Gyp5p and Gyl1p are involved in the control of polarized exocytosis in budding yeast

Laurent Chesneau[1,*], Sophie Dupré[1,*], Anna Burdina[1], Jérôme Roger[1,‡], Sophie Le Panse[2], Michel Jacquet[1] and Marie-Hélène Cuif[1,¶]

[1]Institut de Génétique et Microbiologie, CNRS-UMR 8621, Université Paris XI, 91 405 Orsay Cédex, France
[2]Service de Microscopie Electronique, Institut J. Monod, 75251 Paris CEDEX 05, France

*These authors contributed equally to this work
‡Present address: Laboratoire de physiopathologie moléculaire et cellulaire de la rétine, INSERM U592, 184 rue du Faubourg Saint-Antoine, 75 571 Paris CEDEX 12, France
¶Author for correspondence (e-mail: mhcuif@igmors.u-psud.fr)

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Summary

We report here elements for functional characterization of two members of the *Saccharomyces cerevisiae* Ypt/Rab GTPase activating proteins family (GAP): Gyp5p, a potent GAP in vitro for Ypt1p and Sec4p, and the protein Ymr192wp/APP2 that we propose to rename Gyl1p (GYp like protein). Immunofluorescence experiments showed that Gyp5p and Gyl1p partly colocalize at the bud emergence site, at the bud tip and at the bud neck during cytokinesis. Subcellular fractionation and co-immunoprecipitation experiments showed that Gyp5p and Gyl1p co-fractionate with post-Golgi vesicles and plasma membrane, and belong to the same protein complexes in both localizations. We found by co-immunoprecipitation experiments that a fraction of Gyp5p interacts with Sec4p, a small GTPase involved in exocytosis, and that a fraction of Gyl1p associates at the plasma membrane with the Gyp5p/Sec4p complexes. We showed also that *GYP5* genetically interacts with *SEC2*, which encodes the Sec4p exchange factor. Examination of the *gyp5Δgyl1Δ* mutants grown at 13°C revealed a slight growth defect, a secretion defect and an accumulation of secretory vesicles in the small-budded cells. These data suggest that Gyp5p and Gyl1p are involved in control of polarized exocytosis.

Key words: Gyp5p, Ymr192wp, Sec4p, Polarized exocytosis

Introduction

Ypt/Rab-GTPases are involved in membrane transport regulation in all eukaryotic organisms. Like other members of the small GTPase superfamily, they function in a cyclic manner: they swap between a GTP-bound form, which is able to contact effectors, and an inactive GDP-bound form. The intrinsic rate of conversion between these two states is low, thus regulatory proteins are required for in vivo functioning of this cycle. Guanine nucleotide exchange factors (GEF) catalyse the exchange of GDP for GTP. GTPase activating proteins (GAP) accelerate the hydrolysis from GTP to GDP. Increasing attention has been paid, over the past years, to the regulatory functions of these GEFs and GAPs because their activities can control precise temporal and spatial repartition of functional small GTPases (Zhong et al., 2003).

GAP for Ypt/Rab-GTPases were first cloned in *Saccharomyces cerevisiae* (Du et al., 1998; Strom et al., 1993; Vollmer and Gallwitz, 1995), and they form a family of structurally related proteins extending from yeast to higher eukaryotes (Neuwald, 1997). They display a 200 amino acid GAP domain, containing six shared motifs, involved in catalytic activity (Albert et al., 1999; Rak et al., 2000). Nine GAP for Ypt/Rab-GTPases, most of them named Gyp (GAP for Ypt Proteins), have been identified in *S. cerevisiae*. Among them, Bub2p, which negatively regulates the small GTPase Tem1p, participates in the mitotic exit network (for a review, see Bardin and Amon, 2001). Gyp1p has been characterized as a negative regulator of Ypt1p, localized on Golgi membranes (Du and Novick, 2001). Gyp2p has been described as a negative regulator of Ypt6p, involved in recycling (Lafourcade et al., 2003). Msb3p/Gyp3p and Msb4p/Gyp4p were proposed as regulators of actin polarization and, more recently, as GAPs for Sec4p in vivo (Bi et al., 2000; Gao et al., 2003). Gyp8p was also described as another Ypt1p-GAP in vivo (De Antoni et al., 2002). For others, GAP activity toward small GTPases was determined in vitro, but in vivo functional data are still missing (Albert and Gallwitz, 1999; Will and Gallwitz, 2001).

It is noteworthy that Gyp proteins display a broad substrate specificity in vitro. Therefore, the in vivo function of each Gyp protein cannot be inferred from its in vitro GAP activity. A complete functional characterization requires the determination of protein localization during the cell cycle, and an examination of in vivo interactions with potential GTPase substrates. This is the work we have undertaken for two members of the Gyp family, Gyp5p and Gyl1p/Ymr192wp/App2p.

Gyp5p, encoded by the ORF *YPL249c*, was shown to be a potent GAP in vitro for Ypt1p, involved in ER-to-Golgi transport, and for Sec4p, involved in exocytosis (De Antoni et al., 2002). The authors showed that *GYP5* deletion in an *ypt1*^{Q67L} context leads to cold-sensitive slow growth, accumulation of ER membranes and autophagic processes, and proposed that Gyp5p acts, in combination with Gyp1p and Gyp8p, as an Ypt1p-GAP in vivo. The product of the ORF

YMR192w is the nearest paralog of Gyp5p. It is an uncharacterized protein, with no GAP activity shown to date. The name of *APP2* (actin patches protein) was recently proposed for *YMR192w*, on the basis of a large-scale bioinformatic study predicting a role for Ymr192wp in actin filament organization (Samanta and Liang, 2003). However, as we show in the present paper that Ymr192wp is not colocalized with actin, we propose the name Gyl1p (GYp Like 1) for Ymr192wp, in agreement with the *Saccharomyces* Genome Database (SGD) scientific curators.

Our work was aimed at determining the in vivo localization and function of Gyp5p and Gyl1p. In this report, we show that Gyp5p and Gyl1p associate with the plasma membrane, mainly at the bud emergence site, at the bud membrane during bud growth, and at the bud neck during cytokinesis. Gyp5p and Gyl1p also co-purify with post-Golgi vesicles. We found that Gyp5p and Gyl1p belong to the same protein complexes at the plasma membrane, and that they interact with the small GTPase Sec4p, involved in secretion. Genetic experiments showed that *GYP5* genetically interacts with *SEC2*, the Sec4p exchange factor. The phenotype of both *gyp5Δ* and *gyl1Δ* strains was normal, but the *gyp5Δgyl1Δ* strain displayed slow growth and slow secretion at 13°C. Moreover, electron microscopy showed an accumulation of vesicles in small-budded *gyp5Δgyl1Δ* cells cultured at 13°C. These data suggest that Gyp5p and Gyl1p are involved in Sec4p regulation during polarized secretion.

Materials and Methods

Yeast strains, media and growth conditions

The *gyp5Δ* and *gyl1Δ* strains used in this study were provided by EUROSCARF (S288C derivative genetic context). The wild-type strains (WT) were obtained by crossing BY4741 *cmk1Δ* × BY4742 *cmk2Δ* strains, sporulation and selection of $G418^s$ segregants. The ORT5704-11c *msb3Δmsb4Δ* strain was provided by Francis Fabre (Iwanejko et al., 1999). The MY146 (*Mat a leu2,3-112 ura3-52 sec2-78*) strain was provided by Peter Novick. The JGY86A *sec15-1 sec4Q79L* strain was provided by Erfei Bi (Gao et al., 2003). Combined deletions and mutations were obtained by crosses and sporulation, and the segregants genotype was verified by Southern blot and hybridization with a kan^r probe, essentially as described previously (Sambrook, 1989). All basic yeast manipulations (including culture, sporulation, tetrads dissection and genetics techniques) were carried out according to Guthrie and Fink (Guthrie and Fink, 1991). YPG medium (1% yeast extract, 1% peptone, 2% glucose, plus 2% agar for solid media), supplemented with 400 μg/ml G418, was used for strains bearing the kanMX4 module. YNB (0.17% yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, 2% glucose) plus relevant amino acids and bases was used for vector selection. Transformations of *S. cerevisiae* strains were performed by the lithium acetate method, with single strand carrier DNA and dimethyl sulfoxide (Hill et al., 1991).

