The EMBO Journal vol.13 no.7 pp.1718–1728, 1994

GDI1 encodes a GDP dissociation inhibitor that plays an essential role in the yeast secretory pathway

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Communicated by G.Blobel

GTP binding proteins of the Sec4/Ypt/rab family regulate distinct vesicular traffic events in eukaryotic cells. We have cloned *GDI1*, an essential homolog of bovine rab GDI (GDP dissociation inhibitor) from the yeast *Saccharomyces cerevisiae*. Analogous to the bovine protein, purified Gdi1p slows the dissociation of GDP from Sec4p and releases the GDP-bound form from yeast membranes. Depletion of Gdi1p *in vivo* leads to loss of the soluble pool of Sec4p and inhibition of protein transport at multiple stages of the secretory pathway. Complementation analysis indicates that *GDI1* is allelic to *sec19-1*. These results establish that Gdi1p plays an essential function in membrane traffic and are consistent with a role for Gdi1p in the recycling of proteins of the Sec4/Ypt/rab family from their target membranes back to their vesicular pools.

Key words: GDI/rab/*SEC4*/secretion/yeast

Introduction

The transfer of material between organelles in eukaryotic cells is predominantly mediated by vesicular transport (Palade, 1975; Rothman and Orci, 1992). GTP binding proteins play key roles in the regulation of vesicular traffic at many stages of the exocytic and endocytic transport pathways (Ferro-Novick and Novick, 1993). These proteins include members of the rab and arf subgroups of the ras superfamily of small GTPases as well as members of the heterotrimeric G protein family. Proteins of the ras superfamily have stable GDP- and GTP-bound conformations allowing them to function as molecular switches that control diverse biochemical pathways (Bourne *et al.*, 1990, 1991). The nucleotide state of these proteins is determined by their interaction with guanine nucleotide exchange factors (GEFs) that regulate the dissociation of GDP and binding of GTP as well as GTPase activating proteins (GAPs) that regulate hydrolysis of the bound GTP. The GTP binding proteins and their regulatory components function together in a tightly controlled cycle of GTP binding, hydrolysis and exchange. rab and rho proteins have additional regulatory components known as GDP dissociation inhibitors (GDIs) that act to slow the rate of dissociation of GDP (Sasaki *et al.*, 1990; Ueda *et al.*, 1990). An additional function of rab GDI will be discussed later.

Proper subcellular localization is crucial to the function of ras-related GTPases. Proteins of the rab subgroup, including the Sec4 and Ypt1 proteins of yeast, associate with membranes by a process requiring the covalent attachment of geranylgeranyl lipid moieties to C-terminal cysteine residues (Magee and Newman, 1992). Different members of this family are localized to distinct stages of the exocytic or endocytic pathways. For example, Sec4p, a key regulator of exocytosis in yeast, is found on secretory vesicles and the plasma membrane (Salminen and Novick, 1987; Goud *et al.*, 1988). Ypt1p is found on the Golgi apparatus and on ER–Golgi carrier vesicles and functions to regulate the early stages of the exocytic pathway (Segev *et al.*, 1988; Bacon *et al.*, 1989; Baker *et al.*, 1990; Rexach and Schekman, 1991; Segev, 1991; Lian and Ferro-Novick, 1993). rabs 4 and 5 are associated with endosomes and regulate transport between the cell surface and endosomes (Chavrier *et al.*, 1990; Gorvel *et al.*, 1991; van der Sluijs *et al.*, 1991, 1992; Bucci *et al.*, 1992). In addition to the membrane-bound pools, soluble pools of the Sec4/Ypt/rab proteins have also been detected. A cycle of Sec4p localization has been revealed through pulse–chase studies (Goud *et al.*, 1988). Sec4p rapidly binds to secretory vesicles from the soluble pool. The vesicular pool of Sec4p is brought to the plasma membrane by exocytic fusion. Sec4p then recycles from the plasma membrane, through the soluble pool, onto newly formed vesicles. A similar cycle has been suggested for the synaptic vesicle associated protein, rab3A (Fischer von Mollard *et al.*, 1991).

The cycle of nucleotide binding and hydrolysis may be coupled to the cycle of subcellular localization (Bourne, 1988). In the case of Sec4p, it has been proposed that the GTP-bound form associates with secretory vesicles (Walworth *et al.*, 1989, 1992). Interaction with an effector on the plasma membrane triggers vesicle fusion. Sec4p then interacts with a GAP leaving Sec4p in its GDP-bound form on the plasma membrane. The GDP-bound form recycles through the cytoplasm until interaction with an exchange protein allows GTP binding and reassociation with vesicles. Two recent findings lend support to this cycling model of Sec4p function. One finding concerns the role of GTP hydrolysis in Sec4p function (Walworth *et al.*, 1992). The model predicts that a block in GTP hydrolysis would prevent recycling and thus inhibit function. In contrast, mutations in the ras protein that block GTP hydrolysis result in an increase in the GTP-bound pool and an increase in the activation of the downstream component of the signal transduction pathway (Barbacid, 1987; Bourne *et al.*, 1990, 1991). A *sec4* mutant (*sec4-L*^{79} analogous to *ras-L*^{61}) which strongly inhibits hydrolysis of GTP results in a loss of Sec4p function (Walworth *et al.*, 1992). The second finding in support of the cycling model is the identification and

characterization of the rab GDI (Araki *et al.*, 1990; Sasaki *et al.*, 1990).

Rab GDI was first identified from bovine brain cytosol by its ability to slow the dissociation of GDP from prenylated rab3A (Sasaki *et al.*, 1990). However, rab GDI will also release GDP-bound rab3A, but not GTPγS-bound rab3A from membranes by formation of a soluble heterodimer (Araki *et al.*, 1990). While rab GDI does not act on ras or rho, it will slow the dissociation of GDP from Sec4p and will release a broad range of rab proteins from membranes (Sasaki *et al.*, 1990; Regazzi *et al.*, 1992; Garrett *et al.*, 1993; Ullrich *et al.*, 1993). These activities suggest that rab GDI may play a general role in the recycling of rab proteins (Magee and Newman, 1992; Pfeffer, 1992). In the context of the model, GDI would release the GDP-bound form of the rab protein from the membrane of the target organelle. The soluble complex would remain in the cytoplasm until dissociation of the rab protein from GDI allows binding of GTP and reattachment onto a new vesicle. The prediction of this model is that loss of GDI would block recycling of the rab proteins and thus inhibit transport.

A homolog of rab GDI has recently been isolated from *Drosophila melanogaster* (dDGI; Zahner and Cheney, 1993) and an activity similar to that of rab GDI has been partially purified from the yeast *Saccharomyces cerevisiae* (Garrett *et al.*, 1993). In this paper we report the identification and characterization of Gdi1p, a yeast rab GDI homolog which interacts with Sec4p, and describe the phenotype of Gdi1p depletion. In strong support of the proposed cycling model, we find that depletion of Gdi1p leads to loss of the soluble pool of Sec4p and to blocks in membrane traffic at multiple stages along the secretory pathway.

Results

Isolation and sequence of GDI1

To isolate a cognate of rab GDI from *S.cerevisiae*, yeast genomic DNA was amplified by PCR using degenerate oligonucleotides corresponding to amino acid sequences strictly conserved between rab GDI and dGDI. Two sets of degenerate oligonucleotides were designed. The first pair represented the sequences MDEEYD and DWNVDL corresponding to amino acids 1−6 and 71−76 respectively, of both rab GDI and dGDI (Figure 1B). The second set represented the sequences GELPQG and YGGTYM corresponding to amino acids 232−237 and 245−250 respectively, of both GDI proteins. The first pair of oligonucleotides did not amplify a PCR product of the predicted size (288 bp). The reason for this will be discussed later. In contrast, the second pair generated a fragment of yeast genomic DNA of approximately the anticipated size (77 bp), with a predicted amino acid sequence identical to

