The Small GTP-binding Protein Rholp Is Localized on the Golgi Apparatus and Post-Golgi Vesicles in \textit{Saccharomyces cerevisiae}

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Abstract. In \textit{Saccharomyces cerevisiae} the ras-related protein Rholp is essentially the only target for ADP-ribosylation by exoenzyme C3 of \textit{Clostridium botulinum}. Using C3 to detect Rholp in subcellular fractions, Rholp was found primarily in the 10,000 g pellet (P2) containing large organelles; small amounts also were detected in the 100,000 g pellet (P3), and cytosol. When P2 organelles were separated in sucrose density gradients Rholp comigrated with the Kex-2 activity, a late Golgi marker. Rholp distribution was shifted from P2 to P3 in several mutants that accumulate post-Golgi transport vesicles during fractionation of P3 organelles from wild-type or sec6 cells. Vesicles containing Rholp were of the same size but different density than those bearing Sec4p, a ras-related protein located both on post-Golgi vesicles and the plasma membrane. Immunofluorescence microscopy detected Rholp as a punctate pattern, with signal concentrated towards the cell periphery and in the bud. Thus, in \textit{S. cerevisiae} Rholp resides primarily in the Golgi apparatus, and also in vesicles that are likely to be early post-Golgi vesicles.

\textbf{G-proteins} are regulatory proteins that bind GTP and exhibit slow GTP hydrolysis activity. The two structural states of G-proteins, GTP bound or GDP bound, are finely regulated in response to specific signals. GTP binding results in structural changes that lead to transmission of a signal, while GTP hydrolysis returns the complex to the GDP bound form, the resting state of G-proteins. By this mechanism G-proteins are thought to serve as molecular switches in many regulatory pathways. G-proteins can be divided into four classes. These are (a) the trimeric G-proteins, responding to extracellular signals, (b) certain soluble components of the protein synthetic machinery involved in translational accuracy, (c) proteins related to the ADP-ribosylation factor ARF, and (d) those related to the ras oncprotein.

The superfAMILY of ras-related proteins (Chardin, 1988) is composed of at least three subfamilies called Ras, Rho, and Ypt/Sec4 (called rab in mammalian cells), the last one being the most diverse group of the three. Approximately 30\% of the amino acids are identical among members of different subfamilies, and the GTP binding site comprises most of the conserved residues (De Vos et al., 1988; Pai et al., 1989).

Another important region of the ras-related proteins is the carboxy-terminal domain known to undergo posttranslational modifications essential for attachment of the protein to a membrane (Willumsen et al., 1984; Clarke et al., 1988; Lowy and Willumsen, 1989; Hancock et al., 1989).

ras-related proteins must serve fundamental roles within cells, because most members of this large family are extremely conserved in evolution. For instance, the yeast proteins Ypt1p, Raslp, and Rholp all have counterparts in mammalian cells that are approximately 70\% identical. The specific functions of ras-related proteins are largely unknown. However, members of the ras subfamily are involved in control of cell growth, and in the yeast \textit{Saccharomyces cerevisiae} this function is achieved by stimulation of adenylate cyclase (Kataoka et al., 1984; Broek et al., 1985). Increasing evidence indicates that members of the ypt family are involved in several steps of intracellular transport (Balch, 1989).

At least three rho proteins exist in human cells, rhoA, rhoB, and rhoC, which are more than 90\% identical to each other (Madaule and Axel, 1985; Yeramian et al., 1987; Chardin et al., 1988). \textit{S. cerevisiae} also contains rho genes; \textit{RHO1} is an essential gene that codes for Rholp, the single yeast counterpart of the human rhoA, rhoB and rhoC proteins; \textit{RHO2} codes for a nonessential protein 53\% identical to Rholp (Madaule et al., 1987), and \textit{CDC42} codes for an essential protein 41\% identical to Rholp (Johnson and Prin-
construction, disruption of actin cables (Chardin et al., 1989; Paterson et al., 1989), treatments suffer alteration of the cytoskeleton, notably a disruption of actin cables (Chardin et al., 1989; Quilliam et al., 1990). Modification by C3 is expected to inactivate rho proteins, since Arn41 is located within the presumed effector site. Mammalian cells subjected to C3 treatment suffer alterations of the cytoskeleton, notably a disruption of actin cables (Chardin et al., 1989; Paterson et al., 1990). This result suggests that one function of rho proteins is to control formation/stability of actin microfilaments.