For the determination of doubling time, cells were pregrown at 30°C in YPG medium for 8 hours, then diluted in fresh YPG to an A_{600nm}=0.0005. After overnight culture at 30°C, cells were rediluted in fresh prewarmed YPG to an A_{600nm}=0.1, then shifted to the relevant temperature. The absorbance was thereafter measured every 90 minutes. Doubling time was calculated from the exponential part of the curve. For viability evaluation, Phloxine B (SIGMA) was added at a final concentration of 200 μg/ml to exponentially growing cells. After a 30 minute incubation at 30°C, cells were fixed by addition of formaldehyde (3.4% final concentration), and counted with a hemacytometer. At least 100 cells were counted, and the ratio of unstained cells versus total cells was calculated. Each value for doubling time and viability is the average of results obtained on at least two independent clones.

Gyp5p-Myc and Gyl1p-HA strains, Bgl2p-HA strain

Gyp5p and Gyl1p were tagged at their carboxy-terminus, either by 13 copies of the Myc epitope, or by three copies of the HA epitope, using the PCR-based chromosomal modification described in (Longtine et al., 1998). PCR products were used to transform the WT strains. In-frame fusion was verified by sequencing. Expression of fusion proteins was tested by western blot on total protein extracts, with mouse monoclonal 9E10 anti-Myc (Roche), or rat monoclonal 3F10 anti-HA (Roche). Gyl1p-HA and Gyl1p-Myc migrated on SDS-PAGE as polypeptides of 95 kDa and 115 kDa, respectively, near the predicted molecular weight. Gyp5p-Myc and Gyp5p-HA migrated as polypeptides of 170 and 155 kDa, respectively.

Bgl2p was tagged at its carboxy-terminus by three copies of the HA epitope as described above. Expression of fusion proteins was tested by western blot. Wild-type cells expressing Bgl2p-HA were crossed with *gyp5Δgyl1Δ* cells and sporulated. Segregants disrupted for *GYL1* and *GYP5* and expressing Bgl2p-HA were selected.

Sec4p-GFP and Gyp5p-Myc expression plasmids

The Sec4p-GFP expression vector was obtained by cloning a PCR-amplified Sec4p ORF at the *Bam*H1 site of the pUG36 expression vector (Niedenthal et al., 1996), downstream of the yEGFP3 coding sequence. In-frame fusion was verified by sequencing. Expression of the fusion protein was tested by immunoblotting with monoclonal anti-GFP antibodies (Roche). Sec4p-GFP migrated on SDS-PAGE as a polypeptide of 50 kDa, corresponding to the predicted molecular weight.

To construct the Gyp5p-Myc expression vector, the Gyp5p ORF fused to the C-terminal Myc tag was amplified by PCR on genomic DNA of the strain expressing the Myc-tagged Gyp5p (see above), and subcloned at the *Xba*I and *Sac*I sites into the YCpADH1 vector (Reinders et al., 1998). Expression of the fusion protein was tested by immunoblotting with monoclonal anti-Myc antibodies (Roche).

Yeast extracts

Depending on the experiments, different methods were used. Total protein extracts used for selection of Gyp5p-Myc and Gyl1p-HA expressing strains were prepared from 3×10^7 exponentially growing cells by the addition of 1/10 volume of (NaOH 1.85 M, 1% mercaptoethanol) directly to the culture medium, vortexing and incubation at 4°C. After 10 minutes, 1/10 volume of a cold 50% trichloroacetic acid (TCA) solution was added, samples were shaken and incubated for 10 minutes at 4°C. Protein pellets were collected by a centrifugation at 4000 ***g*** at 4°C, and diluted in Laemmli sample buffer (Laemmli, 1970).

For subcellular fractionation and membrane extraction, exponentially growing cells were harvested by centrifugation, resuspended in 0.1 M Tris-HCl pH 9.4, 10 mM DTT, and shaken for 8 minutes. After centrifugation, yeast cells were spheroplasted with 0.25 mg/ml Zymolyase 20T (ICN Pharmaceuticals), in STC buffer (1 M sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$). Spheroplasts were pelleted at 100 ***g***, washed once with STC buffer and resuspended in lysis buffer (0.2 M Tris-HCl pH 7.6, 6 mM $MgCl_2$, 1 mM EDTA, plus protease inhibitors and 1 mM PMSF). Cells were then broken with glass beads for 5 minutes. The homogenate was transferred to a new 1.5 ml Eppendorf tube and centrifuged at 200 ***g*** for 5 minutes to obtain a clear lysate, without unbroken cells and debris.

Subcellular fractionation and density gradient centrifugation

Subcellular fractionation was performed by differential

centrifugation. The clear lysate described above was first centrifuged for 10 minutes at 13,000 ***g***. The resulting supernatant was centrifuged for 1 hour at 100,000 ***g*** in a Beckmann TL100 ultracentrifuge. Both 13,000 ***g*** and 100,000 ***g*** pellets were resuspended in lysis buffer for subsequent analysis by immunoblot or immunoprecipitation experiments.

For density gradient centrifugation, both 13,000 ***g*** and 100,000 ***g*** pellets obtained as described above were resuspended in STE 10 buffer (10% sucrose, 10 mM Tris-HCl pH 7.6, 10 mM EDTA plus 1 mM PMSF and protease inhibitors), then layered on top of a 11.1 ml 20 to 60% linear sucrose gradient, made up in 10 mM Tris-HCl pH 7.6, 10 mM EDTA. Samples were centrifuged at 100,000 ***g*** for 18 hours at 4°C in a Beckmann SW41 rotor. Fractions were collected from the top of the gradient, and proteins were precipitated by 10% TCA and a 45 minute incubation on ice, pelleted by centrifugation and diluted in Laemmli sample buffer. Mouse monoclonal 5C5 anti Dpm1p (Molecular Probes) and mouse monoclonal 18C8 anti Vps10p (Molecular Probes) were used for immunoblotting.

Membrane extraction, and calf intestine alkaline phosphatase treatment

Membrane extraction were performed by incubating clear lysate samples for 30 minutes on ice with either lysis buffer alone, 5 M urea in lysis buffer, 1% Triton-X100 (TX-100) in lysis buffer, or 0.1 M sodium carbonate pH 11 in lysis buffer. Extracts were separated into membrane and soluble fractions by centrifugation at 100,000 ***g*** for 1 hour at 4°C in a Beckmann TL100 ultra-centrifuge. Proteins were then precipitated by 10% TCA on ice for 30 minutes, pelleted and resuspended in Laemmli sample buffer.

For dephosphorylation by alkaline phosphatase, total protein extracts in Laemmli sample buffer (50 μl) were diluted tenfold in alkaline phosphatase buffer (100 mM Tris-HCl pH 8.5, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$), concentrated again to the initial volume by ultrafiltration and incubated with 3 μl calf intestinal alkaline phosphatase (CIP, 20 U/μl, Roche) for 2 hours at 37°C. The control was incubated under the same conditions without alkaline phosphatase.