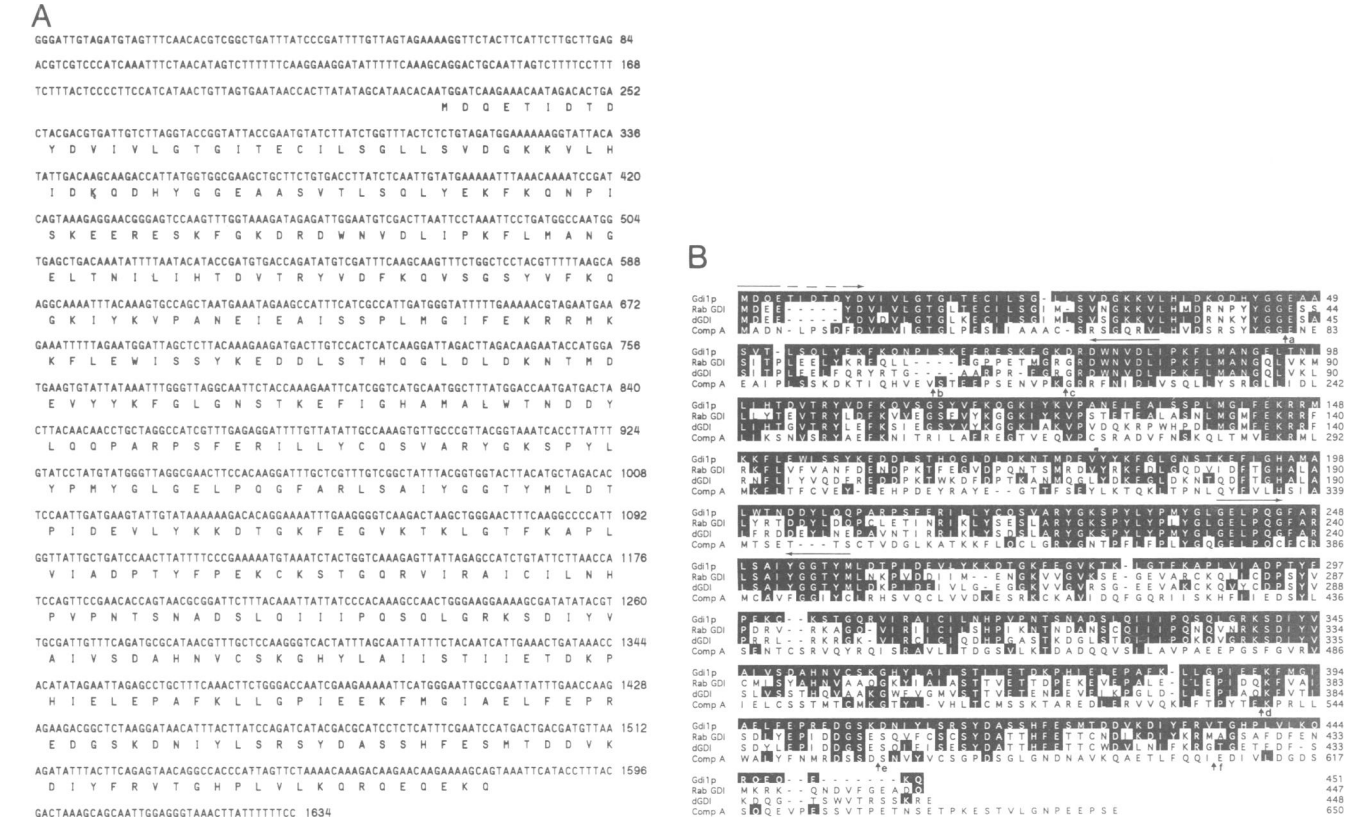

Fig. 1. *GDI1* from *S.cerevisiae* encodes a rab GDI protein. (**A**) Nucleotide and predicted protein sequence of *GDI1*. Nucleotide positions are numbered on the right. The single letter amino acid code is shown below the DNA sequence. (**B**) Comparison of Gdi1p with rab GDI, dGDI and component A of the rat geranylgeranyl transferase type II (Comp A). Sequences were aligned using the Clustal method from the MegAlign program, part of the Lasergene sequence analysis software available from DNASTAR INC. Certain regions of rat component A were deleted to allow easier alignment of the sequences. The deleted amino acid sequences are denoted by an arrow and a letter: a, 46−80; b, 108−188; c, 200−220; d, 531−539; e, 557−566; f, 596−608. Amino acid identity is denoted by a black background. Dashed lines indicate gaps. Lines with arrows indicate stretches of amino acids of rab GDI and dGDI, from which degenerate oligonucleotides were designed for PCR amplification of yeast genomic DNA. The dashed line shows a stretch of amino acids found in Gdi1p but not in rab GDI and dGDI, thus explaining the failure of the first set of degenerate oligonucleotides to PCR amplify a fragment of yeast genomic DNA of the predicted size (see Results).

the comparable region of both rab GDI and dGDI, but showing different codon usage (Matsui *et al.*, 1990; Zahner and Cheney, 1993). The DNA fragment was then used to screen a wild-type yeast genomic DNA library inserted in λ phage EMBL3A under high stringency conditions.

This procedure led to the identification of one intronless open reading frame encoding a predicted protein sequence of 451 amino acids (Figure 1A) with 50% overall identity to both rab GDI and dGDI (Figure1B; Matsui *et al.*, 1990; Zahner and Cheney, 1993). Due to this strong homology, we have named the yeast sequence, *GDI1*. From comparison of Gdi1p with rab GDI and dGDI, it is clear that the extreme N-terminal region of Gdi1p is not conserved with rab GDI and dGDI (Figure 1B). This explains why the first set of degenerate oligonucleotides did not amplify the anticipated PCR product. Component A of the mammalian geranyl-geranyl transferase type II enzyme has been shown to share restricted sequence identity with rab GDI (Andres *et al.*, 1993). This sequence conservation extends to Gdi1p and dGDI (Figure 1B).

Biochemical characterization of Gdi1p

To address whether *GDI1* encodes a protein that exhibits GDI activity towards Sec4p, we overexpressed the gene in *Escherichia coli* and purified the recombinant protein (Gdi1p) to ~85% purity by DEAE Sephacel and MonoQ ion exchange chromatography (see Materials and methods). We found that Gdi1p can inhibit the dissociation of [^{3}H]GDP from both Sec4p (Figure 2) and Ypt1p (not shown) in a dose-dependent manner. This ability to inhibit the dissociation of [^{3}H]GDP from multiple rab proteins *in vitro* has also been described for rab GDI (Sasaki *et al.*, 1990, 1991; Ueda *et al.*, 1991).

In addition to their ability to inhibit the dissociation of [^{3}H]GDP from rab proteins, both rab GDI and dGDI will cause the release of GDP-bound rab proteins from membranes (Araki *et al.*, 1990; Regazzi *et al.*, 1992; Garrett *et al.*, 1993; Soldati *et al.*, 1993; Ullrich *et al.*, 1993). As shown in Figure 3, we found that Gdi1p will cause the dissociation of Sec4p, but not Ras2p, from yeast membranes. Consistent with the membrane dissociation activity of rab GDI, Gdi1p will cause the release of Sec4p from yeast membranes preincubated in the presence of GDP, but not from membranes preincubated in the presence of GTPγS (Figure 3A).

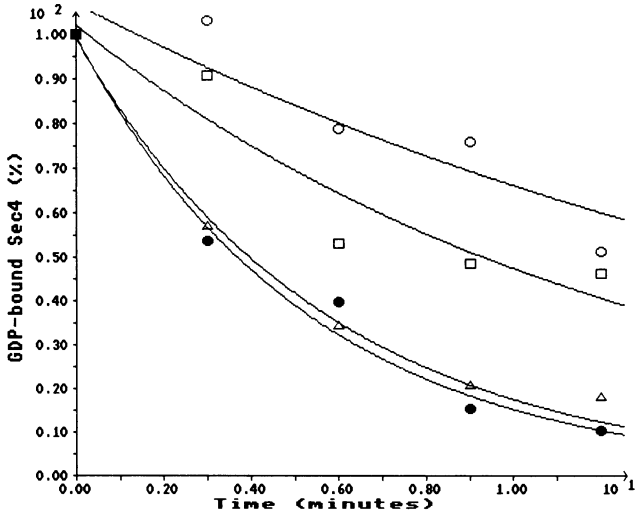

Fig. 2. The effect of Gdi1p on the dissociation of [^{3}H]GDP from Sec4p. Assays were performed as described in Garrett *et al.* (1993). Each assay contained 33 nM Sec4p. Sec4p alone, (filled circles), Sec4p nM + Gdi1p at 33 nM (open triangles), Sec4p + Gdi1p at 150 nM (open squares), Sec4p + Gdi1p at 750 nM (open circles).

GDI1 is essential for cell viability

To determine whether *GDI1* is required for cell viability, a disruption of one chromosomal copy of *GDI1* in a diploid yeast strain was constructed. This was achieved by replacing a portion of *GDI1* (codons 98–350 inclusive) with the *URA3* gene (see Materials and methods). Ura$^+$ transformants were sporulated and the tetrads dissected. For each tetrad only two spores were viable and these were always Ura$^-$. The non-viable spores did germinate but appeared to be arrested at the two or four cell stage. We conclude that *GDI1* is an essential gene.

Cells depleted of Gdi1p show multiple defects in protein transport

The demonstration that Gdi1p can interact with Sec4p suggests that *GDI1* may also be required for protein secretion in yeast. If *GDI1* is required for this function, then depletion of Gdi1p would be predicted to cause a block in protein transport. To test this proposal, a haploid yeast strain [*GAL–GDI* (NY1154)] was constructed in which the only functional copy of *GDI1* was under the control of the *GAL1* promoter (see Materials and methods). In this strain, expression of *GDI1* is induced in the presence of galactose but repressed in the presence of glucose.

