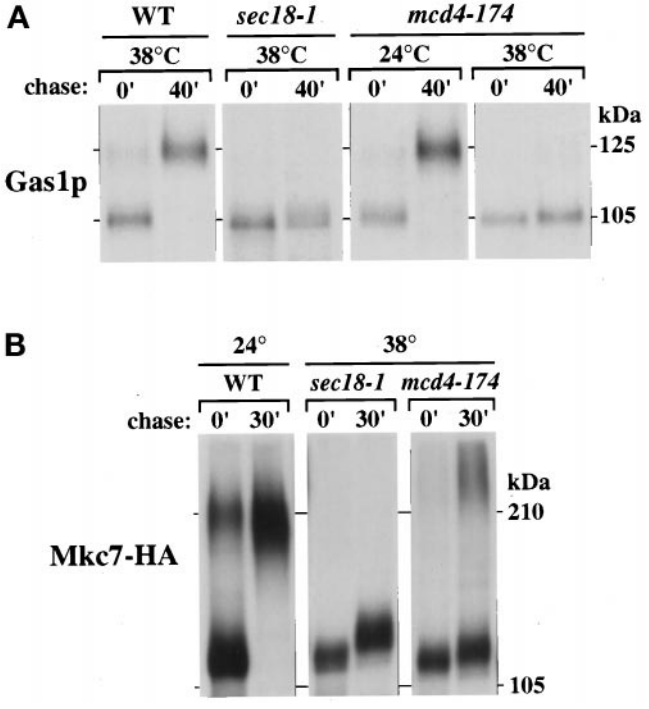

Figure 6. ER-to-Golgi transport of GPI-anchored proteins is defective in mcd4–174. (A) Gas1p transport. Wild-type (WT; GY1446), *sec18–1* (SEY5188), and *mcd4–174* (GY1450) cells were incubated at 24 or 38°C for 15 min, pulse-labeled with ^{35}S-methionine for 10 min, and chased for 40 min. Gas1p was recovered by immunoprecipitation. Shown to the right are the known molecular masses of ER-modified (~105 kDa) and Golgi-modified/mature Gas1p (~125 kDa). (B) Mkc7p transport. Wild-type (WT; GY1446), *sec18–1* (SEY5188), and *mcd4–174* (GY1450) cells harboring pRS425-MKC7HA were incubated at 24 or 38°C for 15 min, pulse labeled with ^{35}S-methionine for 10 min, and chased for 30 min. Mkc7-HA was recovered by immunoprecipitation with the 12CA5 monoclonal antibody. The positions of molecular mass markers are shown to the right.

simplicity, only 38°C is shown), as evidenced by the 105- to 125-kDa mobility shift over the course of a 40-min chase. In contrast, in a *sec18–1* (NSF) mutant, which blocks ER-to-Golgi transport (Conzelmann *et al*., 1988; Graham and Emr, 1991), Gas1p remained as its ER-glycosylated form. In *mcd4–174* cells at a permissive temperature (24°C), Gas1p was matured normally. However, in *mcd4–174* cells at a nonpermissive temperature (38°C), Gas1p was recovered only as the ER-modified form even after 40 min of chase. No Gas1p was secreted into the media or periplasm from *mcd4–174* cells (Gaynor and Emr, unpublished observations). As both N- and O-linked modification are normal in *mcd4–174* (Mondésert *et al*., 1997; Figure 4), these results indicate that the *mcd4–174* mutant is defective for ER-to-Golgi transport of Gas1p under nonpermissive conditions.

To determine whether this transport defect was specific to Gas1p or if other GPI-anchored proteins might also be affected, we assayed transport of an additional GPI-anchored protein, the aspartyl protease Mkc7p (Komano and Fuller, 1995). Like Gas1p, Mkc7p localizes to the plasma membrane, and its ER-to-Golgi transport is accompanied by a significant shift in molecular mass caused by the addition of both N- and O-linked Golgi-specific carbohydrates (Fuller, personal communication). Cells harboring an HA epitope-tagged form of the protein