Immunoprecipitation experiments

Volumes of subcellular fractions corresponding to 5×10^7-10^8 cells, according to the experiments, were diluted in 500 μl of cell lysis buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, plus 1 mM PMSF and protease inhibitors). For P13 and P100 fractions, final concentration of Triton X-100 was adjusted to 2%. After a 1 hour incubation at 4°C with gentle shaking, extracts were centrifuged at 15,000 ***g*** for 5 minutes. 1 μg/10^7 cells of anti-Myc mouse monoclonal antibodies (Roche), or 0.5 μg/10^7 cells of anti-GFP rabbit antibodies (Chemicon) was added to supernatants. Protein G-agarose beads (240 μl of a 50% slurry, prewashed in lysis buffer) were added, and the mixture was incubated for 2 hours at 4°C with gentle shaking. The beads were washed twice with lysis buffer, twice with 100 mM Tris-HCl pH 7.5, 300 mM NaCl, and once with 20 mM Tris-HCl pH 7.5. Beads were then resuspended in 25 μl of Laemmli sample buffer, boiled for 5 minutes and centrifuged for 15 minutes at 20,000 ***g***. The resulting supernatant was submitted to SDS-PAGE. For negative controls, co-immunoprecipitations were performed exactly as described, except that antibodies were omitted.

Invertase secretion assays

Cells were grown overnight at 13°C in YP medium containing 2% glucose. Exponentially growing cells were harvested by centrifugation, washed with precooled water, resuspended in YP medium containing 0.1% glucose and incubated at 13°C. Amounts of cells corresponding to 1 absorbance unit (A_{600nm}) were taken, washed and resuspended in 500 μl of 10 mM NaN_3. External invertase activity was measured on intact cells. Internal invertase activity was measured in spheroplast lysates: cells were spheroplasted; the spheroplasts were then pelleted and lysed in 0.5% Triton X-100. Lysates were centrifuged for 2 minutes at 13,000 ***g*** and supernatants were used to measure internal invertase. External and internal invertase assays were then performed as described in (Goldstein and Lampen, 1975). The percentage of secreted invertase corresponds to external invertase activity divided by total (external plus internal) invertase activity.

Pulse-chase labelling and immunoprecipitation of carboxypeptidase Y

Yeast cells were grown overnight at 13°C in YNB medium plus amino acids. Exponentially growing cells were harvested by centrifugation and resuspended in YNB without methionine during half an hour. Pulse-chase experiment and carboxypeptidase Y (CPY) precipitation was then performed as described in (Belgareh-Touze et al., 2002), except that cells were labelled for 30 minutes.

Bgl2p secretion assays

Bgl2p secretion assay was performed as described in (Gao et al., 2003). WT and *gyp5*Δ*gyl1*Δ cells expressing Bgl2p-HA were grown overnight at 13°C in YPD medium. 10 mM sodium azide was added to exponentially growing cells, and cells were harvested by centrifugation. Spheroplasts were then prepared as described above except that 10 mM sodium azide was added to the STC buffer. Spheroplasts were then pelleted by centrifugation and washed once with STC buffer. The supernatant (S_0 containing external Bgl2p-HA pool) was collected and proteins were precipitated by 10% TCA. Spheroplasts were then resuspended in lysis buffer with 1% TX100 and centrifuged for 10 minutes at 13,000 ***g***. The supernatant (S_1), containing mainly vesicles and cytoplasm, was collected and protein was precipitated by 10% TCA. This S1 fraction contains internal Bgl2p-HA pool. Proteins from S0 and S1 were resuspended in Laemmli buffer, boiled, separated on 10% SDS-PAGE and revealed by immunoblotting with anti-HA antibodies (Roche).

Immunofluorescence and staining

Immunofluorescence staining was performed essentially as described by Pringle et al. (Pringle et al. 1991). The primary antibodies used were mouse monoclonal 9E10 anti-Myc (Roche), rat monoclonal 3F10 anti-HA (Roche), rabbit anti-GFP (Chemicon), mouse monoclonal C4 anti-actin (Chemicon) and rabbit anti-Cdc11 (Santa-Cruz Biotechnology, Inc.). Secondary antibodies were from Jackson Immunoresearch Laboratories. Calcofluor and DAPI staining were performed according to Pringle (Pringle, 1991).

Fluorescence microscopy, acquisition and image treatment

Cells were observed on a Leica DM RXA microscope, and images were captured by a CCD camera 5 MHz Micromax 1300Y (Roper Instruments). Metamorph software (Universal Imaging Corp.) was used to deconvolute Z-series, treat the images and to create projections.

Electron microscopy

Yeast cells were fixed in the culture medium by 10 minutes incubation with 1% aqueous glutaraldehyde, followed by 2 hours incubation at 4°C with fresh fixative. Cells were washed with 0.1 M cacodylate buffer, then with water, and treated with 1% potassium permanganate for 2 hours on ice. After washing in water, cells were resuspended in 2% aqueous uranyl acetate for 1 hour at 4°C, dehydrated in graded series of ethanol, incubated in a mixture of ethanol and Spurr's resin,

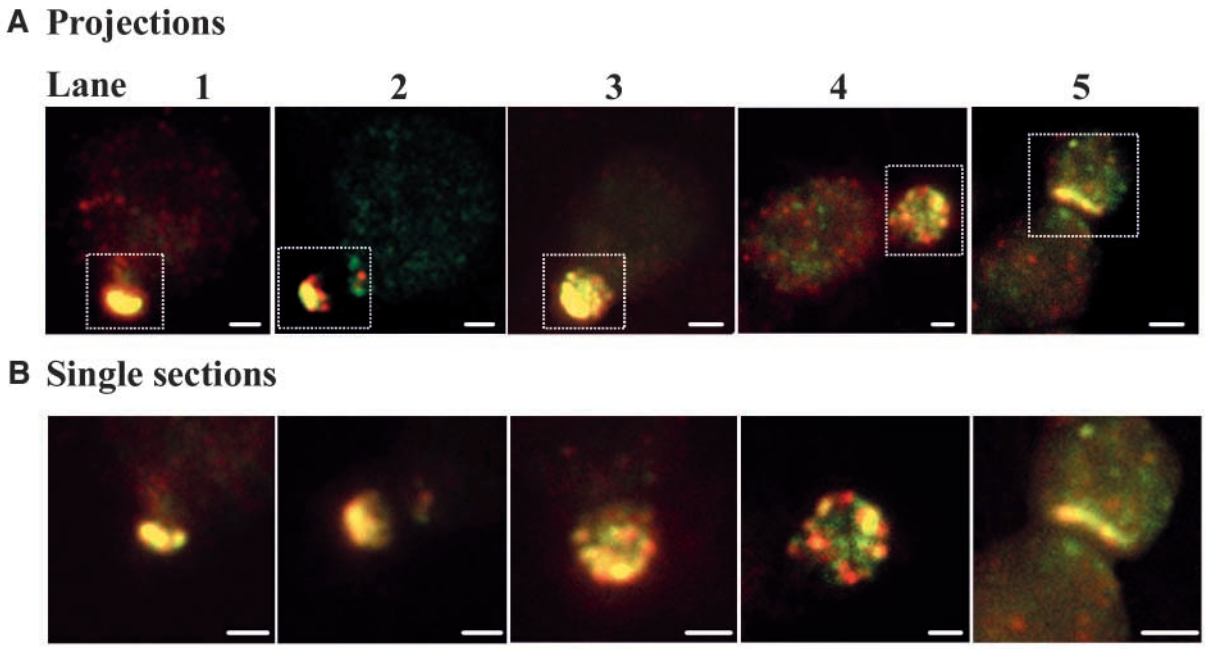

Fig. 1. Gyp5p and Gyl1p localize at the bud tip during polarized growth, at the bud neck during cytokinesis, and are partially colocalized. (A) Yeast cells expressing Gyp5p-Myc (green) and Gyl1p-HA (red) were stained by indirect immunofluorescence and observed with a 3D deconvolution microscopy system. Images shown are projections of deconvoluted Z-series combining green and red signals. Bars, 1 μm. White squares indicate the regions magnified in B. (B) Single sections of part of each image in A. Bars, 1 μm.

and embedded in Spurr's low viscosity medium. Thin sections were cut, stained with uranyl acetate or lead citrate, and observed in a Tecnai 12 electron microscope (Eindhoven, Netherlands).