The growth properties of *GAL–GDI* on YP agar plates containing different carbon sources were examined. As anticipated, *GAL–GDI* grew as well as wild-type yeast

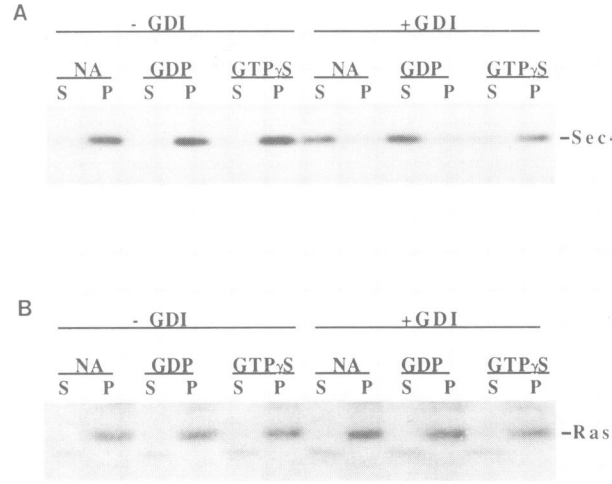

Fig. 3. The effect of Gdi1p on the membrane association of Sec4p and Ras2p. In each case 100 μg of membranes were preincubated in buffer with no addition (NA), with 1 mM GDP (GDP), or 1 mM GTPγS (GTPγS). The incubation was then continued in the presence (+ GDI) or absence (– GDI) of 3.75 μg of Gdi1p. Supernatant (S) and pellet (P) fractions were obtained, subjected to SDS–PAGE on 14% slab gels and transferred to nitrocellulose overnight. (**A**) The nitrocellulose filter was incubated with rabbit polyclonal anti-Sec4p antibody (1:1000), followed by radioiodinated protein A. (**B**) The nitrocellulose filter was incubated in the presence of mouse monoclonal anti-Ras2p antibody (1:3), followed by radioiodinated anti-mouse antibody (from sheep).

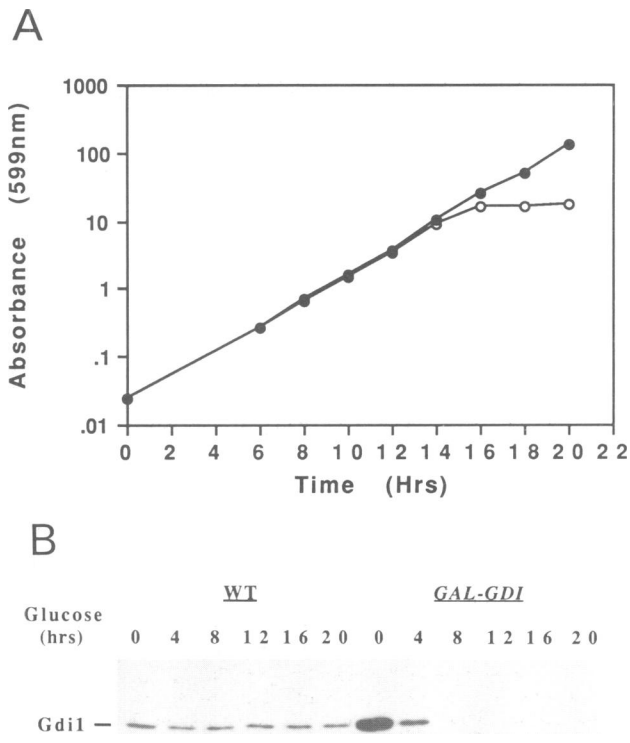

Fig. 4. Growth properties and Gdi1p expression of wild-type and *GAL−GDI* cells grown in YPD. (**A**) Growth properties of wild-type and *GAL−GDI* cells in YPD. Wild-type (closed circles) and *GAL−GDI* (open circles) cells were grown for 10−14 h in YPRG medium at 30°C. The cells were then switched to YPD medium and growth continued at 30°C. The cells were frequently diluted with YPD to allow continuous logarithmic growth. Samples were collected and the OD_{599} (absorbance 599 nm) measured at 2 h time points. The OD_{599} reflects the total cell density, in the original culture volume, having taken the dilution of the culture with YPD into consideration. (**B**) Immunological detection of Gdi1p in wild-type and *GAL−GDI* cells after growth in YPD. Wild-type and *GAL−GDI* cells were grown in YPRG transferred to YPD and growth continued as described for panel A. Samples of 10 OD_{599} units of cells were taken from each culture after 0, 4, 8, 12, 16 and 20 h of growth in YPD. The cells were spheroplasted, lysed, S1 fractions (450 *g* supernatant) prepared, subjected to SDS−PAGE on 14% gels and transferred to nitrocellulose overnight. The nitrocellulose filter was then incubated in the presence of affinity-purified rabbit polyclonal anti-Gdi1p antibody (for antibody production see Materials and methods) followed by radioiodinated protein A, using the procedure described previously for immunological detection of Sec4p (Goud *et al.*, 1988).

(NY1155) on YP agar plates supplemented with 2% raffinose and 0.5% galactose, but failed to grow on YP agar plates supplemented with 2% glucose, since *GDI1* is essential for the vegetative growth of yeast cells (data not shown). The growth properties of both strains were also examined in liquid medium, by initially growing them both in YP containing 2% raffinose and 0.5% galactose (YPRG) for 10−14 h, after which they were switched into YP containing 2% glucose (YPD). The rate of growth of both strains was then monitored by measuring their OD_{599} at 2 h time points over a 20 h period. After 12 h we found that while the wild-type yeast strain continued to grow at a logarithmic rate, growth of *GAL−GDI* started to slow down and after 16 h had completely stopped (Figure 4A). Western blot analysis of S1 fractions (450 *g* lysate supernatant) made from both strains, during growth in YPD, was carried out using a polyclonal antibody raised against a portion of Gdi1p fused to the C-terminus of the glutathione *S*-transferase (GST) protein and affinity-purified using Gdi1p expressed and purified from *E.coli* (see Materials and methods). This revealed that while expression of Gdi1p in wild-type cells remained constant between 0 and 20 h of growth in YPD, after 12 h the *GAL−GDI* cells contained no detectable Gdi1p (Figure 4B).

To study the effect of Gdi1p depletion on the secretory pathway, we examined the transport of two yeast glycoproteins, the secretory protein invertase and the vacuolar protein carboxypeptidase Y (CPY), in Gdi1p-depleted cells and in wild-type cells. Initially, both strains were grown in YPRG for 10−14 h, then transferred into YPD to allow depletion of Gdi1p from the *GAL−GDI* strain. At specific time points, samples were shifted for a further 1 h in YP containing 0.1% glucose to derepress the synthesis of invertase. Cells were then assayed for intracellular (internal) and secreted (external) invertase activity. As shown in Figure 5A, we found that after either 10 or 16 h growth in YPD, wild-type cells efficiently secrete most of the invertase they manufacture and do not accumulate an intracellular pool of invertase. In contrast, when *GAL−GDI* cells were depleted of Gdi1p they accumulated >40% of the invertase they had synthesized, indicating a partial block(s) in secretion.

Since rab GDI can interact with a broad range of rab proteins, it is possible that the accumulation of invertase seen in Gdi1p-depleted cells is due to multiple partial blocks in yeast secretion resulting from the loss of function of several different members of the Sec4/Ypt/rab family. It has previously been shown that glycosylation of invertase occurs at two distinct stages (Esmon *et al.*, 1981; Franzusoff and Schekman, 1989). The first stage is in the ER, since ER accumulating secretory mutants produce core glycosylated invertase with a mobility of 79−81 kDa. Further modifications occur in the Golgi which result in a diffuse form of invertase with a molecular weight of between 100 and 140 kDa. To examine the forms of invertase generated in cells depleted of Gdi1p, the *GAL−GDI* strain and a wild-type control strain were grown for 10−14 h in YPRG, shifted into YPD for 14 h and then switched to 0.1% glucose for 90 min to derepress invertase expression. The intracellular (internal) and secreted (external) forms of invertase were then analyzed by Western blot analysis, using an affinity-purified rabbit polyclonal anti-invertase antibody. In Figure 5B it can be seen that the invertase secreted by the *GAL−GDI* strain is not all fully glycosylated and that the underglycosylated form is similar to that secreted by the *ypt1-1* mutant (NY435). The invertase accumulating within the *GAL−GDI* cells appears to be in several forms. Some resemble the core-glycosylated invertase accumulated by the *sec18-1* mutant (NY431) while the remainder appears to be further glycosylated, although not fully. These findings suggest that invertase transport is partially blocked between the ER and the Golgi and also at a later stage(s).