expressed from a 2μ vector were incubated at 24 or 38°C, pulse-labeled with ^{35}S-methionine, and chased, and Mkc7-HA was immunoprecipitated using the 12CA5 monoclonal antibody directed against the HA epitope (Figure 6B). 2μ expression was necessary because Mkc7-HA expressed from a *CEN* vector could not be observed by immunoprecipitation. Mkc7-HA was matured normally in wild-type cells at 24°C, as its migration rapidly shifted from a ~110-kDa form to a hypermodified, ~200-kDa form during 30 min of chase; in fact, some mature/hypermodified protein was already apparent at the 0-min chase point. Prolonged incubation of wild-type cells at 38°C resulted in proteolysis of the mature, plasma membrane-localized protein, making analysis of the behavior of Mkc7-HA at high temperature in wild-type cells difficult. This was not completely surprising, however, as we and others have observed similar proteolysis for other cell-surface proteins at high temperature (Gaynor and Emr, unpublished observations; Sütterlin, personal communication). In *sec18–1* cells, Mkc7-HA increased slightly in molecular mass after 30 min of chase but was neither hypermodified nor degraded. This slight increase in molecular mass may represent increased carbohydrate modification due to increased residence time in the ER and increased acquisition of O-linked carbohydrates (Gaynor and Emr, 1997). In *mcd4–174* at 38°C, the vast majority of Mkc7-HA also remained as the ~110-kDa form after the 30-min chase (Figure 6B), indicating that its ER-to-Golgi transport was significantly impaired. Some hypermodified Mkc7-HA was also observed; this may be due to the protein's overexpression and/or a slightly less stringent transport block for Mkc7p than for Gas1p in *mcd4–174*. The difference in mobility of the ER-retained forms of Mkc7p in *sec18–1* and *mcd4–174* could reflect a requirement for GPI anchoring for optimal O-linked modification of GPI-anchored proteins in the ER (see next section). That the hypermodified Mkc7-HA migrated at a slightly higher molecular mass in *mcd4–174* than in wild-type cells may reflect a significant delay in export from and consequent acquisition of additional O-linked carbohydrates in the ER; this would then be exaggerated by extension of these additional O-linked sugars in the Golgi. Mkc7p is homologous to another GPI-anchored, plasma membrane-localized aspartyl protease, Yap3p (Ash *et al.*, 1995). Consistent with the Mkc7p and Gas1p results, we found that Yap3p also accumulated as the ER-modified form in *mcd4–174* at 38°C (Gaynor and Emr, unpublished observations). Together, these data indicate that the *mcd4–174* temperature-conditional mutation results in a defect in ER-to-Golgi transport of multiple GPI-anchored proteins.

Mcd4p Is Required for GPI Anchoring

The above data demonstrated that the *mcd4–174* mutant exhibits an ER-to-Golgi transport defect that is specific for GPI-anchored proteins. This defect could be due to one of several distinct possibilities. Of these, the two most likely are either that 1) Mcd4p could act in the GPI-anchoring pathway, or 2) Mcd4p could function in some aspect of sphingolipid/ceramide biosynthesis (see INTRODUCTION). Alternatively, Mcd4p could act as a "transport factor" required for efficient packaging of GPI-anchored proteins into budding COPII vesicles. Although our data suggest that Mcd4p does not cycle through the Golgi (and thus is not likely to function as a "receptor" that is copackaged with its cargo), the ER resident protein Shr3p has been shown to act in this capacity and is thought to be required for the folding and/or packaging of amino acid permeases into COPII vesicles in the ER but is not itself packaged or transported to the Golgi (Kuehn *et al.*, 1996, 1998).

To begin to differentiate among these possibilities, we first investigated whether GPI anchoring is impaired in *mcd4–174*. Yeast mutants that affect GPI synthesis or anchor attachment all exhibit moderate-to-severe defects in inositol incorporation into proteins. This is because GPI anchors contain an inositol moiety (Conzelmann *et al.*, 1988; Leidich *et al.*, 1994), and GPI-anchored proteins are the only proteins known to be covalently attached to inositol (Conzelmann *et al.*, 1990; Benghezal *et al.*, 1995; Hamburger *et al.*, 1995; Leidich *et al.*, 1995a; Schönbächler *et al.*, 1995; Leidich and Orlean, 1996). We therefore tested GPI anchoring in *mcd4–174* by analyzing whether newly synthesized proteins could be labeled with myo-[2-[^{3}H]]inositol. Cells were incubated at 24° or 38°C for 15 min, labeled with myo-[2-[^{3}H]]inositol for 60 min, and processed for SDS-PAGE.

In striking contrast to both wild-type and *sec18–1* cells, little to no inositol labeling of proteins in *mcd4–174* at 38°C was observed (Figure 7). This defect was already partially apparent at 24°C (Gaynor and Emr, unpublished observations) and may account for why *mcd4–174* cells grow more slowly than wild-type cells at a permissive temperature (although there is clearly enough GPI anchor synthesis/attachment in *mcd4–174* at 24°C to allow ER export of GPI-anchored proteins (Figure 6)). Consistent with previous observations (Conzelmann *et al.*, 1990), *sec18–1* cells accumulated different forms of inositol-labeled proteins than did wild-type cells (Figure 7). This occurs because *sec18–1* does not block GPI anchoring but does block ER-to-Golgi transport of newly synthesized GPI-anchored proteins and thus prevents their Golgi-specific carbohydrate modification. If Mcd4p functions as a "transport factor" directing ER export of GPI-anchored proteins, protein forms similar to those observed for *sec18–1* would be expected to accumulate

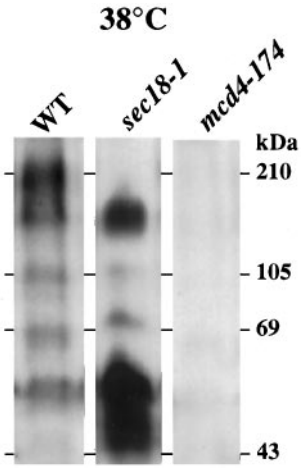

Figure 7. mcd4–174 Cells are defective for incorporation of inositol into proteins. Wild-type (WT; GY1446), *sec18–1* (SEY5188), and *mcd4–174* (GY1450) cells were incubated in inositol-free medium at 38°C for 15 min and then labeled with myo-[2-[^{3}H]]inositol for 45 min; ~1 OD-eq of the total protein extract for each sample was resolved by SDS-PAGE. Positions of molecular mass markers are shown to the right.

in *mcd4–174* as well. However, this was not the case. These data thus strongly indicate that Mcd4p is required for GPI anchoring and that a defect in GPI anchoring accounts for the defect in ER-to-Golgi transport of multiple GPI-anchored proteins in the *mcd4–174* mutant.