Results

Gyp5p and Gyl1p are partly colocalized at the bud emergence site, the bud tip and the bud neck

To determine the cellular localization of Gyp5p and Gyl1p, strains expressing Myc- or HA-tagged versions of both proteins were obtained by modification of chromosomal genes. Immunofluorescence experiments were performed on a strain co-expressing Gyp5p-Myc and Gyl1p-HA, and cells were examined with three-dimensional (3D) deconvolution microscopy. Figure 1A shows yeast cells expressing both Gyp5p-Myc (stained in green) and Gyl1p-HA (stained in red). The predominance of yellow colour indicates that the two proteins localized very closely during the cell cycle: in G1-S they strongly concentrated at the bud emergence site, then at the bud tip (lanes 1 and 2). During S-G2, dispersion of green and red patches in different bud zones was observed (lanes 3 and 4), indicating that the proteins were separated. Later, both Gyp5p-Myc and Gyl1p-HA concentrated at the bud neck during cytokinesis (lane 5). The same results were obtained for Gyp5p-HA and Gyl1p-Myc fusion proteins (data not shown), and similar localizations of Gyp5p-GFP and Gyl1p-GFP fusion proteins were published very recently in a global yeast localization analysis (Huh et al., 2003). A diffuse staining of the mother-cell body was also detected, corresponding probably to a cytosolic pool of both proteins (see below). No other significant localization could be detected using 3D deconvolution microscopy. In Fig. 1B, single sections of the same cells show that colocalization of the two proteins is not absolute. A strict colocalization was observed in some patches, whereas other patches displayed colour variations from red to green, suggesting either some distance between the proteins, a different proportion of the two proteins, or both. Thus, immunofluorescence data indicate that Gyp5p and Gyl1p concentrate in the bud cortical zone, as well as at the cytokinesis site; they significantly colocalize, but this colocalization appears to be transient.

Gyp5p and Gyl1p localize within the septin ring

This localization of Gyp5p and Gyl1p prompted us to examine the colocalization of both proteins with actin and septins. As shown in Fig. 2A, immunostaining of actin (stained in green) and either Gyp5p-Myc or Gyl1p-HA (stained in red) showed that neither Gyp5p nor Gyl1p are colocalized with actin patches. Rather, actin patches seem to be organized around Gyp5p and Gyl1p patches, especially during or immediately after cytokinesis (see arrows). A septin ring is formed at the bud tip during bud emergence. This ring is then split and remains at the bud neck until cytokinesis has occurred (for a review, see Pruyne and Bretscher, 2000). We therefore examined the localization of Gyp5p and Gyl1p with respect to the septin ring (Fig. 2B). Co-immunostaining of Cdc11p, Gyp5p-Myc and Gyl1p-HA showed that both Gyp5p and Gyl1p localize within the septin ring at the bud emergence site and during cytokinesis.

Gyp5p and Gyl1p are present in three main cellular pools

To define the subcellular repartition of Gyp5p and Gyl1p, subcellular fractionation experiments were performed on yeast

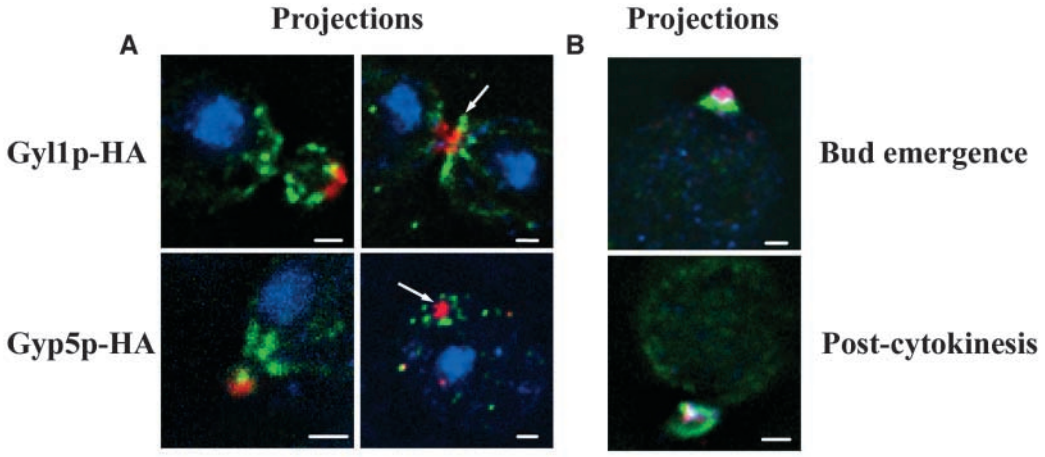

Fig. 2. (A) Gyp5p and Gyl1p do not colocalize with actin. Yeast cells expressing Gyl1p-HA or Gyp5p-HA were stained by immunofluorescence and examined with a 3D deconvolution microscopy system. Images are projections of deconvoluted Z-series combining green, red and blue signals. Bars, 1 μm. First row: Gyl1p-HA is stained in red, actin is stained in green, DNA is stained in blue. Second row: Gyp5p-HA is stained in red, actin is stained in green, DNA is stained in blue. (B) Gyp5p-Myc and Gyl1p-HA localize within the septin ring. Yeast cells expressing Gyl1p-HA and Gyp5p-Myc were stained by immunofluorescence and examined with a 3D deconvolution microscopy system. Images are projections of deconvoluted Z-series combining green, red and blue signals. Cdc11p is stained in green, Gyl11p-HA is stained in red and Gyp5p-Myc is stained in blue. The magenta signal is the result of combination of red and blue signals. Bud emergence and post-cytokinesis stages are shown. Bars, 1 μm.