The yeast vacuolar protease carboxypeptidase Y (CPY) is produced in yeast cells as a 59 kDa polypeptide which receives 8 kDa of N-linked oligosaccharide in the ER and 2 kDa of additional carbohydrate in the Golgi apparatus (Stevens *et al.*, 1982; Franzusoff and Schekman, 1989). This fully glycosylated 69 kDa proenzyme is then proteolytically activated in the vacuole by the *PEP4* gene product which cleaves an 8 kDa propeptide. Yeast secretory mutants that are blocked in transport between the ER and the Golgi

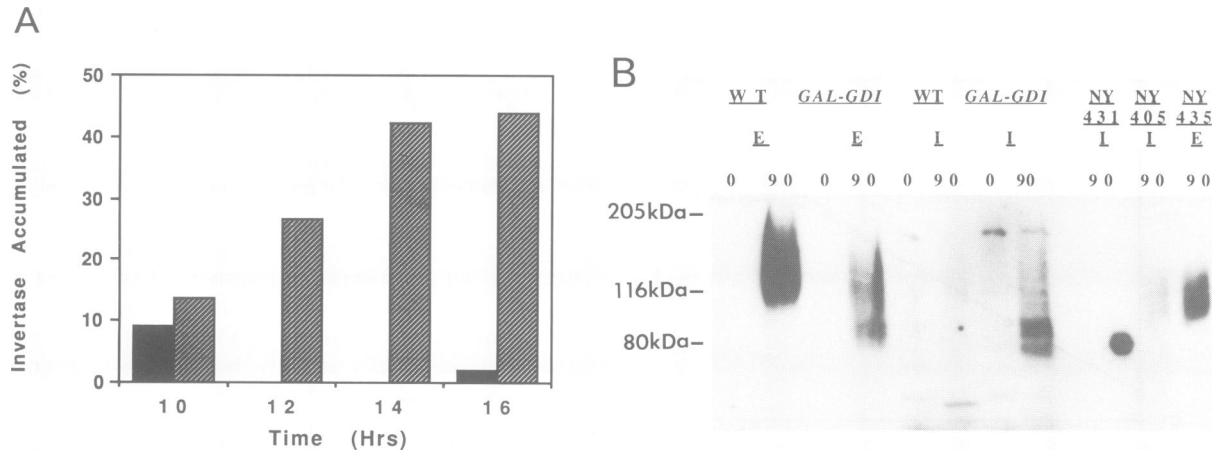

Fig. 5. The effect of Gdi1p depletion on the secretion of invertase by yeast cells. (**A**) Cells depleted of Gdi1p accumulate the secretory protein invertase. Wild-type (closed bar) and *GAL−GDI* (hatched bar) cells were grown for 10−14 h in YPRG at 30°C. Cells were then switched to YPD and growth continued at 30°C. Samples of 2 OD_{599} of *GAL−GDI* cells were collected after 10, 12, 14 and 16 h and of wild-type cells after 10 and 16 h, pelleted and resuspended in 2 ml of YP containing 0.1% glucose. Growth of half the sample was continued for 1 h at 30°C to derepress the synthesis of invertase, while the other half was resedimented and stored on ice in 10 mM NaN_3. Internal and external (secreted) invertase activity were then measured as described by Nair *et al.* (1990). The graph shows the percentage of invertase manufactured during 1 h of growth in 0.1% glucose that accumulated in the cells. This value was calculated by dividing the amount of internal invertase synthesized during 1 h of growth in 0.1% glucose, by the sum of the internal and external invertase synthesized during 1 h of growth in 0.1% glucose. (**B**) Cells depleted of Gdi1p show defects in glycosylation of invertase. Wild-type and *GAL−GDI* cells were grown for 10−14 h in YPRG at 30°C. The cells were then transferred to YPD and growth continued at 30°C. At this point, cultures of yeast strains NY405 (*sec4-8*), NY431 (*sec18-1*) and NY435 (*ypt1-1*) were started in YPD at 25°C. After 14 h, cells from all strains were pelleted, resuspended in YP + 0.1% glucose and growth continued for a further 90 min [time = 90 (min) on the figure], at 30°C for the wild-type and *GAL−GDI* strains and at 37°C for NY405, NY431 and NY435, to derepress the synthesis of invertase, after which 10 OD_{599} units of each strain were removed for analysis. 10 OD_{599} units of cells from all strains were also pelleted, resuspended in ice cold 10 mM NaN_3 and stored on ice [time = 0 (min) on the figure]. Each sample was spheroplasted and the spheroplasts (I = internal invertase) and the spheroplast supernatant (E = external invertase) subjected to SDS−PAGE on 10% slab gels and immunoblotted with affinity-purified anti-invertase antibody (1:2000) as described in Materials and methods. Molecular weight markers are shown on the left hand side of the figure.

accumulate a 67 kDa partially glycosylated form of the proenzyme (termed p1), while Golgi accumulating mutants and mutants defective in transport to the vacuole show a build-up of the 69 kDa fully glycosylated proenzyme (termed p2) (Stevens *et al.*, 1982; Franzusoff and Schekman, 1989). To determine the effect of Gdi1p depletion on CPY transport, *GAL−GDI* cells and a wild-type control strain were grown in YPRG for 10−14 h, shifted to YPD for 14 h and metabolically radiolabelled with [^{35}S]cysteine and [^{35}S]methionine for 4 min in minimal medium. The cells were then pelleted, resuspended in chase medium and samples removed at 0, 10 and 30 min. The samples were lysed and CPY was immunoprecipitated from the lysate with a rabbit polyclonal anti-CPY antibody. In wild-type cells the p1 and p2 forms of CPY can be seen at 0 min, while in the *GAL−GDI* cells only the p1 form is visible (Figure 6). By 10 min, most of the p2 form of CPY has been chased to the mature form in wild-type cells and by 30 min only the mature form is present. In the *GAL−GDI* strain both the p1 and p2 forms of CPY are retained at 10 and 30 min, with little accumulation of the mature form of CPY. These results are consistent with a slowing of the processing of CPY from the p1 to the p2 form and a further inhibition of the processing of p2 to the mature 61 kDa form of CPY. These findings suggest that depletion of Gdi1p leads to multiple defects in the transport and processing of CPY.

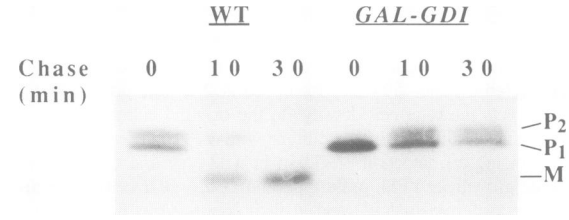

Fig. 6. Cells depleted of Gdi1p are defective for the processing of CPY. Wild-type and *GAL−GDI* cells were grown for 10−14 h in YPRG at 30°C. The cells were then transferred to YPD and growth was continued at 30°C. After 14 h the cells were metabolically radiolabelled for 4 min with [^{35}S]methionine and [^{35}S]cysteine, pelleted, resuspended in chase media and samples removed after 0, 10 and 30 min. The samples were lysed and the forms of CPY immunoprecipitated from the lysates with a rabbit polyclonal anti-CPY antibody and analyzed by SDS−PAGE on 10% slab gels (see Materials and methods).

Gdi1p-depleted cells accumulate ER, Golgi and secretory vesicles

To investigate the consequences of Gdi1p depletion on cell morphology, the *GAL−GDI* strain and a wild-type control strain were grown for 10−14 h in YPRG and then switched to YPD for 14 or 16 h and examined by thin section electron microscopy. To check the morphological effects of growth in YPRG, thin section electron microscopy was also carried out on *GAL−GDI* cells grown in YPRG. These cells exhibited no obvious build-up of any membrane structure (Figure 7A) and resembled wild-type cells grown under the same conditions (data not shown). However, when the same strain was depleted of Gdi1p by growth for 14 (Figure 7B) or 16 h (Figure 7C and D) in YPD, there was exaggeration of the ER, along with accumulation of cup-shaped bodies, previously reported to be Golgi-related structures (Novick *et al.*, 1981), and a build-up of vesicles. The vesicles appear to be predominantly 100 nm in diameter, although some 40−60 nm vesicles are occasionally seen. Build-up of

extensive ER and accumulation of 40–60 nm vesicles is observed in the numerous secretory mutants that block transport between the ER and the Golgi, while mutants blocked in transport between the Golgi and the plasma membrane accumulate 100 nm vesicles (Novick *et al.*, 1980; Kaiser and Schekman, 1990). The fact that cells depleted