Genetic analyses of mutations in yeast rho genes also indicate a role of rho proteins in the determination of cell morphology and stability of the actin cytoskeleton. Inactivation of CDC42 or hyperactivation of RHO1 causes similar morphological defects, namely prevention of bud formation, disruption of actin cables, and greatly enlarged cells (Johnson and Pringle, 1990; Johnson, J. S., A. M. Myers, M. McCaffrey, P. Boquet, and P. Madaule, manuscript in preparation). This phenotype is very similar to that caused by conditional alleles of the actin structural gene ACT1 (Novick and Botstein, 1985). To gain further insight into the molecular mechanisms by which rho proteins control the cytoskeleton and cell morphology, the present work was undertaken to determine the location of Rholp within yeast cells.

In the present study we demonstrate that Rholp is by far the most prevalent C3 substrate in yeast, and most likely is the only target of this exoenzyme. Using C3 modification as a means of detection, Rholp was localized primarily to the Golgi apparatus, with a small proportion also found in post-Golgi vesicles, suggesting that Rholp functions at the junction between these two compartments. Immunofluorescence microscopy, performed on yeast, using antibodies directed against Rholp is then likely to depict the Golgi apparatus.

Materials and Methods

Media and Genetic Manipulations

Nonselective media for yeast contained 1% Bacto yeast extract (Difco Laboratories Inc., Detroit, Michigan), 2% Bacto peptone (Difco Laboratories Inc.), and 2% carbon source, either glucose (YPD) or galactose (YPGal). YPD supplemented with 20 mg/liter adenine (YPAD) was used for growth of adenine auxotrophs. Selective media contained 0.7% yeast nitrogen base without amino acids (Difco Laboratories Inc.). 2% glucose, and auxotrophic requirements (uracil, histidine, adenine, leucine, tryptophan) at 20 mg/liter as required. Solid media contained 2% agar. Sporulation medium contained 0.1% glucose and 1% potassium acetate. Dissection of tetrads was performed by standard methods (Sherman et al., 1986). Yeast strains were transformed with 1-5 pg purified linear or circular plasmid DNA as described (Sanger et al., 1977). E. coli strains HB101 and TG-1 were used for amplification of plasmids and/or production of single stranded DNA. Construction of the RHO1 null allele rho1::HIS3 has been described (Madaule et al., 1987). The conditional allele GAL-RHO1 was constructed as follows. The 1.6-kb EcoRI fragment containing the entire RHO1 gene (Madaule et al., 1987) was ligated into plasmid vector YEp352 (Hill et al., 1986), and this recombinant plasmid, pMM102, was linearized 350-bp upstream of the RHO1 coding sequence by digestion with BamHI. The promoter region of RHO1 was digested with exonuclease Bal31, and EcoRI linkers were ligated to the products. Plasmids containing EcoRI inserts of the appropriate size were identified by restriction mapping. One such insert was transferred to pUC18 to form plasmid pREL-6. Nucleotide sequence analysis of this plasmid revealed that the upstream EcoRI site was located six nucleotides from the RHO1 initiation codon (5'GATTCCTCGAGAAATGATG..-3'). The EcoRI fragment was then ligated into the unique EcoRI site of the centromeric plasmid pMD100 (Johnston and Davis, 1984), which is located in the GAL10 promoter eight nucleotides upstream of the GAL10 initiation codon. The resultant allele is called GAL-RHO1, and the plasmid is pMM105, which bears the URA3 marker gene.

Strain MM50 was constructed as follows. The RHO1/rho1::HIS3 heterozygous diploid W303 - RHO1 (Madaule et al., 1987) was initially transformed with pMM105. The resulting strain was induced to sporulate, and products were separated on YGal, and colonies were identified that required galactose for growth. As expected, these colonies were both histidine independent (rho1::HIS3) and uracil independent (pMM105). The genotype of one such strain, denoted MM50, was verified by Southern blot analysis (data not shown).