As it was initially somewhat surprising that *mcd4–174* cells were defective for GPI anchoring, we also investigated whether other known yeast *gpi* mutants exhibit *mcd*-like phenotypes. We first analyzed *gpi1*Δ, *gpi2*^{ts}, and *gpi3*^{ts} cells under restrictive and semirestrictive conditions by microscopy and found that 80–90% of these cells arrested as large, round, mostly unbudded cells, similar to *mcd4* and other *mcd* mutants. In addition, analysis of DNA content in these cells by propidium iodide staining and fluorescence-activated cell sorter analysis demonstrated that the number of *gpi* mutant cells with a 2C DNA content was greater than twice the number of budded cells. This is very similar to the *mcd4* mutant budding phenotype and indicates that the *gpi* mutants are severely delayed for budding. These results demonstrate that GPI-anchoring defects can clearly cause *mcd*-like phenotypes and are also consistent with the *mcd* screen yielding a mutant defective for GPI anchoring.

mcd4–174 Cells Synthesize All Classes of Inositolphosphoceramides (IPCs) but Accumulate Multiple Base-labile Potential GPI Anchor Precursors

Although the above data strongly argue that the GPI protein transport block in *mcd4–174* is due to a defect in GPI anchoring (see above), we also needed to test whether ceramide synthesis might also be affected. Conveniently, this analysis would also allow us to determine whether *mcd4–174* cells accumulate inositol-containing GPI anchor precursors, an expected phenotype for a GPI-anchoring mutant.

IPCs and their derivatives are the major yeast sphingolipids (Smith and Lester, 1974). The first step in their synthesis is the condensation of palmitoyl-CoA with serine to form 3-ketosphinganine. 3-Ketosphinganine is subsequently converted to sphinganine, which is then hydroxylated to become phytoceramide (Nagiec *et al.*, 1997). The addition of phosphatidylinositol (PI) to phytoceramide generates IPC. Yeast cells synthesize three major classes of IPCs, which differ in the type of long chain base and in hydroxylation and chain length of the fatty acids. One of the IPC classes can also be mannosylated to become mannosylinositolphosphate (MIPC); MIPC can then be modified further with another inositol phosphate to become mannosyl(inositolphosphate)$_2$ceramide (M(IP)$_2$C) (Steiner *et al.*, 1969; Smith and Lester, 1974; Becker and Lester, 1980).

Ceramide synthesis was assayed by incubating wild-type, *sec18–1*, and *mcd4–174* cells at 24 or 38°C for 15 min, pulse-labeling cells with myo-[2-[^{3}H]]inositol for 20 min, and chasing for 60 min. Lipids were extracted, desalted, and resolved by TLC using a solvent system that optimizes resolution of IPCs (solvent 1, $CHCl_3/CH_3OH$/0.25% KCl, 55:45:10). All three strains exhibited approximately similar lipid profiles at 24°C; therefore, only the 38°C results are shown (Figure 8).

As synthesis of at least one class of IPC, as well as the formation of MIPC and M(IP)$_2$C, is blocked in mutants defective for ER-to-Golgi transport (i.e., *sec18–1* [Puoti *et al.*, 1991]), we used the lipid profile of *sec18–1* together with both a phosphatidylinositol (PI) standard and extensive previously published analyses of yeast ceramides (Puoti *et al.*, 1991; Leidich *et al.*, 1994, 1995b; Benghezal *et al.*, 1995; Schönbächler *et al.*, 1995) to establish the identities of most of the lipid moieties (i.e., PI, IPCs, MIPC, lysophosphatidylinositol [lyso-PI], and M(IP)$_2$C) observed in our experiments. This analysis indicated that all classes of IPCs were synthesized in *mcd4–174* at 38°C (Figure 8). This analysis also suggested that *mcd4–174* may accumulate GPI anchor precursors at 38°C. For example, the additional dark spot that migrates below MIPC in the *mcd4–174* lane but is only marginally visible in the WT and *sec18–1* lanes (Figure 8, **) could correspond to an accumulated GPI intermediate, and the fact that the IPC spot for *mcd4–174* was much thicker that that of wild-type cells (IPCs, *) may reflect an accumulating GPI precursor(s) comigrating with IPCs using this solvent system. Nevertheless, these data clearly demonstrate that the defect in ER-to-Golgi transport of GPI-anchored proteins in *mcd4–174* is not due to reduced ceramide synthesis.