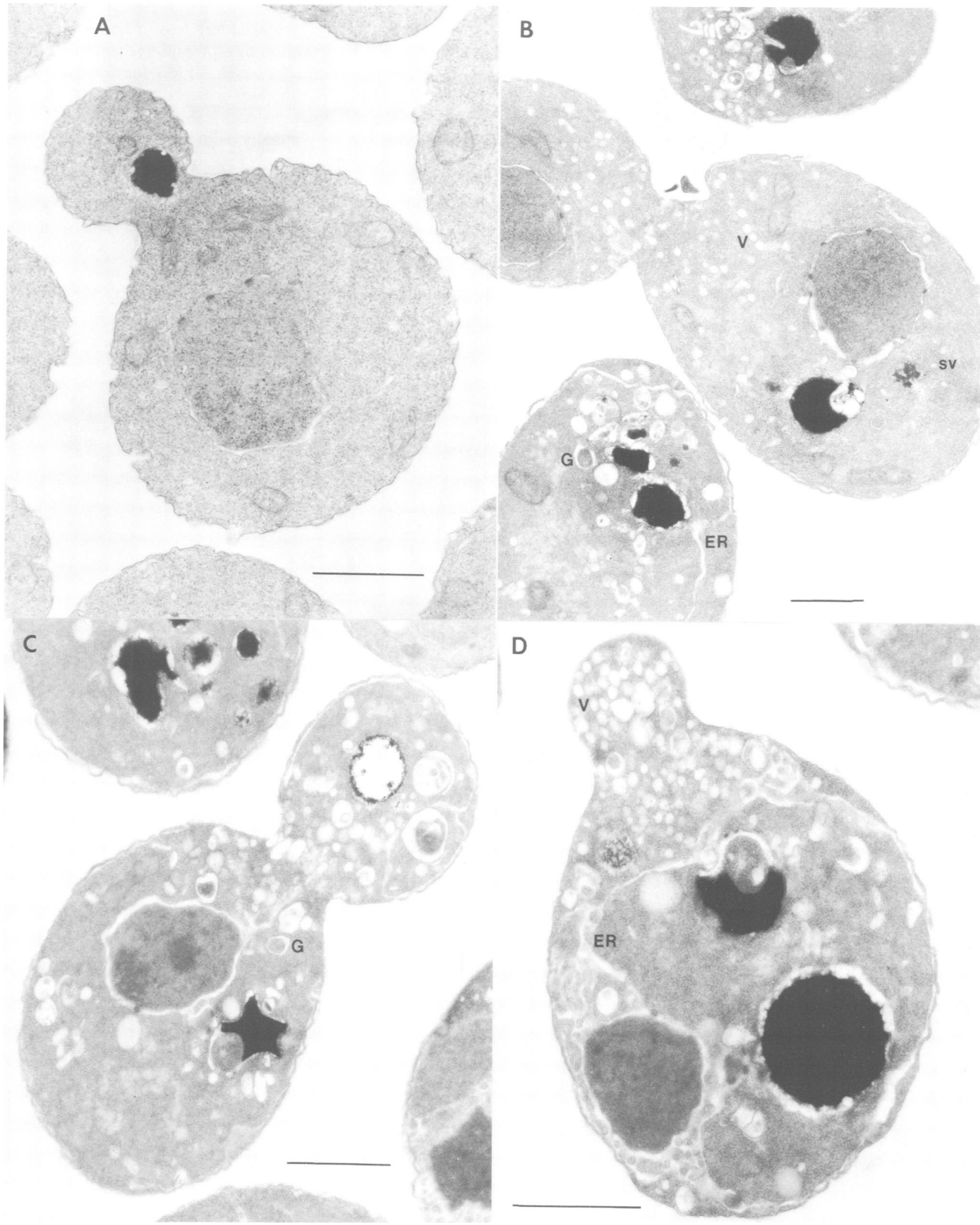

Fig. 7. Thin section electron micrographs of Gdi1p-depleted cells. (**A**) *GAL–GDI* cells grown in YPRG; (**B**) *GAL–GDI* cells 14 h after transfer to YPD; (**C**) and (**D**) *GAL–GDI* cells 16 h after transfer to YPD. The labelled structures are: ER, endoplasmic reticulum; G, Golgi-related structure; V, 100 nm vesicles; sv, 40–60 nm vesicles. The bar equals 1 μm.

of Gdi1p show accumulation of all these structures supports the suggestion that these cells display partial blocks in protein transport at multiple stages of the secretory pathway.

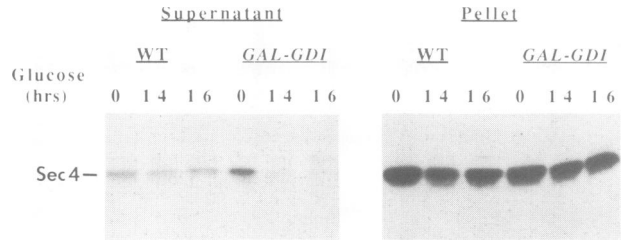

Fig. 8. Gdi1p depletion causes a loss of Sec4p from the soluble pool. Wild-type and *GAL–GDI* cells were grown for 10–14 h in YPRG at 30°C. The cells were then transferred to YPD and growth continued at 30°C. Cells were removed after 0, 14 and 16 h of growth in YPD, 100 000 *g* supernatants and pellets prepared and assayed for their Sec4p content by immunoblotting using a rabbit polyclonal anti-sec4p antibody, followed by radioiodinated protein A, as described in Materials and methods.

Depletion of Gdi1p causes a loss in the soluble pool of Sec4p

We have shown *in vitro* that Gdi1p will cause the release of Sec4p from yeast membranes. If Gdi1p serves to recycle Sec4p from yeast membranes back into the cytosol *in vivo*, depletion of Gdi1p should lead to a loss of Sec4p from the soluble pool. To examine this possibility, wild-type and *GAL–GDI* cells were grown in YPRG for 10–14 h and then shifted to YPD. Samples were taken at 0, 14 and 16 h, 100 000 *g* supernatants and pellets were prepared and analyzed for Sec4p by immunoblotting. Although the soluble pool of Sec4p represents ~15% of the total in wild-type cells, it can clearly be seen that after either 14 or 16 h growth in YPD the *GAL–GDI* cells have lost their soluble pool of Sec4p (Figure 8). These findings are consistent with the proposal that Gdi1p functions to recycle Sec4p from membranes back into the soluble pool in yeast.

GDI1 is allelic to sec19-1

The accumulation of ER, Golgi and secretory vesicles in Gdi1p-depleted cells is strikingly reminiscent of the

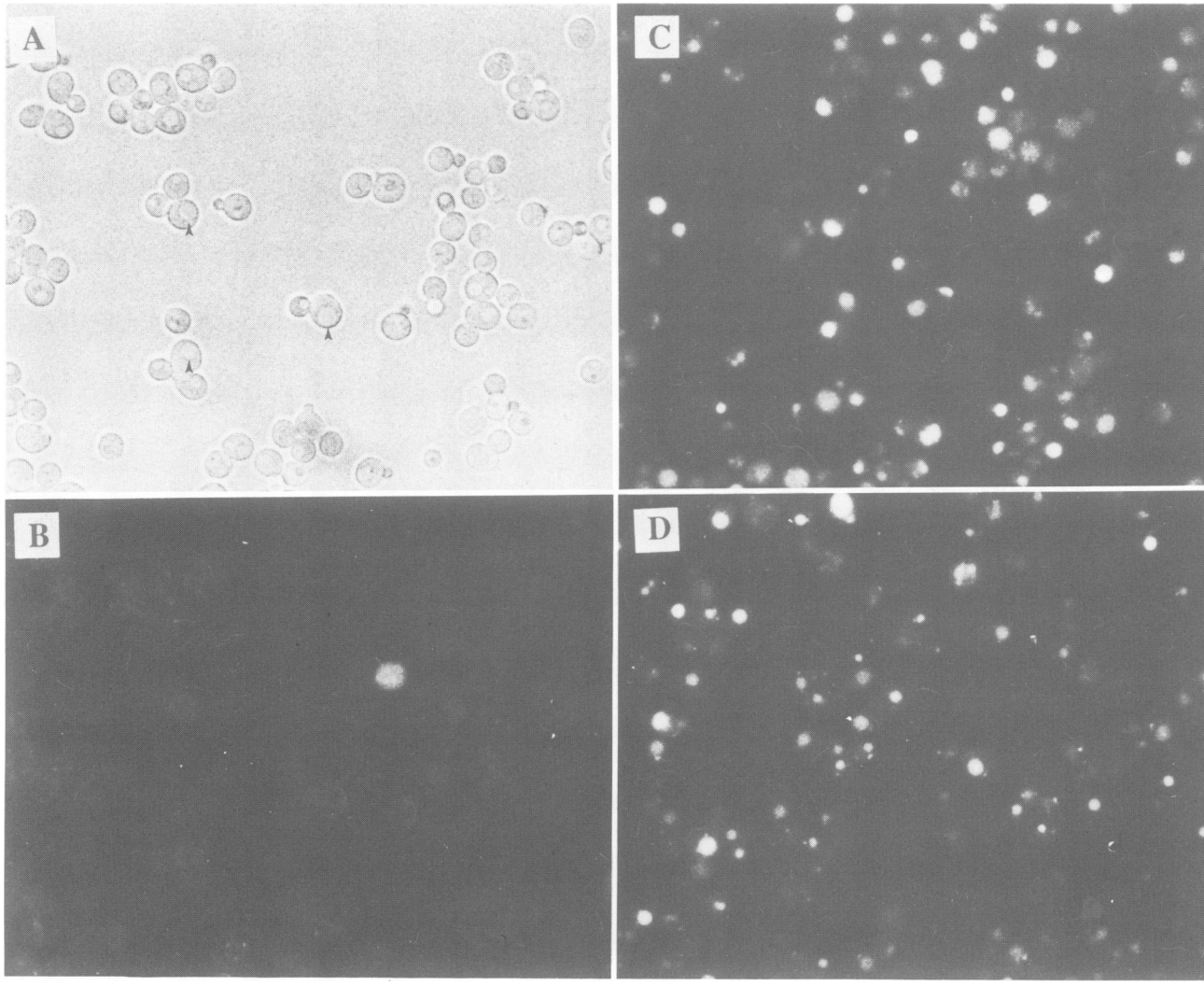

Fig. 9. Fluid phase uptake of Lucifer yellow into the *sec19-1* vacuoles. Wild-type (NY13) and *sec19-1* (NY420) cells were grown in YPD at 25°C, then incubated in the presence of 8 mg/ml Lucifer yellow for 1 h at either 25°C or 37°C. (**A**, **B**) *sec19-1* cells at 37°C. (**C**) *sec19-1* cells at 25°C. (**D**) Wild-type cells at 37°C. Cells were photographed using the fluorescein channel (B–D) or by Nomarski optics (panel A). Panels A and B show the same field of cells. The arrow heads in panel A point to vacuoles visible by Nomarski optics in *sec19-1* cells. In panel B, one brightly stained dead cell is visible.

phenotype of *sec19-1* (Novick *et al.*, 1980). Furthermore, this temperature-sensitive secretory mutant, like Gdi1p-depleted cells, accumulates transport intermediates of invertase and CPY (Esmon *et al.*, 1981; Stevens *et al.*, 1982). We therefore performed complementation and linkage analyses. A cross of a *GAL−GDI* strain (NY1154) with a *sec19-1* strain (NY420; *MAT***a**, *ura3-52, sec19-1*) resulted in a diploid strain that was inviable on glucose-containing medium at 37°C, a condition which is restrictive for both mutations. This indicates that these two mutations fail to complement. Dissection of tetrads derived from this cross yielded 29 parental ditypes, 0 tetratypes and 0 non-parental ditypes, indicating tight linkage between Δ*gdi1* and *sec19-1*. Furthermore, expression of Gdi1p from the *GAL1* promoter suppressed, in a galactose-dependent manner, the temperature-sensitive growth defects of *sec19-1* strains. Together, these data establish that *GDI1* is allelic to *sec19-1*.