Strain JII was constructed by integration of GAL-RHO1 into the genome of wild-type strain W303. A BamHI-EcoRI partial digestion fragment containing GAL-RHO1 was prepared from pMM105 and ligated to the integrative plasmid vector Yip352 (Hill et al., 1986) to form plasmid pMM107. This plasmid was linearized by digestion at the unique Ncol site within URA3, and used to transform strain W303 to uracil independence. Southern hybridization analysis confirmed that GAL-RHO1 had integrated in the URA3 locus, as a tandem repeat of 5-10 copies (data not shown).

Subcellular Fractionation Methods and Enzyme Assays

Yeast cells were fractionated essentially as described by Goud et al. (1988). Lysis buffer contained 10 mM triethanolamine pH 7.2, 0.8 M sorbitol, 1 mM phenylmethylsulphonylfluoride, and a cocktail of protease inhibitors (leupeptin, chymostatin, pepstatin, and antipain; Sigma Chemical Co., St. Louis, MO). EDTA was omitted from the lysis buffer of Goud et al. (1988) because of its inhibitory effect on the efficiency of Rholp labeling by C3. For C3 labeling of particulate fractions, PI, P2, and P3 were resuspended in lysis buffer so that equal volumes of all differential centrifugation fractions contained the equivalent number of cells. Fractionation of P3 pellets by Sephacryl S-1000 gel filtration chromatography was as described (Walworth and Novick, 1987). The procedure of Ruohola and Ferro-Novick (1987) was used for fractionation of P2 or P3 organelles in the site of the density gradient. NADPH cytochrome c reductase, vanadate sensitive Mg2+ ATPase, cytochrome c oxidase, and z-mannosidase were assayed in the various fractions as described (Ruohola and Ferro-Novick, 1987; Walworth and Novick, 1987). Ca2+-dependent GPDase (cdGPDase) was assayed by the method of Abejon et al. (1989). Ket-2 activity was measured as described by Cunningham and Wickner (1989).

[32P]JADP-Ribosylation of Rholp by Exoenzyme C3

Exoenzyme C3 was prepared from the culture supernatant of C. botulinum strain 1873-D as described (Rubin et al., 1988). C3 labelling reactions containing 5 µl of labeling mixture (60 mM Hepes pH 8.0, 1 mM MgCl2, 100 mM AMP, 2.5 mM [32P]NAD1 [50 Ci/mmol; DuPont-NEN, Cambridge, MA], 0.3 µg/ml exoenzyme C3) and 5-15 µl of yeast fraction were incubated for 1 h at 37°C. The yeast fractions were total yeast lysates, supernatants SI, S2, and S3, pellets PI, P2, and P3 suspended in spheroplast lysis buffer (or suspended in and dialysed against lysis buffer), sucrose gradient fractions, or Sephacryl S-1000 gel filtration fractions. After the reactions, proteins were denatured and separated by SDS-PAGE in 15% acrylamide. Gels were stained with Coomassie blue, destained, dried, and subjected to autoradiography. Radioactive bands were excised from the gel and cpm in each band were measured by scintillation counting. NADase activity in each sample was assayed by measuring the hydrolysis of [32P]NAD followed by TLC on cellulose plates to identify the reaction products.
Table 1. Yeast Strains