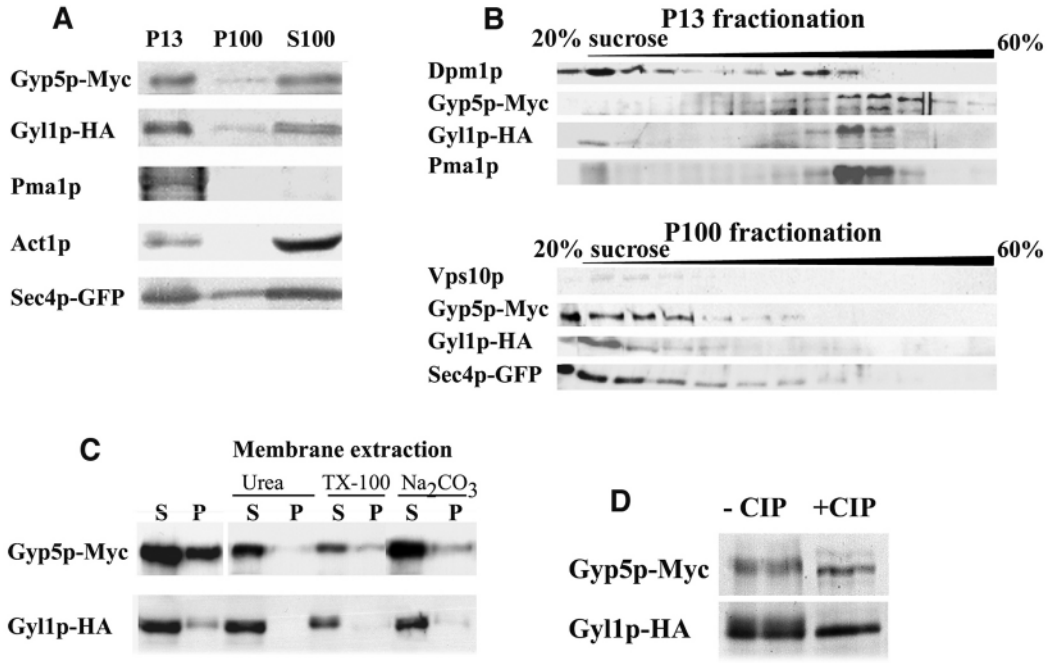

Fig. 3. (A) Gyp5p and Gyl1p are present in three main cellular pools: yeast cells expressing Gyp5p-Myc, Gyl1p-HA and Sec4p-GFP were submitted to subcellular fractionation to generate P13, P100 and S100 fractions. The volumes of fractions loaded on each lane of SDS-PAGE correspond to 2×10^7cells. The nitrocellulose membranes were incubated with either anti-Myc, anti-HA or anti-GFP antibodies, then stripped from the antibodies and incubated again with anti-Pma1p antibodies (from R. Serrano) and anti-actin antibodies. (B) Gyp5p and Gyl1p co-fractionate with plasma membrane and vesicles markers: the P13 and P100 fractions shown in A were loaded on linear 20-60% sucrose gradients. Fractions were probed by immunoblotting with different antibodies as described in A. Dpm1p is an ER membrane dolichol-phosphate mannose synthase. (C) Gyp5p and Gyl1p behave as peripheral membrane proteins: yeast cells expressing Gyp5p-Myc and Gyl1p-HA were submitted to membrane extraction, as described in Materials and Methods. S, supernatant; P, membrane pellet. (D) Gyp5p and Gyl1p are phosphorylated proteins: total protein extracts of yeast cells expressing Gyp5p-Myc and Gyl1p-HA were submitted to calf intestine alkaline phosphatase (CIP) treatment, as described in Material and Methods, resolved on SDS-PAGE and revealed with either anti-Myc or anti-HA antibodies.

cells co-expressing Gyp5-Myc and Gyl1p-HA fusion proteins. Figure 3A shows immunoblotting experiments performed on cell fractions. P13 corresponds to the pellet recovered after 10 minutes 13,000 ***g*** centrifugation of the total cell lysate. This fraction is known to be enriched in cytoskeleton (Wittenberg et al., 1987), as well as plasma membrane and organelle membranes (Goud et al., 1988). Indeed, Pma1p, an integral plasma membrane protein, as well as actin, were found in the P13. P100 and S100 correspond respectively to the pellet and supernatant recovered after 1 hour 100,000 ***g*** centrifugation of the first supernatant (S13). P100 is known to be enriched in late Golgi and vesicle membranes. P13 and P100 fractions were loaded onto sucrose gradients and submitted to centrifugation. Repartition of Gyp5p and Gyl1p in the sucrose gradients is shown in Fig. 3B.

Both Gyp5p-Myc and Gyl1p-HA were abundant in the S100 fraction, indicating an important cytosolic pool, as already described (De Antoni et al., 2002). Significant

amounts of Gyp5p-Myc and Gyl1p-HA were present in the P13 fraction, and co-fractionated mainly in high-density fractions with the plasma membrane protein Pma1p. These results indicate that a large fraction of Gyp5p and Gyl1p is associated with the plasma membrane. Small amounts of Gyp5p-Myc and Gyl1p-HA were also found in the P100 fraction. Moreover, P100 fractionation in sucrose gradients showed that Gyp5p-Myc and Gyl1p-HA co-fractionated with Vps10p, a protein known to cycle between Golgi and endosomes (Marcusson et al., 1994), and with a GFP-tagged version (described below) of Sec4p, a small GTPase associated with secretory vesicles (Goud et al., 1988). These results suggest that post-Golgi vesicles contain a pool of Gyp5p and Gyl1p.

Membrane-associated Gyp5p-Myc and Gyl1p-HA were submitted to different protein extraction methods. As shown in Fig. 3C, Gyp5p-Myc and Gyl1p-HA were efficiently extracted from membranes not only by a 5 M urea treatment, but also by 1% Triton-X100 or 0.2 M Na_2CO_3, pH 11 treatments. Thus, Gyp5p and Gyl1p behave as peripheral membrane proteins.

Taken altogether, these data indicate that Gyp5p and Gyl1p are present in three main pools: one is cytosolic, the second is peripherally associated with the plasma membrane and the third is associated with post-Golgi vesicles.

Gyp5p and Gyl1p are phosphorylated proteins

Western blotting of Gyp5p-Myc and Gyl1p-HA often resulted in doublet bands (a typical migration profile can be seen for Gyl1p-HA in Fig. 3A, S100). This suggested that both proteins might undergo phosphorylation. Total lysates of yeast cells co-expressing Gyp5p-Myc and Gyl1p-HA were therefore incubated with calf intestine alkaline phosphatase (CIP). As shown in Fig. 3D, CIP treatment of both proteins resulted in upper band disappearance and lower band reinforcement. This result indicates that both Gyp5p and Gyl1p undergo phosphorylation events. As the phosphorylated and nonphosphorylated forms were present in the different fractions, these do not seem to be associated with a specific cellular distribution.

Gyp5p and Gyl1p are co-immunoprecipitated, both at the plasma membrane and on post-Golgi vesicles

Immunoprecipitation experiments were performed in subcellular fractions (Fig. 4). In the P13 and P100 fractions, extensive precipitation of Gyp5p-Myc led to an almost complete co-precipitation of Gyl1p-HA. Reverse experiments using anti-HA antibodies gave the same results (data not shown). Thus, a large part of Gyp5p and Gyl1p associated either with the plasma membrane or with post-Golgi vesicles belongs to the same protein complex. By contrast, only a small fraction of Gyl1p-HA was co-precipitated with Gyp5p-Myc in the S100 fraction. These results suggest that Gyp5p and Gyl1p are mainly separated in the cytosol.

Part of Gyp5p and Gyl1p are involved in Sec4p-containing complexes

To examine interactions between Gyp5p, Gyl1p and Sec4p, we constructed a plasmid expressing a Sec4p-GFP fusion protein.

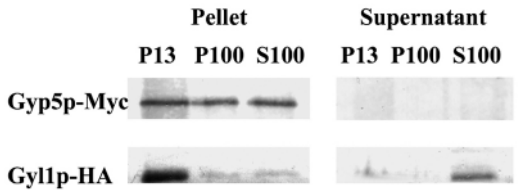

Fig. 4. Gyl1p-HA co-immunoprecipitates with Gyp5p-Myc. Volumes of subcellular fractions corresponding to 5×10^7 yeast cells expressing Gyp5p-Myc and Gyl1p-HA were submitted to immunoprecipitation with anti-Myc antibodies. Proteins bound to Protein G-agarose beads (Pellet) or remaining in the supernatant (Supernatant) were separated on SDS-PAGE, and revealed with either anti-Myc or anti-HA antibodies. One fifth of the total amount of proteins was loaded in supernatant lanes.

The cellular distribution of Sec4p-GFP along the cell cycle was examined by deconvolution microscopy on living cells (four-dimensional). Sec4p-GFP concentrated strongly at the bud emergence site, at the bud tip and at the bud neck during cytokinesis (data not shown), in a manner consistent with previous indirect immunofluorescence data (Walch-Solimena et al., 1997). Yeast strains co-expressing Gyp5p-Myc and Gyl1p-HA were transformed with this plasmid, and immunoprecipitation experiments were performed in total cell extract and in subcellular fractions (Fig. 5A). Immunoprecipitation of Sec4p-GFP led to Gyp5p-Myc co-precipitation in the total cell extract and in the P13 and P100 fractions. In each fraction, the amount of co-precipitated Gyp5p-Myc was lower than one tenth of the total amount of Gyp5p-Myc (note that protein amounts loaded in supernatant lanes represent one fifth of total). Smaller amounts of Gyl1p-HA were recovered in the P13 fraction, and minute amounts only in the P100 fraction.