Fluid phase uptake of Lucifer yellow is blocked in sec19-1

To assess the effect of loss of Gdi1p function on the endocytic pathway, we examined the uptake of the fluorescent fluid phase marker Lucifer yellow into the vacuoles of *sec19-1* and wild-type cells. At the permissive temperature (25°C), the vacuoles of *sec19-1* stained intensely after a 1 h incubation with 8 mg/ml Lucifer yellow (Figure 9C), as did the vacuoles of the wild-type strain (data not shown). At the restrictive temperature (37°C), the vacuoles of wild-type cells were clearly labelled (Figure 9D). However, the *sec19-1* cells showed only weak staining of the cell wall and small internal structures that did not co-localize with the vacuoles visible by Nomarski optics (Figure 9A and B). Thus, this temperature-sensitive allele of *GDI1* is defective for fluid phase uptake.

Discussion

We have identified Gdi1p, a rab GDI homolog from yeast. By analogy with bovine rab GDI, we found that Gdi1p will cause the inhibition of dissociation of GDP from Sec4p and will also cause the release of the GDP-bound form of Sec4p from yeast membranes. These two activities could, in principle, be used in a number of ways. In the context of a signal transduction pathway, a protein which blocks dissociation of GDP would, in turn, block binding of GTP. Since binding of GTP is essential for 'activation' of a signal transducer, a GDI could act as an inhibitor of signal transduction. Our results demonstrate that loss of Gdi1p function leads to disappearance of the soluble pool of Sec4p and to the inhibition of protein transport at multiple stages of the secretory pathway. These findings are not consistent with the signal transduction model but instead support a role for Gdi1p in the recycling of Sec4p and perhaps other proteins of the Sec4/Ypt/rab family from the membranes of their target organelles through the cytoplasm onto new carrier vesicles.

In this paper we report that *sec19-1* is allelic to *GDI1*. The *sec19-1* mutant completely blocks the secretion of invertase and acid phosphatase and leads to the accumulation of CPY in the p1 form, immediately following a shift to the restrictive temperature (Novick *et al.*, 1980; Stevens *et al.*, 1982). In contrast, depletion of Gdi1p leads to multiple partial transport blocks along the secretory pathway and vacuolar biosynthetic pathway. The partial nature of the blocks may reflect the method of depletion by dilution during growth in glucose. However, the number of blocks suggests that Gdi1p may have more than one site of action in the cell. The *sec19-1* mutant is also defective in uptake of the fluid phase marker Lucifer yellow into the vacuole at the restrictive temperature, indicating that loss of Gdi1p function also has an effect (direct or indirect) on the endocytic pathway. Furthermore, this temperature-sensitive mutant, like the Gdi1p-depleted cells, accumulates several types of membrane-bound structures, reflecting multiple transport blocks (Novick *et al.*, 1980). The multiplicity of transport blocks and the variety of structures seen to accumulate upon loss of Gdi1p function is consistent with Gdi1p regulating different members of the Sec4/Ypt/rab family, which function in both the endocytic (Ypt7p) and exocytic (Sec4p, Ypt1p) pathway in yeast (Segev *et al.*, 1988; Bacon *et al.*, 1989; Baker *et al.*, 1990; Salminen and Novick, 1987; Wichmann *et al.*, 1992; Schimmoller and Reizman, 1993).

Prenylation of mammalian rab proteins is mediated by geranylgeranyl transferase type II (Seabra *et al.*, 1992a,b). This enzyme consists of two components: component B contains two polypeptides, α and β, that share sequence similarity to the subunits of farnesyl transferase and geranylgeranyl transferase type I (Armstrong *et al.*, 1993), and component A, which shares limited sequence similarity with rab GDI proteins (Figure 1B; Andres *et al.*, 1993), recognizes the unmodified rab protein and presents it to component B (Andres *et al.*, 1993). In yeast, the α and β subunits of component B are encoded by *MAD2* and *BET2* respectively (Li and Murray, 1991; Rossi *et al.*, 1991; Jiang *et al.*, 1993; Li *et al.*, 1993). Reconstitution of transferase activity requires, in addition to these gene products, an activity analogous to component A (Jiang *et al.*, 1993). Purified Gdi1p will not function as component A in this assay (Y.Jiang, M.D.Garrett, P.J.Novick and S.Ferro-Novick, unpublished results). Therefore, despite the sequence similarity, *GDI1* does not encode the yeast component A.

After Sec4p is released from the target membrane into the cytosol by Gdi1p, the question arises, how does Sec4p reattach to a new round of vesicles, if it is complexed to Gdi1p? One possibility is that a reversible modification of either Sec4p or Gdi1p causes dissociation of the complex, allowing attachment of Sec4p to a new vesicle. In that light it is interesting to note that in *Drosophila*, dGDI is subject to a basic shift in isoelectric point that is dependent on the action of the *quartet* gene product (Zahner and Cheney, 1993). In addition, it has recently been shown that rab GDI is phosphorylated when complexed to rab proteins in the cytosol (Steele-Mortimer *et al.*, 1993). Such a modification could regulate the interaction of rab proteins with GDI. A second possibility is that the Sec4p−Gdi1p complex interacts with a vesicle associated protein which can compete with Gdi1p for Sec4p. Sec4 p would then dissociate from Gdi1p and become attached to the vesicle membrane. An extension of this model is that the competing protein (or an associated protein) is an exchange factor for Sec4p, thus coupling the reattachment of Sec4p to vesicles with guanine nucleotide exchange. With regard to Sec4p and Gdi1p, a possible candidate for this protein is Dss4p, an exchange factor for Sec4p, which is predominantly found in the insoluble fraction of yeast lysates. (Moya *et al.*, 1993). With the identification and isolation of Gdi1p, these questions can now be examined both genetically and biochemically in yeast.

Materials and methods

General materials and techniques

Molecular biology techniques were used as described by Sambrook *et al.* (1989). The *E.coli* strain TG1 was used for all cloning and subcloning experiments (Gibson, 1984). All DNA for sequence analysis was subcloned into the pUC118 and pUC119 vectors (Viera and Messing, 1987). DNA sequencing was by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp.) in the presence of [α-^{35}S]dATP (>1000 Ci/mmol; Amersham International plc) (Williams *et al.*, 1986), using the M13 universal sequencing primer or specific internal primers. Host bacteria for the λ phage was *E.coli* strain LE392 (Borck *et al.*, 1976; Murray *et al.*, 1977). λ Phage DNA was isolated using the Lambda Starter kit (Qiagen Inc.).

Yeast cells were grown in rich medium (YP) containing 1% Bacto yeast extract, 2% Bacto-peptone (Difco Laboratories) and either 2% glucose (YPD) or 2% raffinose and 0.5% galactose (YPRG) or in minimal medium (SD) containing 0.7% yeast nitrogen base without amino acids (Difco), 2% glucose and supplemented for auxotrophic requirements as described by Sherman *et al.* (1974) where needed. Yeast transformations were performed by alkali cation treatment as described previously (Ito *et al.*, 1983).

For SDS–PAGE, samples were boiled for 5 min at 100°C in sample buffer containing 2% SDS and run on 10 or 14% slab gels according to Laemmli (1970). Protein estimations were performed according to Bradford (1976) using bovine immunoglobulin (Sigma) as the standard.