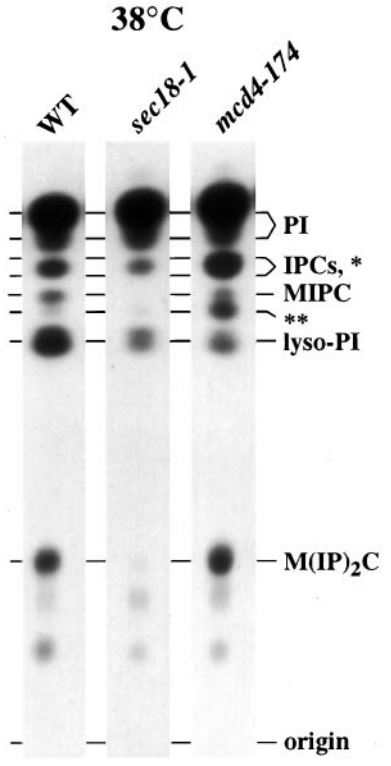

Figure 8. IPC synthesis in mcd4–174. Wild-type (WT; GY1446), *sec18–1* (SEY5188), and *mcd4–174* (GY1450) cells were incubated in inositol-free medium at 38°C for 15 min, labeled with myo-[2-[^{3}H]] inositol for 20 min, and chased with cold myo-inositol for 60 min. Lipids were extracted and ~0.5 OD-eq per sample were resolved by TLC using solvent 1. The positions of PI, IPCs, MIPC, lyso-PI (lyso-phosphatidylinositol), $M(IP)_2C$, and the origin are shown to the right. ** Denotes a lipid that accumulates in *mcd4–174* but not wild-type or *sec18–1* cells, and the IPCs; * Indicates that IPCs may mask another lipid species accumulating in *mcd4–174*.

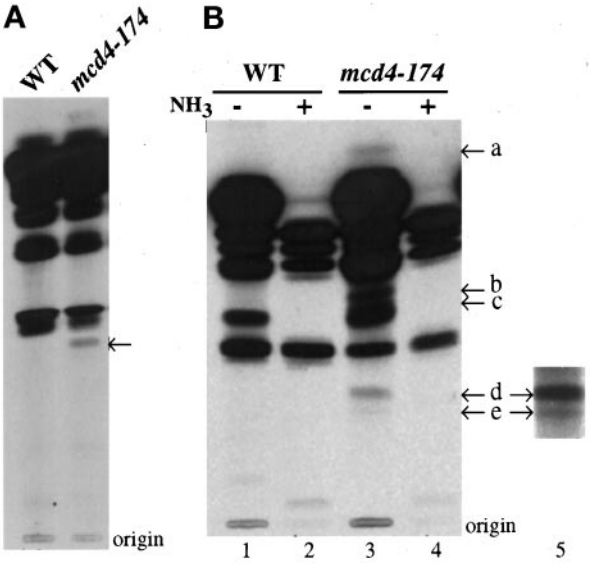

Figure 9. Accumulation of potential GPI anchor precursors in mcd4–174. Wild-type (WT; SEY6210) and *mcd4–174* (GY1450) cells were labeled at 38°C with myo-[2-[^{3}H]]inositol and lipids were extracted as described for Figure 8. (A) Labeled lipids were resolved by TLC using solvent 2. The position of a lipid that accumulates in *mcd4–174* but not wild-type cells is indicated by the arrow (←). (B) Labeled lipids were either treated with the mild base methanolic NH_3 (lanes 2 and 4, "+") or left untreated (lanes 1 and 3, "−"; lane 5) and resolved by TLC using solvent 3. The positions of base-labile lipids (potential GPI precursors) that accumulate in *mcd4–174* but not wild-type cells (lipids a, b, c, d, and) are indicated by the arrows (←). Lane 5 shows lipids d and e from a ~3.5-fold longer exposure than that used for lanes 1–4.

Further investigations to determine whether GPI-anchor biosynthetic intermediates accumulate in *mcd4–174* were carried out by 1) separating the myo-[2-[^{3}H]]inositol–labeled lipids using two additional solvent systems standard to the field of GPI precursor analysis (solvent 2, $CHCl_3/CH_3OH/H_2O$, 10:10:2.5 [vol/vol]; solvent 3, $CHCl_3/CH_3OH$/13 M NH_4OH/1 M NH_4OAc/H_2O, 180:140:9:9:23 [vol/vol]), and 2) testing them for sensitivity to methanolic NH_3, a treatment that cleaves GPI-linked but not ceramide-linked inositol glycolipids (Costello and Orlean, 1992; Sipos *et al.*, 1994). As before, no major differences between *mcd4–174* and wild-type cells were observed at 24°C; therefore, all data shown are from cells labeled at 38°C.