The distribution of Gyp5p-Myc, Gyl1p-HA and Sec4p-GFP was examined by indirect immunofluorescence and 3D deconvolution microscopy. Gyp5p-Myc and Gyl1p-HA colocalize with Sec4p-GFP at the bud emergence site (Fig. 5B, lane 1). In a small-budded cell (lane 2), colour variations indicate that essentially all combinations of proteins can be found at the bud membrane, with one patch at the bud tip where the three proteins colocalize. At the time of cytokinesis (lane 3), a significant colocalization of the three proteins is found at the bud neck.

Taken together, these results show that a fraction of Gyp5p belongs to Sec4p-containing complexes, both at the plasma membrane and on post-Golgi vesicles. At the plasma membrane, a fraction of the Gyp5p/Sec4p complex may associate with Gyl1p. As judged from immunofluorescence data, interactions between Gyp5p, Gyl1p and Sec4p would occur mainly at sites of bud emergence, at the bud tip and at the bud neck during cytokinesis.

GYP5 genetically interacts with *SEC2*

Gyp5p was shown to be a potent GAP for Sec4p in vitro (De Antoni et al., 2002). In an attempt to assess the in vivo function of Gyp5p, we examined genetic interactions between *GYP5* and *SEC2*, which encodes the Sec4p exchange factor (GEF) (Walch-Solimena et al., 1997). Sec2-78p was described as a temperature-sensitive mutant form of Sec2p: *sec2-78* cells

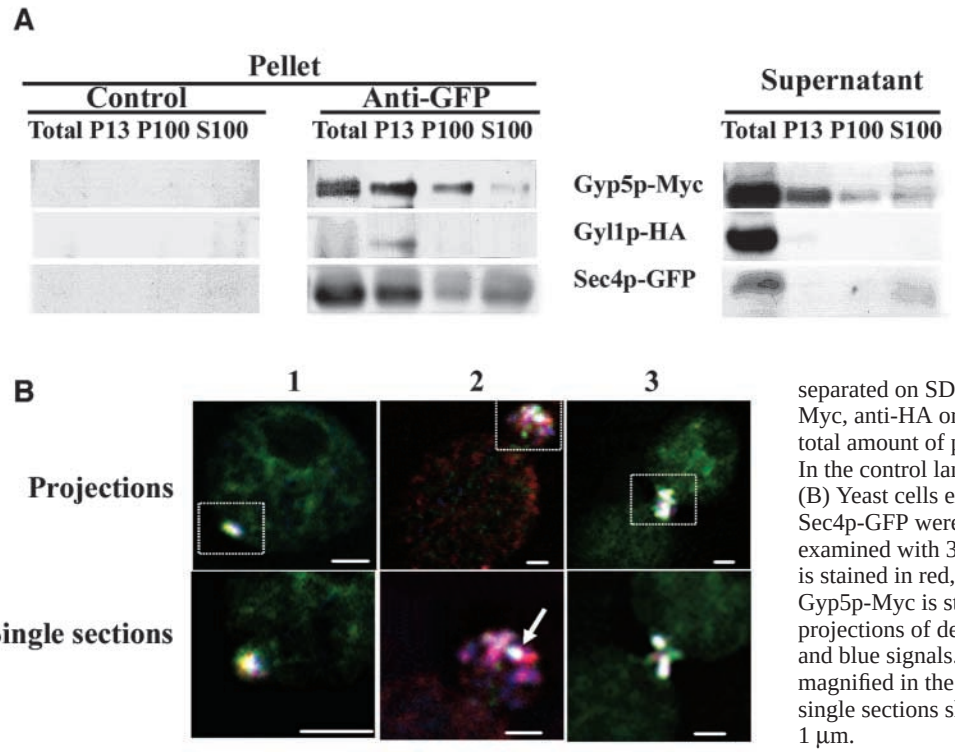

Fig. 5. Gyp5p-Myc and Gyl1p-HA interact with Sec4p-GFP. Lane 1, bud emergence; lane 2, small-budded cell; lane 3, cytokinesis. (A) Subcellular fractions obtained from 10^8 cells expressing Gyp5p-Myc, Gyl1p-HA and Sec4p-GFP were submitted to immunoprecipitation with anti-GFP antibodies, as described in Materials and Methods. Proteins bound to the agarose beads (Pellet), or remaining in the supernatant (Supernatant), were separated on SDS-PAGE and revealed with either anti-Myc, anti-HA or anti-GFP antibodies. One fifth of the total amount of proteins was loaded in supernatant lanes. In the control lanes, anti-GFP antibodies were omitted. (B) Yeast cells expressing Gyp5p-Myc, Gyl1p-HA and Sec4p-GFP were stained by immunofluorescence and examined with 3D deconvolution microscopy. Gyl1p-HA is stained in red, Sec4p-GFP is stained in green and Gyp5p-Myc is stained in blue. Images in the first row are projections of deconvoluted Z-series combining red, green and blue signals. White squares indicate the region magnified in the second row. Images in the second row are single sections shown with higher magnification. Bars, 1 μm.

grow at 30°C, but are unable to grow at 37°C. At this temperature, they display both accumulation and delocalization of secretory vesicles (Walch-Solimena et al., 1997). We transformed the *sec2-78* strain with an YCpADH1 plasmid expressing a Myc-tagged form of Gyp5p, and incubated the transformed cells plated on selective medium for two days, either at 26°C, 30°C or 37°C. Growth of a *sec2-78* strain overexpressing Gyp5p is strongly inhibited at 30°C, compared with *sec2-78* cells transformed with the empty YCpADH1 vector (Fig. 6). This result suggests that Gyp5p is involved in the control of polarized exocytosis, and that Gyp5p and Sec2p might exert in vivo opposite effects on polarized secretion.

Co-deletion of *GYP5* and *GYL1* leads to cold-sensitive slow growth, accumulation of vesicles and reduced secretion

We searched for functional evidences for involvement of Gyp5p and Gyl1p in the regulation of exocytosis. It has been recently shown that Msb3p and Msb4p, two others members of the Gyp family, are involved in the control of exocytosis through their GAP activity (Gao et al., 2003). We concluded that deletion of *GYP5* and/or *GYL1* could therefore lead only to a reduction of the GAP activity regulating exocytosis. A very similar case has been already described about the *sec4-leu79* mutation: Walworth and co-workers showed that Sec4-leu79p is about three times less sensitive to GAP activity than wild-type Sec4p. *Sec4-leu79* yeast cells display slow growth, slow invertase secretion and accumulation of secretory vesicles, all of these phenotypes revealed at 14°C only (Walworth et al., 1992). We therefore examined growth, morphology and invertase secretion in *gyp5Δ*, *gyl1Δ* and *gyp5Δgyl1Δ* strains.

Growth rate in rich medium of the *gyp5Δ* and *gyl1Δ* strains was normal at any temperature. However, as shown in Fig. 7A, the *gyp5Δgyl1Δ* strain displayed a slight but reproducible increase of doubling time at 30°C (around 100 minutes versus 90 minutes for WT cells). In the first 24 hours after a 13°C shift, doubling time increased to 600 minutes, versus 480 minutes for WT cells. At any temperature, Phloxine B staining showed percentages of Phloxine B-positive cells (nonviable cells) similar to the WT strain, indicating that the viability of the *gyp5Δgyl1Δ* cells was normal (data not shown). These results indicate that the *gyp5Δgyl1Δ* strain displays a cold-sensitive slow growth phenotype.