Cloning of GDI1

The rab GDI and dGDI amino acid sequences were compared to identify regions of strict amino acid conservation from which degenerate oligonucleotides could be designed to allow the PCR amplification of the homologous sequence from *S.cerevisiae*. Two sets of degenerate oligonucleotides were manufactured on a DNA Synthesizer (Model 394, Applied Biosystems Inc.). The first set, 5′-GACTGGATCCATGGA(C/T)GA-(A/G)GA(A/G)TA(C/T)GA-3′ (upstream) and 5′-GACTTCTAGAAA-(G/A)(G/A)TCIAC(G/A)TTCCA(G/A)TC-3′ (downstream) represented the sequences MDEEYD (amino acids 1–6) and DWNVDL (amino acids 71–76) respectively of both rab GDI and dGDI. The second set, 5′-GACTGGATCCGGIGA(A/G)(C/T)TICCICA(A/G)GG-3′ (upstream) and 5′-GACTTCTAGACAT(G/A)TAIGTICCICC(G/A)TA-3′ (downstream) represented the sequences GELPQG (amino acids 232–237) and YGGTYM (amino acids 245–250) respectively of both GDI protein sequences. For PCR amplification, each reaction (100 μl) contained 50 mM KCl, 10 mM Tris (pH 8.3, 20°C), 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.5 μg of each oligonucleotide and 1 μg of genomic DNA prepared from the yeast strain ANY 122 (*MATa, ura3-52, bet2-1*) as the template (Philippsen *et al.*, 1991). Each reaction was subjected to 96°C for 5 min, followed by 45°C for 10 min, during which time 5 U of Taq DNA polymerase from *Thermus aquaticus* BM (Boehringer Mannheim) were added to the reaction and then 2 min at 60°C. The next 10 cycles were 1 min at 92°C, 5 min at the annealing temperature of 45°C, followed by 2 min at the extension temperature of 72.1°C. The final 30 cycles of amplification were 1 min at 92°C, 1 min at 55°C for annealing and 2 min at 72.1°C for extension.

Using this procedure, the second set of oligonucleotides amplified a strong band of approximately the predicted size (77 bp) which was subcloned into the pUC118 and pUC119 vectors and six subclones were sequenced. All the subclones contained the same DNA sequence after the 3′ end of the upstream degenerate oligonucleotide, yielding the predicted amino acid sequence of FARLSAI. This amino acid sequence was identical to the same region of rab GDI and dGDI between the two degenerate PCR primers, but it showed different codon usage. A 378 bp *Pvu*II fragment containing this yeast sequence, from one of these subclones, was labelled with [α-^{32}P]dCTP (6000 Ci/mmol, Amersham International plc) using the Multiprime DNA labelling system (Amersham International plc) to generate a probe for screening a wild-type yeast genomic DNA library inserted in phage λ EMBL3A (Snyder *et al.*, 1986).

Approximately 9×10^3 plaques were transferred to duplicate filters. Prehybridization was for at least 1 h at 65°C in 6×SSC and 0.05×Blotto. 2.25×10^7 d.p.m. of probe was diluted with 400 μl of TE and 100 μl of calf thymus DNA (5 mg/ml) heated to 100°C for 5 min and added to the prehybridization solution to give a final concentration of 5×10^5 d.p.m./ml. The filters were hybridized overnight at 65°C, then washed at room temperature in 2×SSC and 0.5% SDS for 30 min, 1×SSC and 0.5% SDS for 30 min, 0.5×SSC and 0.5% SDS for 30 min and finally 0.5×SSC for 10 min, air-dried and autoradiographed. Thirteen positive spots were identified. To isolate single plaques from eight of these spots, each was rescreened by hybridization on duplicate filters at ~200 plaques per filter. Six of the original eight spots were positive after this rescreening. Further analysis was carried out by restriction digest of DNA from the λ phage single plaques (clones) with a selection of enzymes and Southern blotting using the ^{32}P-labelled 378 bp *Pvu*II fragment as a probe. Appropriate DNA fragments were then subcloned for SS DNA sequencing.

Production of GDI1 protein expressed and purified from E.coli

The *GDI1* gene was introduced into the pET11d expression vector (Studier *et al.*, 1990). Using PCR, a *Nco*I site was introduced at the initiating methionine and a *Bam*HI site introduced at codon 138 of *GDI1*. This PCR product was cut with *Nco*I and *Bam*HI and ligated into pET11d that had been digested with the same enzymes. The new construct was digested with *Kpn*I and *Bam*HI which removed codons 17–138 of *GDI1* from the plasmid but leaving codons 1–16. Into this was ligated a 2 kb DNA fragment isolated from clone 1.2 (one of the eight original clones) digested with *Kpn*I and *Bcl*I containing codons 17 to 451, starting from the *Kpn*I site to generate pNB620. This procedure was carried out because *GDI1* contains an internal *Nco*I site which meant that the whole gene could not be inserted into pET11d by one subcloning step.

The *E.coli* strain BL21(DE3) was transformed with pNB620 to give strain NRB620, the cells grown, induced with isopropyl-β-D-thiogalactopyranoside (IPTG), cells harvested and a 13 800 *g* supernatant prepared as described previously except the lysis buffer contained 25 mM NaCl (Novick *et al.*, 1992). The bacterial supernatant was loaded onto a 10 ml DEAE Sephacel column (Pharmacia LKB Biotechnology). After washing with buffer A (25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM DTT) containing 25 mM NaCl, bound protein was eluted with a 75 ml gradient of 25–200 mM NaCl in buffer A and 2.2 ml fractions collected. Gdi1p eluted between fractions 22 and 26 (~80–110 mM NaCl). These fractions were pooled and dialyzed overnight in 4 l of buffer A containing 25 mM NaCl. The dialyzed protein was loaded onto a MonoQ HR5/5 column (Pharmacia) which was washed with 5 ml of buffer A containing 25 mM NaCl. Elution was with a 20 ml gradient of 25–200 mM NaCl in buffer A. Gdi1p eluted in fractions 20 and 21 (~117–127 mM NaCl). These fractions were pooled and stored at 4°C until required.

Biochemical assays for Gdi1p

The Sec4p membrane dissociation assay and the GDP-off rate assay were performed as described previously (Garrett *et al.*, 1993), except that duplicate samples were transferred to nitrocellulose filters for the Sec4p membrane dissociation assay, one filter being probed for Sec4p as described previously (Garrett *et al.*, 1993) and one being probed with cell supernatant of a mouse monoclonal anti-ras2 antibody (1:3), followed by an anti-mouse antibody (from sheep, 19 μCi/μg, Amersham International plc).

Disruption of GDI1

To construct the plasmid for disruption of *GDI1*, two DNA fragments of *GDI1* were subcloned into a yeast integrating vector containing the *URA3* gene. DNA from clone 1.2 was cut with *Fsp*I and *Eco*RI to give a 1.36 kb fragment containing codons 351–451 of *GDI1* and the 3′ downstream sequence. This fragment was ligated into the yeast integrating vector pRS306 (Sikorski and Hieter, 1989), which had been cut with *Kpn*I, incubated with T4 DNA polymerase (New England Biolabs, Inc.) for 15 min at 12°C in the presence of 0.1 mM dNTPs to remove the 3′ protruding ends and then cut with *Eco*RI, to generate the plasmid, pNB621. This ligation recreated the *Eco*RI site but removed the *Kpn*I site. DNA from clone 1.2 was digested with *Kpn*I to give a 6 kb DNA fragment containing codons 17–451 of *GDI1* and 4 kb of 3′ downstream sequence and ligated into pUC118 to generate pNB622. This construct was then cut with *Bam*HI and *Ssp*I to give a 249 bp fragment, where *Bam*HI had cut next to *Kpn*I inside the polylinker and *Ssp*I had cut inside *GDI1* between codons 97 and 98. This fragment was ligated into pNB621 that had been cut with *Xba*I, incubated in the presence of 0.1 mM dNTPs and Klenow (New England Biolabs, Inc.) for 15 min at 20°C to fill in the 3′ recessed ends and then cut with *Bam*HI to give the construct pNB623. This recreates the *Bam*HI site but removes the *Xba*I site. For transformation into yeast, the plasmid was linearized by cutting with *Eco*RI and *Kpn*I. This digest removed the remaining polylinker between the two *GDI1* fragments and left one end of each available for recombination into the *GDI1* locus. The linearized DNA was transformed into NY1060 (*MATa/α, leu2-3,112/leu2-3,112, ura3-52/ura3-52, GAL*$^+$), whereupon it disrupted one chromosomal copy of *GDI1* by integration of the linearized DNA at the *GDI1* locus, causing replacement of codons 98–350 (inclusive) of *GDI1* with the *URA3* gene. Transformants were selected on SD plates supplemented with leucine and one of the transformants designated NY1151 (*MATa/α, leu2-3,112/leu2-3,112ura3-52/ura3-52, gdi1Δ::URA3/GDI1, GAL*$^+$).

Construction of GAL–GDI

Using PCR, a *Bam*HI site was generated 10 bp upstream of the initiating ATG of *GDI1* and a *Hin*dIII site generated 87 bp downstream of the

terminating TAA of *GDI1*. The PCR product was cut with *Bam*HI and *Hin*dIII and subcloned into pNB527 (a yeast integrating vector containing the *LEU2* gene as an auxotropic marker and the *GAL1* promoter for inducible expression of a gene inserted into the plasmid, P.J.Brennwald, personal communication) which had been cut with the same enzymes to generate pNB624. This plasmid was sequenced to verify that PCR had not introduced any mutations into the *GDI1* coding sequence. The construct was digested with *Eco*RV, which cut within the *LEU2* gene and transformed into NY1151. Transformants were selected on SD plates, grown on YPD, and sporulated. Dissection of tetrads yielded strain NY1154 (*MATα, ura3-52, leu2-3,112::LEU2, GAL1-GDI, gdi1Δ::URA3, GAL*$^+$).