| Strain | Genotype | Source |
|--------|----------|--------|
| MM50   | Mat α, ura 3, leu 2, his 3, trp 1, ade 2, can 1, rhol::HIS3 | This study |
| aW303  | Mat a, leu2, his3, ura3, trpl, ade2 (URA 3, CEN 4, gal RHO I) | R. Rothstein |
| J11    | Mat a, leu 2, his 3, trp 1, ade 2, can 1, URA 3::gal RHO1+ | This study |
| NY 13  | Mat a, ura 3-52 | P. Novick |
| NY 3(secl-1)† | Mat a, ura 3-52, sec1 | P. Novick |
| NY 130(sec2-56)† | Mat a, ura 3-52, sec2 | P. Novick |
| NY 412(sec3-2)† | Mat a, ura 3-52, sec3 | P. Novick |
| NY 405(sec4-2)† | Mat a, ura 3-52, sec4 | P. Novick |
| NY 176(sec7-1)† | Mat a, ura 3-52, sec7-1 | P. Novick |
| NY 410(sec8-1)† | Mat a, ura 3-52, sec8 | P. Novick |
| NY 430(sec14-3)† | Mat a, ura 3-52, sec14 | P. Novick |
| NY 64(sec15-1)† | Mat a, ura 3-52, sec15 | P. Novick |
| NY 432(sec18-1)† | Mat a, ura 3-52, sec18-1 | P. Novick |

* Approximately 10 copies of GAL-RHO, are integrated at the URA3 locus.
† These strains are called respectively sec1, sec2, sec3, etc. in text.

Production and Affinity Purification of Anti-Rholp Antibodies

Polyclonal antisera were raised in rabbits injected with a β-anthranilate (component I) synthase-Rholp fusion protein produced in E. coli. Using the Bal31 digestion method described above, an EcoRI site was placed within the coding region of RHO1, 27 nucleotides downstream from the ATG initiation codon. The resultant EcoRI fragment, containing most of the RHO1 coding sequence, was ligated in frame into the E. coli gene trpE of the plasmid vector pATH1 (Koerner et al., 1990). The trp promoter of the recombinant plasmid was induced as described (Koerner et al., 1990), the fusion protein was size fractionated by SDS-PAGE, and acrylamide strips containing this protein were crushed in complete Freund's adjuvant and injected subcutaneously into New Zealand white rabbits. Immunoblots were performed as described (Schmidt et al., 1984).

Immunofluorescence Microscopy

Cells were grown to early log phase in YPD medium and fixed by addition of formaldehyde (3.7% final concentration) directly to the culture. Cells were then treated for immunofluorescence microscopy as described (Adams and Pringle, 1984; Pringle et al., 1989), using FITC-conjugated goat anti–rabbit IgG (Sigma Chemical Co.) as the second antibody. Anti–Rholp was bound to the cells at a final concentration of 50 μg/ml in PBS, 0.2% Triton X-100, 0.1% BSA. Preadsorption of anti–Rholp was performed as follows. Appropriate cells were washed once and resuspended in H2O (1 ml/g wet weight cells). The suspension was boiled for 40 min, chilled on ice, adjusted to pH 7.3 by addition of NaOH, and adjusted to PBS by addition of a 20x PBS stock solution (0.025 ml per 1.0 ml lysate). A sample of the boiled cell suspension was diluted 10-fold in PBS, 0.2% Triton X-100, 0.1% BSA, and anti–Rholp was added to a final antibody concentration of 40 μg/ml. The mixture was incubated for 16 h at 4°C with gentle agitation, after which insoluble cell material was separated from the supernatant by three successive 5 min spins at 12,000 g. The preabsorbed antibody solution was then added to a pellet of cells fixed for immunofluorescence analysis, and the remainder of the staining and visualization procedures was performed by the standard method.