Thin-section electron microscopy was performed on *gyp5Δgyl1Δ* cells grown at 13°C for 16 hours. A large number (n=129) of *gyp5Δgyl1Δ* cells from two different clones were examined and compared with WT cells (Fig. 7B). This analysis revealed that *gyp5Δgyl1Δ* cells display a frequent accumulation of secretory vesicles, specific to small-budded cells. In small buds (defined here by a ratio – bud width/bud neck width –

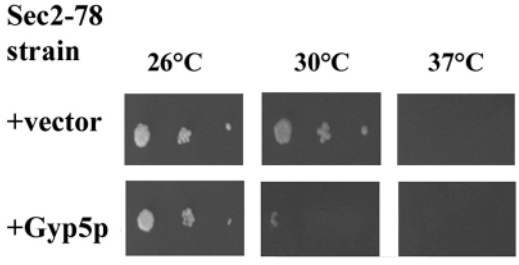

Fig. 6. Overexpression of Gyp5p-Myc enhances the *sec2-78* phenotype. An YCpADH1 vector, empty or carrying Myc-tagged Gyp5p, was transformed into a *sec2-78* strain. Successive dilutions of transformed cells in exponential growth phase were spotted onto selective medium and incubated for 2 days at 26°C, 30°C or 37°C.

A Doubling time in early log phase (minutes)

	13°C	30°C
WT	476 +/- 25	88 +/- 2
gyp5Δgyl1Δ	603 +/- 15	102 +/- 3

B % of cells with secretory vesicles

	Emerging bud	Small bud*	Large bud
WT	31.5% (n=19)	35% (n=22)	14% (n=28)
gyp5Δgyl1Δ	40 % (n=30)	58.6% (n=46)	15% (n=53)

*Significant p<0.001

C

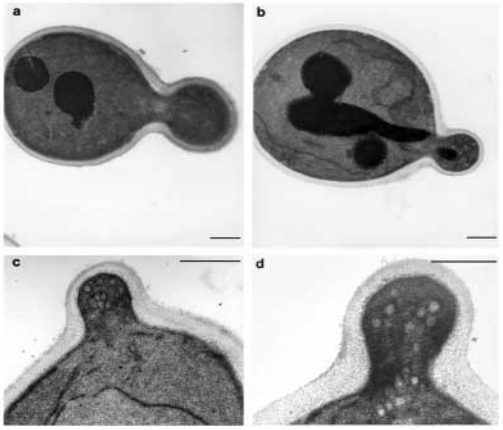

Fig. 7. *gyp5Δgyl1Δ* cells display a cold-sensitive slow growth phenotype and a frequent accumulation of secretory vesicles in small buds. (A) Doubling time of WT and *gyp5Δgyl1Δ* strains in rich medium was determined in early log phase at 13° and 30°C. Values are the average of results obtained with four independent clones. (B) Thin section electron microscopy was performed on WT and *gyp5Δgyl1Δ* cells cultured at 13°C for 16 hours. Small buds correspond to a ratio (bud width/bud neck width) <2, large buds to a ratio of 2 and higher. The % of WT cells and *gyp5Δgyl1Δ* cells containing at least five distinct secretory vesicles were determined. (C) Thin section electron microscopy of WT (a) and *gyp5Δgyl1Δ* cells (b-d). Bars, 500 nm.

lower than 2), 35% of the WT cells displayed at least five vesicles in the bud, whereas 58% of *gyp5Δgyl1Δ* cells exhibited a large accumulation of vesicles. This difference is statistically significant, with a *P* value <0.001. Figure 7C shows representative images of WT cells (a) and small-budded *gyp5Δgyl1Δ* cells (b, c, d). Vesicles are not delocalized into the mother-cell body, but remain concentrated into the bud. Images (b) and (c) also show that *gyp5Δgyl1Δ* cells accumulate significant amounts of endoplasmic reticulum membrane, a feature that has already been described for the *gyp5Δ ypt1*^{Q67L} (De Antoni et al., 2002).

Invertase secretion assays were performed after the induction of invertase expression by a shift in low glucose medium. At various times after the shift, invertase activity was measured in both the periplasm and an internal fraction after elimination of large membranes, and the percentage of secreted invertase was calculated. Invertase secretion was normal in *gyp5Δ* and *gyl1Δ* strain at any temperature, and no defect was detected in the *gyp5Δgyl1Δ* strain at 30°C. As shown in Fig. 8A, in the *gyp5Δgyl1Δ* strain grown at 13°C, the percentage of secreted invertase was only 81% of the WT at 15 minutes after the shift. This difference is small, but statistically significant (P<0.025), and it is reproducible as it was observed on six independent *gyp5Δgyl1Δ* clones in two different genetic backgrounds. Pulse-chase experiments showed that kinetics of maturation of carboxypeptidase Y is similar in *gyp5Δgyl1Δ* cells and in WT cells (Fig. 8B), a result indicating that proximal steps of the secretory pathway are normal in the *gyp5Δgyl1Δ* cells. Therefore, this slowing of invertase secretion reveals a slower distal step of the secretory pathway, that is exocytosis.

Bgl2p, an endo-β-1,3-glucanase required for cell wall organization (Mrsa et al., 1993), is often used for evaluation of secretory process, as it was proven that the major population of secretory vesicles carry Bgl2p (Harsay and Bretscher, 1995). We created a HA-tagged form of Bgl2p and transferred it by crosses in a *gyp5Δgyl1Δ* strain. External and internal pools of Bgl2p were collected from cells cultured at 13°C. The amount of secreted Bgl2p was significantly reduced in *gyp5Δgyl1Δ* cells compared with WT cells, with a corresponding increase of the internal amount of Bgl2p-HA (Fig. 8C). Quantification of the signals indicated that internal Bgl2p figures out at 88% of the total (internal plus external) amount of Bgl2p in *gyp5Δgyl1Δ* cells, versus 45% of the total amount of Bgl2p in WT cells. This result confirms that *gyp5Δgyl1Δ* cells display a partial secretion defect.

Taken together, the slower invertase secretion, the Bgl2p secretion defect and the accumulation of secretory vesicles found in small-budded *gyp5Δgyl1Δ* cells suggest that Gyp5p and Gyl1p are involved in the control of polarized exocytosis.

Discussion

We present in this paper new elements for the functional characterization of Gyp5p, a member of the Gyp family. Gyp5p was already known to be a cytosolic protein involved in the regulation of Ypt1p, thus in the control of the endoplasmic reticulum-to-Golgi traffic (De Antoni et al., 2002). We show here that Gyp5p is also peripherally associated with the plasma membrane, present on post-Golgi vesicles, and is associated with its nearest paralog Gyl1p in these localizations. We show that Gyp5p and Gyl1p concentrate at the bud emergence site, the bud tip and the bud neck, i.e. at sites of polarized exocytosis. Co-immunoprecipitation experiments show that Gyp5p and Gyl1p interact with Sec4p-containing complexes at the plasma membrane. We show also that *GYP5* genetically interacts with *SEC2*, the Sec4p exchange factor. Moreover, co-deletion of *GYP5* and *GYL1* induces a slow growth, an accumulation of vesicles restricted to small-budded cells, a reduced Bgl2p secretion and a slower invertase secretion in cells grown at 13°C. Taken all together, these results suggest that Gyp5p and Gyl1p are involved in the regulation of polarized exocytosis.