TLC resolution of [[^{3}H]]inositol-labeled lipids using solvent 2 revealed at least one additional, low-abundance, somewhat polar lipid present in extracts of *mcd4–174* but not wild-type cells (Figure 9A, arrow). The chromatographic mobility of this lipid differs from those of the "complete precursor" GPIs (CPs) that accumulate in transamidase mutants such as *gaa1* and *gpi8*, as CPs migrate much closer to the origin in similar solvents (Benghezal *et al.*, 1995, 1996; Hamburger *et al.*, 1995). This new lipid also did not have the mobility of the Man_2, EtN-P–containing species that accumulates in *gpi10* mutants (Benghezal *et al.*, 1995; Canivenc-Gansel *et al.*, 1998, Sütterlin *et al.*, 1998), nor does it comigrate with either of the two Man_4, EtN-P-containing species that accumulate in a mutant deficient in the yeast homologue of PIG-F, a human gene required for addition of EtN-P to the third mannose of the GPI precursor (Taron and Orlean, unpublished observations). The mobility of the lipid in Figure 9A is comparable to that of the lipid that accumulates in the *gpi7* mutant (Benghezal *et al.*, 1995, 1996), which is defective for adding the terminal EtN-P to the anchor. However, the *mcd4–174* lipid is labeled much less intensely than the *gpi7* species. If *gpi7* and *mcd4–174* were allelic, our labeling result would not be expected, because in contrast with the tight temperature sensitivity of *mcd4–174*, *gpi7* isolates are only slightly temperature sensitive and might

therefore be predicted to accumulate less GPI precursor than *mcd4–174*.

Although it remains possible that the *mcd4–174* and *gpi7* lipids may prove to be the same, further analysis using solvent 3 revealed that the biochemical phenotype of *mcd4–174* is different from that of *gpi7*. Interestingly, TLC using solvent 3 "uncovered" five [^{3}H]-inositol-labeled lipids that accumulate in *mcd4–174* but not wild-type cells (Figure 9B, lane 3, arrows). Of these, species b and c are the most abundant, whereas a, d, and e are minor species. Lipid c is clearly visible on film but is difficult to see on the scan due to its near comigration with the base-labile lipid just beneath it. A longer exposure that more clearly demonstrates the presence of lipids d and e is shown to the right (Figure 9B, lane 5, arrows). Treatment of the [^{3}H]inositol-labeled lipids with mild base (methanolic NH_3) prior to TLC resolution caused all five of the *mcd4–174*-specific bands to disappear (Figure 9B lanes 1–4, compare "−" vs. "+" lanes). This base lability is a feature of all known yeast GPI precursors (Costello and Orlean, 1992; Sipos *et al.*, 1994) and is consistent with the possibility that these new lipids are indeed GPI biosynthetic intermediates. As with the TLC developed in solvent 2, the *mcd4–174*–specific lipids visualized using solvent 3 are labeled less intensely than lipids that accumulate in other *gpi* mutants.

Finally, the accumulation of multiple potential GPI precursors raised the possibility that these species are counterparts of normal yeast GPI precursors lacking the acyl group that becomes esterified to inositol at the beginning of the anchor synthesis pathway (Costello and Orlean, 1992; Sipos *et al.*, 1994). This is unlikely to be the case, however, as *mcd4–174* membranes are fully capable of making GlcN-(acyl-Ins)PI, GlcNAc-PI, and GlcN-PI in vitro (Kostova and Orlean, unpublished observations). Taken together, these analyses indicate that *mcd4–174* mutants accumulate multiple candidate GPI biosynthetic intermediates and suggest that Mcd4p may have an as-yet-uncharacterized role in the assembly of GPI precursors.

DISCUSSION

We have shown that *MCD4* encodes an essential, novel, and highly conserved component of the eukaryotic GPI anchor synthesis pathway. The multimembrane-spanning Mcd4 protein localizes to the ER, with most of the N-terminal half of the protein extending into the ER lumen. We have also cloned the human *MCD4* gene and found that Mcd4 proteins exist in multiple eukaryotic systems, are strikingly well conserved, have additional yeast homologues, and contain motifs found in mammalian phosphodiesterases, pyrophosphatases, and certain sulfatases. The *S. cerevisiae mcd4–174* mutant, originally identified in a screen to isolate mutants defective for bud emergence and polarized growth, exhibits specific defects for ER-to-Golgi transport of multiple GPI-anchored proteins, aberrantly secretes proteins into the growth medium, and accumulates ER-like and other abnormal membrane structures. *mcd4–174* is also severely defective in incorporation of inositol into newly synthesized proteins and accumulates multiple candidate GPI anchor precursors that, when resolved by TLC, migrate in a pattern unlike that seen with any known *gpi* mutants. Our observations are consistent with 1) Mcd4p participating in a critical yet poorly understood step in the GPI anchor synthesis pathway and 2) an intimate connection between GPI anchoring, polarized secretion, bud emergence, and cell wall function. This work also lends increased insight into regulatory feedback mechanisms controlling each of these essential cellular functions.