Results

C3 Detects Rholp in S. cerevisiae

ADP-ribosylation of Rholp by C3 was examined in totally lysed cells from the yeast cultures. Rholp was detected by western blot analysis using antibodies specific for Rholp. Anti-Rholp antibodies were affinity purified from the crude supernatant by affinity purification using β-galactosidase-Rholp fusion protein produced in E. coli. The affinity-purification matrix was prepared by coupling anti-β-galactosidase antibodies (Sigma Chemical Co.) to cyanogen bromide activated Sepharose CL-4B (Pharmacia, Uppsala, Sweden), as described by the manufacturer. The affinity-purification matrix was prepared by coupling anti-β-galactosidase antibodies (Sigma Chemical Co.) to cyanogen bromide activated Sepharose CL-4B (Pharmacia, Uppsala, Sweden), as described by the manufacturer. The affinity-purification matrix was prepared by coupling anti-β-galactosidase antibodies (Sigma Chemical Co.) to cyanogen bromide activated Sepharose CL-4B (Pharmacia, Uppsala, Sweden), as described by the manufacturer. The affinity-purification matrix was prepared by coupling anti-β-galactosidase antibodies (Sigma Chemical Co.) to cyanogen bromide activated Sepharose CL-4B (Pharmacia, Uppsala, Sweden), as described by the manufacturer. SDS-PAGE gels for immunoblotting using anti-Rholp were run with 8 M urea included in the gel. These gels were transferred to nitrocellulose filters and probed with anti–Rholp as described (Schmidt et al., 1984).
The localization of Rholp in *S. cerevisiae* was studied initially by subcellular fractionation of wild-type strain NY13. Cells in late log phase (200 Aboo units) were converted to spheroplasts and lysed osmotically. Lysates were spun at 450g to collect unlysed cells and nuclei, yielding the P1 pellet and S1 supernatant. S1 was then centrifuged at 10,000 g yielding the P2 pellet, enriched in large organelles, and S2 supernatant. Finally, centrifugation of S2 at 100,000 g gave rise to the S3 cytosol fraction and P3 pellet, enriched in small organelles. Each subcellular fraction (S1, P1, S2, P2, S3, P3) was labeled by ADP-ribosylation with C3 (either before or after dialysis to remove endogenous NAD) separated by SDS-PAGE, visualized by autoradiography, and spots quantitated by scintillation counting. All subcellular fractions were also assayed for their enzyme marker content, namely, vanadate sensitive Mg++ ATPase (Bowman and Slayman, 1979; Willsky, 1979) for the plasma membrane; Ca++-dependent GDPase (cdGDPase) (Abeijon et al., 1989) for the Golgi apparatus and Kex-2 as a late Golgi marker (Cunnigham and Wickner, 1989; Julius et al., 1984). As shown in Table I, taking values in the S1 fraction as 100%, 76% of Rholp is present in P2 and 9% in P3. A significant signal is observed in P1, but since this fraction contains unbroken cells and nuclei it was not included in partition calculations. A signal is also present in S3 (16%), indicating that a proportion of Rholp is cytosolic. The fact that the sum of Rholp detected in P2 + S2 equals the S1 value indicates that the C3 ADP-ribosylation of Rholp is a quantitative method. Kex-2 marker was found to distribute primarily in the P2 fraction. Plasma membrane ATPase and cdGDPase show an equal distribution between S2 and P2. A substantial proportion (44%) of the PM ATPase was found in the cytosolic S3 fraction due probably to solubilization of this enzyme from the particulate form during fractionation.

Rholp was also detected by Western blot analysis using affinity-purified antibodies reactive with Rholp (anti-Rholp; see Materials and Methods). Induction of the GAL-RHO1 gene in galactose medium resulted in a strong increase in abundance of a 23-kD protein recognized by anti-Rholp (Fig. 2A), indicating that the antibodies detect Rholp in total cell extracts. Differential centrifugation fractions from wild-type yeast were analyzed by Western blot (Fig. 2B). In agreement with the C3 labeling results described above, the majority of Rholp was detected in P2 with a somewhat smaller amount observed in S2. A minor signal was also de-

### Table II. Percent Rholp and Marker Distribution in Wild-type Yeast NY13

| Fraction | Rholp | PM ATPase | cdGDPase | KEX-2 |
|----------|-------|-----------|----------|-------|
| S1       | 100   | 100       | 100      | 100   |
| P2       | 76    | 38        | 47       | 82    |
| S2       | 21    | 60        | 57       | 41    |
| P3       | 9     | 15        | 41       | 40    |
| S3       | 16    | 44        | 0        | 41    |
| Total for |       |           |          | 123   |

P2 + S2 97

89

Estimation of Rholp content by C3 ADP-ribosylation was identical after dialysis to remove endogenous NAD. Weak NADase activity could be detected in S1, S2, and S3 fractions.
Figure 2. Detection of Rhop by Western blot analysis. (A) JJi cells were grown to mid-log phase (0.5-1.0 × 10⁸ cells/ml) in liquid YPD medium. Cells were then pelleted from the medium, washed once in YPGal, resuspended in YPGal at the original cell concentration, and returned to a shaker at 30°C. Samples of the culture were removed at various times thereafter, and total protein extracts were prepared and separated by SDS-PAGE in the presence of 8 M urea (1014g protein/lane). The proteins were transferred to nitrocellulose filters, and probed with anti-Rhop. (B) Differential centrifugation fractions were prepared from wild-type strain AW303 and analyzed as in A. Extracts from an equivalent number of cells were loaded in each lane, with 30 μg protein loaded for the S1 fraction.