Production of anti-Gdi1p antiserum

Anti-Gdi1p antibody was produced against a Gdi1p-GST fusion protein that contains the C-terminal 261 amino acids of *GDI1* (amino acids 191-451). The gene fusion was constructed by subcloning a 1.88 kb DNA fragment containing the C-terminal 261 amino acids of *GDI1* and 3′ untranslated sequence into the *Eco*RI site of the GST fusion vector pGEX1 (Smith and Johnson, 1988). The fusion protein was then isolated from the insoluble fraction of strain NRB626, a TGI transformant containing the pNB626 plasmid. NRB626 was grown overnight at 37°C in 100 ml of LB-AMP (LB containing 0.1 mg/ml ampicillin). The overnight culture was diluted 10-fold with 900 ml of LB-AMP and growth continued for 1 h. The fusion protein was then induced by the addition of 0.2 mM IPTG and growth continued for 3 h. The cells were harvested by centrifugation, resuspended in 7 ml of PBS (phosphate-buffered saline) and sonicated for 3×2 min on ice, with cooling on ice for 1 min between each sonication. The sonicate was centrifuged at 13 800 *g* for 15 min and the pellet resuspended in PBS containing 1% Triton X-100. The resuspended pellet was recentrifuged at 13 800 *g* and this second pellet resuspended in 7 ml of sample buffer. The sample was then run on two 10% preparative gels. The gels were stained with 0.05% Coomassie blue (R-250) and destained with water to visualize the correct band. The fusion protein band was excised from the gel, chopped into small pieces and electroeluted onto dialysis membrane at 50 V overnight (Hunkapillar *et al.*, 1983). This procedure yielded 1.5 mg of protein in 5 ml of dialysis buffer which was stored at 4°C. The immunization protocol for two rabbits was carried out by Cocalico Biologicals Inc. Each rabbit was initially injected at subcutaneous (axillary and inguinal) and intramuscular (hind leg) sites with a total of 100 μg of protein emulsified in Freund's complete adjuvant. Each subsequent boost (days 14, 21, 42 and 68) was subcutaneously in the back, and intramuscularly in the hind leg with 50 μg of protein in Freund's incomplete adjuvant.

For affinity purification of anti-Gdi1p antibodies, 2 mg of Gdi1p were prepared from *E.coli* strain NRB620 as described earlier and dialyzed overnight in 0.1 M sodium borate (pH 8.5) containing 0.01% SDS. The protein was then cross-linked to 2 ml of Reacti-gel (1,1′ carbonyldiimidazole-activated agarose; Pierce Chemicals) according to the manufacturer's instructions. Gdi1p antibody was affinity-purified from 5 ml of Gdi1p antiserum as described by Potenza *et al.* (1992), except that eluted fractions were neutralized with 300 μl of 1 M K_2HPO_4 and no NaCl, and BSA (to a final concentration of 0.5 mg/ml) was added to the pooled eluted fractions before dialysis to stabilize the antibody. The antibody yield was estimated as 0.227 mg in 3.5 ml.

Invertase secretion

Wild-type [NY1155 (*MATα, ura3-52, leu2-3,112, GAL*$^+$)] and *GAL-GDI* (NY1154) cells were grown for 10-14 h in YPRG at 30°C, transferred to YPD and growth continued at 30°C. 2.0 OD_{599} units of *GAL-GDI* cells were removed after 10, 12, 14 and 16 h of growth in YPD and 2.0 OD_{599} units of wild-type cells removed after 10 and 16 growth in YPD. The cells were sedimented and resuspended in 2 ml of YP + 0.1% glucose. Half of each sample was immediately sedimented, resuspended in 1 ml of ice-cold 10 mM NaN_3 and stored on ice. The other 1 ml of cells was incubated at 30°C for 1 h. Measurement of internal and external invertase activity was then performed on all samples as described by Nair *et al.* (1990).

To analyze the internal and external forms of invertase, wild-type and *GAL-GDI* cells were grown for 10-14 h in YPRG at 30°C, transferred to YPD and growth continued at 30°C. At this point yeast strains NY405 (*MAT***a**, *ura3-52, sec4-8*), NY431 (*MAT***a**, *ura3-52, sec18-1*) and NY435 (*MAT***a**, *ura3-52, ypt1-1*) were grown in YPD at 25°C. After 14 h, 10 OD_{599} units of cells from each culture were removed, pelleted, resuspended in 1 ml of 10 mM NaN_3 and stored on ice. Fifty OD_{599} units of cells from each culture were then pelleted, resuspended in 50 ml of YP + 0.1% glucose and growth continued for 90 min at 30°C for the wild-type and *GAL-GDI* cells and at 37°C for the NY405, NY431 and NY435 yeast strains to derepress the synthesis of invertase. Ten OD_{599} units of cells from each culture were sedimented and resuspended in 1 ml of ice-cold 10 mM NaN_3. Spheroplasts were prepared by addition of 1 ml of spheroplast mix [2.8 M sorbitol, 100 mM KPi (pH 7.5), 10 mM NaN_3, 50 mM β-mercaptoethanol] and 0.3 mg/ml Zymolyase-100T (ICN Biomedicals, Inc.) to the suspension and incubation for 45 min at 37°C. The spheroplasts were sedimented and resuspended in 1 ml of 0.5×spheroplast mix. Sample buffer was then added to the spheroplast supernatant (external invertase) and the resuspended spheroplasts (internal invertase) and samples subjected to SDS-PAGE on 10% slab gels. After transfer onto nitrocellulose (BA 83, 0.22 mM; Schleicher & Scheull, Inc.) overnight at 4°C, invertase was probed with an affinity-purified rabbit polyclonal anti-invertase antibody (1:2000; Segev *et al.*, 1988) and radioiodinated protein A (30 mCi/mg; Amersham International plc) as described previously for immunological detection of Sec4p (Goud *et al.*, 1988).

In vivo labelling and immunoprecipitation

Cells were grown in YPRG for 10-14 h at 30°C and then transferred to YPD for a further 14 h growth at 30°C. *In vivo* labelling, immunoprecipitation of samples with rabbit polyclonal anti-carboxypeptidase Y antiserum and analysis of immunoprecipitated samples were carried out as described by Govindan and Novick (B.Govindan, personal communication, manuscript in preparation).

Thin section electron microscopy

Cells were grown for 10-14 h in YPRG at 30°C. One portion of each culture was transferred to YPD for growth for a further 14 or 16 h at 30°C. A second portion of each culture was transferred back into YPRG and growth continued for a further 16 h in YPRG. Thin section electron microscopy was carried out by the method of Salminen and Novick (1987) as modified by Rossi *et al.* (G.Rossi, K.Kolstad, F.Pallauault and S.Ferro-Novick, personal communication, manuscript in preparation).

Cell fractionation

Cells were grown for 10-14 h in YPRG at 30°C, transferred to YPD and growth continued at 30°C. From each culture, 20 OD_{599} units of cells were sedimented and spheroplasted as described for the analysis of internal and external forms of invertase after 0, 14 and 16 h of growth in YPD. The spheroplasts were sedimented and resuspended in 1 ml of ice-cold yeast lysis buffer [0.8 M sorbitol, 10 mM triethanolamine, 1 mM EDTA (pH 7.2), 1 mM PMSF and 1 μg/ml of leupeptin, pepstatin, chymostatin, aprotinin and antipain]. The lysate was homogenized by 20 strokes in a 2 ml homogenizer and centrifuged at 450 *g* for 3 min. The homogenization procedure was repeated on the pellet (P1) and the supernatants pooled (S1) and centrifuged for 70 min at 100 000 *g* in a 50Ti rotor (Beckman instruments Inc). The supernatants were removed and the pellets resuspended in 1 ml of yeast lysis buffer. Protein estimations were performed on the initial lysates and the protein content of the pellets and supernatants normalized to account for differences in protein concentration of the lysates. Equal samples were then subjected to SDS-PAGE on 14% slab gels. Proteins were electrophoretically transferred to nitrocellulose, probed with rabbit polyclonal anti-Sec4p antiserum (1:1000) and radioiodinated protein A (Amersham International plc) as described by Goud *et al.* (1988).

Uptake of Lucifer yellow

The uptake of Lucifer yellow was visualized as described by Dulic *et al.* (1991).

Acknowledgements

We thank P.J.Brennwald for helpful advice and discussions, and specifically for advice on designing the oligonucleotides for PCR and also for the pNB527 construct and the CPY antibody. We also thank M.Talmor for oligonucleotide synthesis, Y.Jiang and S.Ferro-Novick for the yeast genomic DNA from the *bet2-1* strain, M.Snyder for the wild-type yeast genomic DNA library in λ phage EMBL3A, J.Mulligan for helpful advice, J.Broach for the Ras2p antibody, B.Dunn and D.Botstein for the invertase antibody, B.Govindan for advice on the CPY pulse-chase experiments, F.Palluault, L.Vanacore and P.Webster for help with thin section electron microscopy and H.Tan for photographic assistance. This work was supported by grants CA46128 and GM35370 to P.N. from the NIH and grant MCB9205697 to C.M.C. from the NSF. M.D.G. is a Lucille P.Markey Visiting Scholar and is supported by the Lucille P.Markey Charitable Trust.

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Received on November 9, 1993; revised on January 21, 1994