Mcd4p and the GPI-anchoring Pathway

GPI anchoring is an essential, complex process requiring the action of multiple ER-localized proteins that function in various aspects of anchor synthesis and attachment. Some of these proteins have already been identified in yeast: for example, 1) Gpi1p, Gpi2p, and Gpi3p (Spt14p), all of which act at an early stage of anchor synthesis to catalyze the addition of GlcNAc to inositol (Leidich *et al.*, 1994, 1995a,b; Schönbächler *et al.*, 1995; Vossen *et al.*, 1995); 2) the yeast homologue of PIG-Lp, the mammalian GlcNAc-PI deN-acetylase (Nakamura *et al.*, 1997); 3) Dpm1p and Sec53p (*sec53* was also identified as an *mcd* mutant), each of which help provide the ER with mannose for anchor synthesis as well as for N- and O-linked carbohydrate modification (Kepes and Schekman, 1988; Orlean *et al.*, 1988; Orlean, 1990); 4) Gpi7p, which may be involved in addition of phosphoethanolamine near the end of the synthesis pathway (Benghezal *et al.*, 1995); 5) the yeast homologue of PIG-Fp, a human protein also required for addition of phosphoethanolamine to the GPI precursor (Taron and Orlean, unpublished observations); and 6) Gpi8p and Gaa1p, required for the transamidation reaction that attaches the GPI anchor onto newly synthesized proteins (Hamburger *et al.*, 1995; Benghezal *et al.*, 1996). The *mcd4–174* mutant phenotypes are entirely consistent with Mcd4p acting specifically in the GPI-anchoring pathway. For instance, all GPI-anchoring mutants thus far characterized are defective for incorporation of inositol into proteins, and the GPI anchoring-specific mutants (i.e., *gpi1*, *gpi2*, *gpi3*, *gpi7*, *gpi8*, and *gaa1*) are defective for ER-to-Golgi transport of GPI-anchored but not other secretory proteins. Many GPI anchoring-specific mutants also exhibit similar bud emergence defects as *mcd4–174* (Reed, unpublished observations; Leidich and Orlean, 1996; Vossen *et al.*, 1997), and at least one GPI mutant (*gpi3*) has also been shown to accumulate

ER-like membranes and to secrete cell wall proteins into the medium (Vossen *et al.*, 1997).

Interestingly, each of the aforementioned GPI anchoring-specific proteins already cloned are, like Mcd4p, also highly conserved throughout eukaryotes. In addition to the yeast PIG gene homologues described above, strikingly well-conserved human homologues have now been identified for Gpi1p (Watanabe *et al.*, 1998), Gpi2p (Inoue *et al.*, 1996), Gpi3p (Leidich *et al.*, 1995b; Schönbächler *et al.*, 1995), Gpi8p (Benghezal *et al.*, 1996), and Gaa1p (Hiroi *et al.*, 1998). As with the *S. cerevisiae*, *S. pombe*, and human Mcd4 proteins (Figure 2A), the region of highest homology is almost always within the ER lumenal domains. Each of the GPI anchoring-specific proteins are also transmembrane proteins, usually multimembrane spanning, with at least one (Gaa1p) being topologically quite similar to Mcd4p (albeit with fewer TMDs) (Hamburger *et al.*, 1995). Like Mcd4p, Gaa1p also contains a C-terminal KKXX (KXKXX) motif, which mediates Golgi-to-ER retrieval of membrane proteins by directing their packaging into retrograde COPI-coated vesicles (Letourneur *et al.*, 1994). COPI is also required for ER export of certain cargo proteins (i.e., vacuolar hydrolases, the mating pheromone α-factor, and GPI-anchored proteins (Gaynor and Emr, 1997; Sütterlin *et al.*, 1997); in fact, GPI-anchored proteins are the only anterograde cargo thus far shown to be affected in the *ret1–1* (α-COP) mutant. As the *ret1–1* mutant is likely to be specifically defective for recognition or packaging of KKXX-containing proteins into COPI vesicles (Letourneur *et al.*, 1994), GPI-anchored proteins may require KKXX-mediated retrieval for their anterograde transport. However, Mcd4p does not appear to cycle between the Golgi and ER, nor does it require its KKXX motif for proper function, indicating that the requirements for Mcd4p and COPI in GPI protein transport are likely to be distinct. Mcd4p is thus retained in the ER either by an as-yet-undescribed mechanism or, as with other essential ER resident proteins (i.e., Wbp1p [te Heesen *et al.*, 1993; Gaynor *et al.*, 1994]), via a physical interaction with another component(s) of the GPI-anchoring pathway.

Speculations on a Role for Mcd4p in GPI Anchor Synthesis

Assuming that Mcdp participates in a step in GPI precursor assembly for which the gene(s) involved have not yet been identified, which remaining step might be Mcd4p dependent and involve a reaction consistent with the observed amino acid sequence similarities that Mcd4p shows to other proteins? Genetically uncharacterized steps include inositol acylation of GlcN-PI, addition of the first (α1,4-linked), second (α1,6-linked), and fourth (α1,2-linked) mannoses to the GPI precursor, and the recently reported addition of phosphoethanolamine to the α1,4-linked mannose (Canivenc-Gansel *et al.*, 1998; Sütterlin *et al.*, 1998). Mcd4p is not likely to participate either in inositol acylation of GlcNAc-PI or in transfer of the first, α1,4-linked mannose to GlcN-(acyl-Ins)PI, as *mcd4–174* cells competently synthesize, but do not accumulate, GlcN-(acyl-Ins)PI. Likewise, a role in addition of the fourth, α1,2-linked mannose also seems unlikely, because a mutation in a different gene blocks this step (Grimme and Orlean, unpublished observations), and the *mcd4–174* lipids do not have the same mobility as the lipid that accumulates in this mutant. Mcd4p could transfer the second, α1,6-linked mannose to the anchor, in which case one would expect EtN-P-Man-GlcN-(acyl-Ins)PI or Man-GlcN-(acyl-Ins)PI to accumulate. However, this too seems unlikely, since a mutant that is defective for addition of a specific mannose to the anchor core would be expected to accumulate one predominant GPI anchor precursor, and we observe accumulation of multiple precursor moieties in *mcd4–174* cells. Finally, because "complete precursor" GPI anchors do not accumulate in the *mcd4–174* mutant, Mcd4p is not likely to function as an additional component of the transamidase complex.