Rhop accumulates on Post-Golgi Vesicles in certain Secretion-defective Mutants

Temperature-sensitive yeast mutants defective in specific steps of the protein transport pathway from the ER to the cell surface (sec mutants; see Table I) were used to characterize further the association of Rhop with the Golgi apparatus. Three mutants were analyzed in detail both by differential centrifugation and equilibrium density gradient fractionation. These are sec18, blocked early in secretion (Novick et al., 1980); sec7, blocked at a step in the Golgi apparatus; and sec6, blocked late in the secretory pathway and known to accumulate post-Golgi vesicles (Novick et al., 1981). Cells were grown to late log phase at 25°C in YPD (2% glucose), then shifted to YP medium (0.1% glucose) for 2 h at the non-permissive temperature 37°C. Secretion is blocked at 37°C because of the sec mutation, while the decrease in glucose concentration induces synthesis of invertase; thus, the secretory block can be followed by assaying invertase activity. In control experiments, growth of wild-type cells under these conditions did not affect the observed distribution of Rhop in differential centrifugation fractions, nor its migration during sucrose density gradient fractionation (data not shown).

Differential centrifugation fractions were prepared from the three mutants as described above, and Rhop abundance in each fraction was determined by C3 labeling. In all three mutants the total amount of Rhop in the particulate fractions (ie., P2 + P3) was about the same as that found in wild-type cells (Table III). Migration of Rhop and the enzymatic markers during density gradient fractionation of P2 from sec6, sec18, or sec7 was very similar to that shown for wild-type cells in Fig. 3 (data not shown). In sec18 and sec7, the distribution of Rhop between P2 and P3 was similar to wild-type (Table III). However, in the sec6 mutant a large increase in the abundance of Rhop was found in the P3 fraction, with a concomitant decrease in P2 (Table III). This result suggests that Rhop is located on post-Golgi vesicles as well as in the Golgi apparatus.

To test this hypothesis, P3 fractions from sec6 were fractionated further by centrifugation to equilibrium in sucrose density gradients. Each gradient fraction was assayed for (a) Rhop, (b) invertase, which accumulates in post-Golgi vesicles in the thermosensitive mutant strain sec6 (Novick et al.,
and (c) Sec4p, a marker of post-Golgi vesicles (Goud et al., 1988). Sec4p was identified in the gradient fractions by Western blot analysis. The invertase peak lies just between the peaks of Rholp and Sec4p consistent with a post-Golgi vesicle localization for these proteins (Fig. 4). Rholp clearly migrated to a slightly lighter density than that of Sec4p (Fig. 4), suggesting that these two proteins are associated with different kinds of secretory vesicles. The density of Rholp-bearing vesicles was identical in sec6 and N73 (data not shown).

Material in the P3 pellet of sec6 also was fractionated on the basis of size using gel exclusion chromatography (Walworth and Novick, 1987). Fractions were assayed as described above for invertase, cdGDPase, Kex-2 activity,
Rholp, and Sec4p, as well as for total protein concentration. The post-Golgi vesicle marker invertase eluted from the column in a peak reaching a maximum at fraction 24 (Fig. 5). The Rholp elution profile followed exactly that of invertase which suggests that Rholp is indeed bound to post-Golgi vesicles. The Kex-2 peak was shifted slightly towards the smaller size range compared to that of both Rholp and Sec4p (Fig. 5).