The small GTPase Sec4p is known to be a crucial protein for the control of exocytosis. Sec4p is present on secretory vesicles in its GTP-bound conformation and interacts with the Sec15p unit of the exocyst complex, thereby targeting secretory vesicles to sites of exocytosis (Guo et al., 1999). The

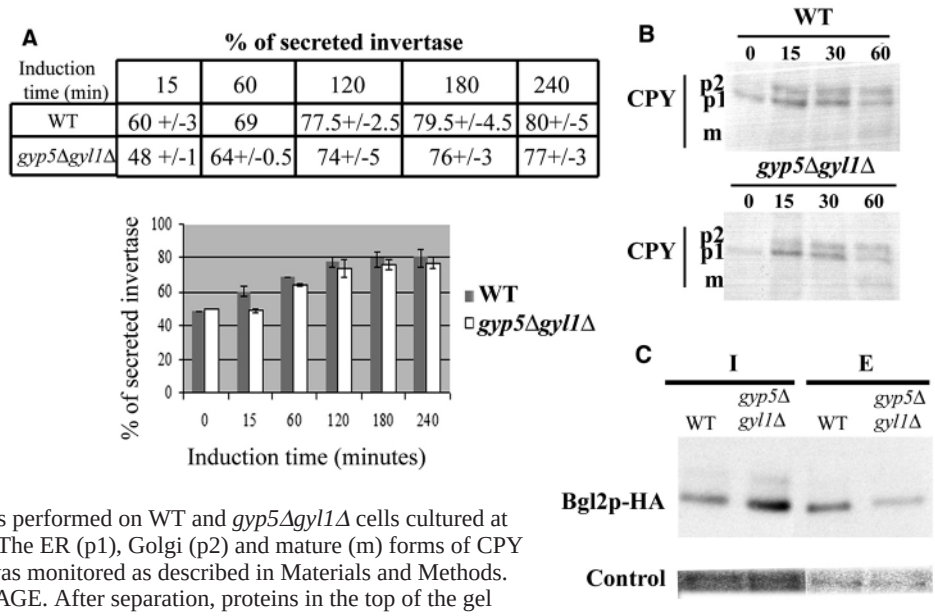

Fig. 8. *gyp5Δgyl1Δ* cells display a distal secretion defect at 13°C. (A) Invertase expression was induced in WT and *gyp5Δgyl1Δ* cells cultured at 13°C, and invertase secretion was monitored as described in Materials and Methods. The % of secreted invertase is the ratio (external invertase/total invertase). Values are the average of three experiments performed on two independent clones. One invertase unit is defined as the enzyme quantity producing 1 μmol of glucose/minute/10^7 cells. (B) Proximal steps of secretory pathway are normal in *gyp5Δgyl1Δ* cells. Pulse-chase labelling and immunoprecipitation of CPY was performed on WT and *gyp5Δgyl1Δ* cells cultured at 13°C, as described in Materials and Methods. The ER (p1), Golgi (p2) and mature (m) forms of CPY are indicated. (C) Bgl2p-HA secretion assay was monitored as described in Materials and Methods. Equal amounts of cells were loaded on SDS-PAGE. After separation, proteins in the top of the gel were stained by Coomassie blue and proteins of the bottom of the gel were transferred onto nitrocellulose membrane. Immunodetection of this membrane with HA antibody was then performed. The control is one protein of the top of the gel stained by Coomassie blue.

control of Sec4p on secretion is exerted through a cyclic mechanism (Walworth et al., 1989). Sec2p was shown to be a nucleotide exchange factor for Sec4p (Walch-Solimena et al., 1997), and Dss4p was identified as a nucleotide release factor (Collins et al., 1997). More recently, it was shown that Sec2p function is regulated by the small GTPases Ypt31/Ypt32, suggesting the existence of a second cycle of regulation (Ortiz et al., 2002).

These two intricated cycles of Sec4p regulation require GAP activities. The results we present here raise the possibility that Gyp5p could act in vivo as a GAP for Sec4p, in association with Gyl1p. We show in this paper that *GYP5* genetically interacts with *SEC2*: overexpression of Gyp5p inhibits the growth of a *sec2-78* strain at 30°C. This result strongly suggests that Sec2p and Gyp5p exert in vivo opposite effects in Sec4p regulation. Moreover, we show that part of Gyp5p and Gyl1p are present in Sec4p-containing complexes, mainly at the plasma membrane. This interaction might allow Gyp5p to exert its GAP activity toward Sec4p.

Recently, Msb3/Gyp3p and Msb4/Gyp4p, two other members of the Gyp family, were shown to act in vivo as GAPs involved in the regulation of exocytosis (Gao et al., 2003). It is therefore interesting to examine the functional relationships of Gyp5p, Gyl1p, Msb3p and Msb4p. Genetics experiments were used to obtain first insights into this question. First, we crossed *gyp5Δgyl1Δ* and *msb3Δmsb4Δ* cells to obtain quadruple deletants: morphological defects, growth retardation and invertase secretion defects appeared equivalent in the *msb3Δmsb4Δ* cells and in the *gyp5Δgyl1Δmsb3Δmsb4Δ* cells (data not shown). This similarity of defects in *msb3Δmsb4Δ* and *gyp5Δgyl1Δmsb3Δmsb4Δ* cells suggests that Gyp5p/Gyl1p and Msb3p/Msb4p are involved in the same biological process. But two lines of evidence indicate that the Gyp5p/Gyl1p pair is not equivalent to the Msb3p/Msb4p pair. As shown by Gao et al., overexpression of Msb3p is able to rescue a *sec4Q79L sec15-1* mutant, possibly by decreasing the level of GTP-bound Sec4p (Gao et al., 2003). We found that overexpression of Gyp5p is not able to rescue growth of the *sec4Q79L sec15-1* strain (data not shown). Moreover, electron microscopy shows an accumulation of vesicles in small-budded *gyp5Δgyl1Δ* cells only, whereas an accumulation of vesicles is found also in large-budded *msb3Δmsb4Δ* cells (Gao et al., 2003) (our own results). Therefore, if Gyp5p, Msb3p and Msb4p act in vivo as GAPs toward Sec4p, these results suggest that the Gyp5p/Gyl1p pair and the Msb3p/Msb4p pair are not fully redundant. Indeed, a strong colocalization of Gyp5p, Gyl1p and Sec4p is seen only at stages of bud emergence, small bud and cytokinesis. This suggests that their function in the control of exocytosis might be restricted to these stages, which are the periods of polarized growth, whereas Msb3p and Msb4p appear to be functional during both polarized and isotropic growth. Also, the full control of Sec4p probably requires a GAP activity toward Ypt31/32, in order to allow a permanent recruitment of the exchange factor Sec2p to the vesicles. Msb3p and Msb4p, which display a GAP activity toward Ypt31/32 in vitro, might act also at this step.

Another puzzling question brought about in this study is the role of Gyl1p, the nearest Gyp5p paralog. Gyl1p displays a typical Ypt/Rab GAP domain, with six shared amino acid motifs (Neuwald, 1997), but it is devoid of the arginine and aspartate residues that were shown to be critical for Gyp1p catalytic activity (Albert et al., 1999; Rak et al., 2000), so that it probably does not display any GAP activity. In this report, co-immunoprecipitation experiments indicated that Gyp5p and Gyl1p associate, mainly at the plasma membrane and on post-Golgi vesicles. Immunofluorescence data indicated that they get separated at the time of isotropic growth. However, although belonging to the same protein complex, Gyp5p and Gyl1p might be involved in distinct cellular processes. But *gyp5Δgyl1Δ* strains display a cold-sensitive slow growth and a reduced secretion, whereas *gyp5Δ* and *gyl1Δ* single mutants are normal. These results support a functional cooperation between Gyp5p and Gyl1p. Therefore, if Gyl1p would possess a GAP activity, it might cooperate directly with Gyp5p GAP activity.

A Gyl1p GAP activity might also be directed towards Ypt31p/Ypt32p. Alternatively, Gyl1p might be devoid of GAP activity, and contribute to spatio-temporal regulation of Gyp5p localization and/or activity. In this case, Gyl1p would be an interesting member of the Gyp family, and its characterization might allow other functions of this family of proteins to be revealed.

Functional significance in vivo of the broad substrate specificity established in vitro is one of the main questions about Gyp proteins. It was shown previously that several Gyps are involved in the regulation of Ypt1p in vivo. Conversely, the results we present here show that Gyp5p displays distinct localizations, and strongly suggest that it could exert its GAP activity in two different biological processes – that is, endoplasmic reticulum to Golgi traffic and polarized exocytosis. Since broad substrate specificity in vitro is a general phenomenon for Gyp proteins, it might be that other Gyp proteins are involved in different biological functions in vivo. Spatio-temporal studies including a combination of imaging and biochemical methods will therefore be necessary to fully understand Gyp localization kinetics, and functions.

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