Our results are, however, consistent with Mcd4p being involved in addition or removal of the side-branching phosphoethanolamine (EtN-P) moiety to the GPI glycan. This step in the anchor synthesis pathway is particularly interesting because of its species specificity: as mentioned in INTRODUCTION, side chain EtN-P modification has been known for several years to occur in mammalian cells but not parasitic eukaryotes such as *Trypanosoma*, *Leishmania*, and *Plasmodium* spp. and was only recently described to occur in *S. cerevisiae*. Several lines of evidence suggest that Mcd4p participates in this step of the yeast GPI anchor synthesis pathway. First, unlike the core mannose residues, the side-branching EtN-P moieties do not act as "building blocks" onto which the rest of the anchor must be constructed. Consequently, failure to add this side chain modification may not completely hinder further addition of core mannose molecules; rather, this may simply make the growing GPI anchor a much less efficient substrate for further modification, resulting in the accumulation of a series of aberrant GPI precursors that all lack the same substituent (i.e., EtN-P). This would explain why the *mcd4–174* mutant accumulates multiple GPI biosynthetic precursors, none of which are overly abundant. The most abundant band, lipid b (Figure 9B, lane 3), may represent the GPI precursor that most requires EtN-P modification for further anchor construction. Nevertheless, the strong blocks in GPI anchor addition and GPI protein transport in *mcd4–174* indicate that these precursors never fully "mature" into anchors that are competent to be added to protein.

Our sequence data demonstrating that 1) Mcd4p shares homology and motifs with known mammalian phosphodiesterases and nucleotide pyrophosphatases and 2) the *mcd4–174* mutation is in a conserved amino acid residue of motif 2 also support the idea that Mcd4p participates in modification of GPI anchors with a phosphodiester-linked substituent (i.e., EtN-P). The most straightforward explanation for how Mcd4p might affect this step is that it is directly involved in addition of EtN-P to GPI glycan, by creating (perhaps in concert with another protein) a phosphodieseter bond between EtN-P and mannose. The existence of yeast Mcd4p homologues suggests that EtN-P side branches may be added at more than one position; indeed, in addition to the EtN-P residue found on the first, α1,4-linked mannose, EtN-P is also likely to be present on the second, α1,6-linked mannose in at least one GPI precursor (Taron and Orlean, unpublished observations). Alternatively, the resemblance of Mcd4p to certain nucleotide pyrophosphatases also raises the possibility that Mcd4p might cleave a nucleotide-bound EtN-P precursor (i.e., CDP-ethanolamine) to generate the EtN-P moiety that is then added to the GPI anchor. Lastly, Mcd4p may instead act to remove EtN-P from the GPI anchor, a role that is consistent both with Mcd4p's homology to phosphodiesterases and with a previous suggestion that EtN-P side branches may have a transient role in anchor assembly and transfer (Canivenc-Gansel *et al.*, 1998). The low abundance of inositol-labeled lipids that accumulate in *mcd4–174* precludes an easy analysis of their head groups to establish whether either they lack the normal branching EtN-Ps or retain ones at positions from which they are normally removed.

mcd *Mutants, Bud Emergence, GPI Anchoring, and Glycosylation: The Cell Wall Connection*

Why was a gene required for GPI anchoring identified in a screen for bud emergence-defective mutants? Cell wall remodeling and integrity are both required in order for bud emergence to proceed normally (Klis, 1994; Cid *et al.*, 1995; Orlean, 1997); therefore, a likely common denominator linking the two processes of bud emergence and GPI anchoring is the cell wall. Our media secretion and CWP33 results clearly indicate a defect in cell wall integrity (and up-regulation of CWP33 synthesis) in *mcd4–174* mutants; a similar cell wall-integrity defect was also observed in the *gpi3* mutant (Vossen *et al.*, 1997). Furthermore, screens designed to isolate cell wall-defective mutants often identify genes encoding either GPI-anchoring components or GPI-anchored proteins (Ram *et al.*, 1995; Van der Vaart *et al.*, 1995; Vossen *et al.*, 1995). Yeast cell walls are composed primarily of mannoproteins and glucan. Some cell wall mannoproteins are initially synthesized and transported to the plasma membrane as GPI-anchored proteins; at the plasma membrane, the GPI anchor is replaced with β1,6-glucan that links the protein to the wall (Cid *et al.*, 1995; Orlean, 1997). A defect in synthesis or transport of such proteins would be likely to lead to a defect in cell wall structure or integrity. Alternatively or additionally, a failure to transport plasma membrane-localized GPI-anchored proteins such as Gas1p to their final destination may also yield cell wall defects sufficient to account for many of the *mcd4* mutant phenotypes. Although a precise function for Gas1p has not yet been established, *gas1*Δ cells contain at least 50% less β-glucan than wild-type cells (Ram *et al.*, 1995; Kapteyn *et al.*, 1997; Popolo *et al.*, 1997), exhibit similar morphological characteristics as *mcd4* mutants (i.e., enlarged and round cells, with cell separation defects [Popolo *et al.*, 1993; Ram *et al.*, 1995]), and exhibit a similar media protein secretion profile as observed for *mcd4–174* (Gaynor and Emr, unpublished observations). Five homologous *GAS1* genes have recently been identified in the yeast genome (Caro *et al.*, 1997); analyses of these genes should lend insight into the precise role of this abundant GPI-anchored protein.