Since the normal subcellular location of Rholp is altered in sec6, other secretory mutants known to accumulate post-Golgi vesicles were examined for the distribution of Rholp in the S1, S2, P2, S3, and P3 fractions. P1, P2, and P3 pellets were prepared as described, except that 40 OD units of cells were used for each strain. Invertase activity was determined in the P2 and P3 fractions from each mutant, demonstrating that in all cases an efficient secretory block had been obtained (data not shown). C3 labeling indicated that, in addition to sec6, the sec2, and sec4 mutants also underwent a major redistribution of Rholp to the P3 pellet (Table IV). A less striking redistribution between P2 and P3 was also observed in the sec8, sec15, and sec1 mutants. The secretion

Table III. Percent Rholp Distribution in Mutants of the Secretory Pathway

| Strain       | Secretony block point | Fraction | S1  | S2  | P2  | S3  | P3  |
|--------------|-----------------------|----------|-----|-----|-----|-----|-----|
| NY 13(WT)    | None                  |          | 100 | 21  | 80  | 9   | 12  |
| sec18        | ER to Golgi           |          | 100 | 22  | 78  | 10  | 16  |
| sec7         | Golgi                 |          | 100 | 28  | 71  | 4   | 24  |
| sec6         | Golgi to PM           |          | 100 | 47  | 47  | 9   | 43  |

Figure 4. Analysis of Rholp and Sec4p in P3 sucrose density gradients of sec6. P3 pellets were prepared by differential centrifugation from sec6 strain (shifted to 37°C in 0.1% glucose) and resuspended in 2 ml of 60% sucrose and loaded on the bottom of a 35–60% sucrose gradient. Rholp was assayed by C3 ADP-ribosylation, 15% SDS-PAGE analyses, and autoradiography using 15-μl aliquots as described in Materials and Methods. Sec4p was monitored by Western blot and quantified by measurement of radioactive spots. Invertase was measured as described previously (Goldstein and Lampen, 1975). Sucrose density is expressed in percent values.

Figure 5. Rholp bearing vesicles are of the same size as invertase containing vesicles from sec6 strain. 35 4-ml fractions were collected from Sephacryl S-1000 column as described in Materials and Methods. Each fraction was assayed for Rholp, Ca++-dependent GDPase, Sec4p, invertase, and protein content (Bradford). The results are expressed as cpm or OD.
block in sec3, as measured by invertase accumulation was only twice that of the wild-type strain. Thus, a redistribution of Rholp to the vesicle fraction is observed in at least six out of seven mutants known to accumulate post-Golgi vesicles.

**Localization of Rholp by Immunofluorescence Microscopy**

Anti-Rholp was used to detect Rholp in wild-type strains NY13 and aW303 by indirect immunofluorescence microscopy. Fig. 6 (A and B) shows that the antibodies detect a pattern of dots which appear to be concentrated towards the periphery of the cell. As a control experiment to test specificity of this staining pattern, anti-Rholp was preadsorbed with yeast lysates from cells that either were depleted of Rholp or overaccumulated this protein (see Materials and Methods). As shown in Figs. 1 and 2, growth of strain MM50 in glucose medium resulted in depletion of Rholp to trace levels, while growth of strain JJ1 in galactose medium caused accumulation of Rholp to very high levels. Preadsorption of the antibodies with glucose-grown MM50 cell material did not alter the immunofluorescence staining pattern from that found with untreated antibodies (Fig. 6D). However, preadsorption with the same concentration of galactose-grown JJ1 cell material completely eliminated the dotted staining pattern (Fig. 6C). Therefore, the dots appear to be specific to Rholp, and not due to nonspecific interactions such as antibodies adhering to the cell wall.