Cell wall-specific requirements for bud emergence would also be consistent with the *mcd* screen yielding multiple genes required for Golgi-specific glycosylation. Some cell wall mannoproteins are >95% carbohydrate by mass, and the carbohydrate content of the cell wall has long been known to affect its function and integrity (Klis, 1994; Cid *et al.*, 1995; Orlean, 1997). Interestingly, a screen to isolate mutants synthetically lethal in combination with an *OCH1* (initiating α1,6-mannosyltransferase) deletion identified both *MCD4* and *GPI2* (Waters and Harris, personal communication). This is not only consistent with Mcd4p acting in the GPI-anchoring pathway but also strengthens the idea that the critical common requirement for both GPI anchoring and Golgi-specific glycosylation in bud emergence may be in preserving cell wall integrity and function. Alternatively or additionally, a specific defect in glycosylation of GPI-anchored proteins such as Gas1p might affect their transport and/or function sufficiently to account for the common phenotypes observed for both the *mcd4* and glycosylation-defective *mcd* mutants. Given the strong implication that cell wall components are almost certainly affected in *mcd* mutants, it is also interesting to speculate that perhaps exocyst mutants were isolated in the *mcd* screen because of their inability to deliver components to the bud tip that are specifically required for cell wall remodeling activities to occur normally.

Potential Feedback Responses in mcd4–174 *Cells*

Finally, our data imply that *mcd4–174* cells are also likely to initiate regulatory feedback mechanisms in response to the cell wall integrity and GPI-anchoring defects. For

example, proteins like CWP33 were clearly much more abundant in the media from *mcd4–174* cells than from wild-type cells; however, similar amounts of CWP33 were observed in the cell-associated fraction in both cell types. This indicates that not only is the *mcd4–174* GPI anchoring defect likely to weaken the cell wall and compromise its ability to retain mannoproteins, but that these cells may also up-regulate cell wall protein synthesis as a compensatory mechanism to maintain near-normal levels of protein in the wall. Consistent with this idea, it has previously been shown that overexpression of another cell wall protein, Cwp2p, partially suppresses the pH sensitivity of a yeast sphingolipid/ceramide synthesis mutant (Skrzypek *et al.*, 1997).

It is interesting to note that a clear connection has already been established between the cell wall and at least two signal transduction pathways, one of which involves the yeast protein kinase C (Pkc1p) (Cid *et al.*, 1995). *pkc1* mutants have cell integrity defects (Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992), exhibit defects in bud emergence (Costigan *et al.*, 1992; Mazzoni *et al.*, 1993; Marini *et al.*, 1996; Gray *et al.*, 1997), and are synthetically lethal in combination with deletion of *GAS1* (Popolo *et al.*, 1997). Pkc1p acts at the plasma membrane to initiate a MAP kinase cascade (Cid *et al.*, 1995), and this *PKC1*-MAP kinase pathway is already known to influence transcription of several genes involved in cell wall biosynthesis (i.e., *FKS1*, encoding a subunit of the β1,3-glucan synthase; *MNN1*, encoding the α1,3-mannosyltransferase, and *CSD2*, encoding chitin synthase III [Igual *et al.*, 1996]). Interestingly, Pkc1p activation as well as transcription of *GAS1* and several cell wall components are also cell cycle dependent (Mazzoni *et al.*, 1993; Ram *et al.*, 1995; Igual *et al.*, 1996; Marini *et al.*, 1996; Gray *et al.*, 1997), with both events peaking at the G1–S transition where polarized growth and bud emergence are initiated. The increase in synthesis of cell wall proteins observed in *mcd4–174* cells is thus entirely consistent with an intimate connection between the cell cycle, bud emergence, cell wall integrity, and signal transduction-feedback pathways.

Our findings clearly highlight the complexity of the GPI biosynthetic pathway, raise the possibility that Mcd4p participates in the modification of GPI anchors with side-branching EtN-P, and provide direct evidence that the distinct processes of GPI anchoring and bud emergence in yeast are actually highly interconnected. Future studies to elucidate the precise biochemical function of Mcd4p and its homologues will yield insight into the precise role these proteins play in the GPI-anchoring pathway not only in yeast but in multicellular eukaryotic systems as well.

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