**Discussion**

Exoenzyme C3 from *C. botulinum* strains C and D is known to ADP-ribosylate the mammalian GTP binding proteins rhoA and rhoC, and presumably also modifies rhoB (Charadin et al., 1989). Amongst the three rho proteins known in *S. cerevisiae*, Rholp, Rhop2p, and Cdc42p, the former is by far the most closely related to rhoA, rhoB, and rhoC, with identities at ~70% of the amino acids (Madaule et al., 1987). Therefore, Rholp was expected to be the 23-kD protein previously identified as a substrate of C3 in this organism (Charadin et al., 1989). A recent report (Didsbury et al., 1989) suggests that C3 also modifies two newly discovered human proteins related to rho, termed rac1 and rac2 (rac stands for “ras substrate for C3”). Rac proteins share 70% identities with the Cdc42p protein of *S. cerevisiae* (Johnson and Pringle, 1990) and thus may be considered as the mammalian counterparts of this yeast protein. Thus, Cdc42p was also expected to be modified by C3. However, almost no ADP-ribosylation by C3 was observed in strain MM50 grown in conditions where expression of the RHO1 coding sequence is repressed, demonstrating that Rholp is the sole detectable substrate for C3 in yeast. Other substrates, if they exist, are either present at very low levels compared to Rholp, or are very poor substrates for C3 in our experimental procedure.
In any case, C3-mediated ADP-ribosylation is a reliable means of assaying Rholp in yeast lysates.

Using C3 to measure the abundance of Rholp in subcellular fractions, most of the Rholp in wild-type yeast appeared to be located within the Golgi apparatus. In support of this observation a small GTP binding protein ADP-ribosylated by a preparation defined as "botulinum toxin" has been shown to be associated with the Golgi apparatus in rat liver cells (Toki et al., 1989). Rholp was detected primarily in the P2 pellet which is enriched in large organelles, and in density gradient fractionation of these organelles Rholp was found to colocalize with Kex-2 activity, a late Golgi marker. Rholp in the density gradients was not associated with enzymatic markers of mitochondria, vacuole, plasma membrane, or endoplasmic reticulum. Mutations that cause accumulation of secretory vesicles, derived from the Golgi, result in the redistribution of Rholp to the vesicle fraction. These observations suggest strongly that Rholp resides in the Golgi apparatus. The data do not rule out, however, that a minor fraction of Rholp may also be associated with another membrane in addition to the Golgi.

About 9% of the particulate Rholp in wild-type cells was detected in the P3 pellet, which is enriched in small organelles. For the following reasons the Rholp-bearing material appeared to be an authentic vesicle, as opposed to broken Golgi that contaminates P3. The Rholp-bearing organelle in P3 has a size similar to that of secretory vesicles. In the secretory mutants sec2, sec4, sec6, sec8, and sec15, grown in conditions where they accumulate post-Golgi vesicles, a large amount of Rholp was found in the P3 pellet. Conceivably, these sec mutations could alter the Golgi apparatus indirectly, so that partial breakage of this organelle occurs during fractionation. However, artificial breakage of the Golgi apparatus is unlikely to account for the significant increase in Rholp found in the P3 fractions of sec2, sec4, or sec6. In addition, the vesicle bearing Rholp that accumulates in sec6 has precisely the same elution profile as the invertase containing vesicle. Thus, Rholp appeared to be bound to a physiological vesicle that accumulates in these late secretory mutants.

What is the nature of the Rholp-bearing vesicles? The most likely explanation is that these are post-Golgi vesicles, since they accumulate in some late secretory mutants. However, not all of the post-Golgi sec mutants exhibit the same shift of Rholp from P2 to P3. The precise defects leading to accumulation of post-Golgi vesicles are not known, however, it is possible that some sec genes, code for proteins that function shortly after secretory vesicles leave the Golgi apparatus (early post-Golgi vesicles). These mutations could also affect the localization for example of Rholp on early post-Golgi vesicles.

A second possibility is that Rholp resides entirely within the Golgi apparatus, and that Rholp-bearing vesicles are involved in transitions between Golgi saccules. However, the fact that Rholp accumulates in the vesicular fraction of certain late secretory mutants along with Sec4p and invertase, favors the interpretation that Rholp-bearing vesicles are post-Golgi vesicles.

A model for Rholp localization is depicted in Fig. 7. A substantial amount of Rholp would reside on trans-Golgi membranes. A small proportion of the Rholp pool would leave this organelle bound to membranes, as part of the early post-Golgi vesicles. In what could be envisaged as a maturation process, Rholp would return to the Golgi apparatus, possibly as a cytosolic intermediate, while the post-Golgi vesicles would acquire their final size and density, and would bear Sec4p. This model is consistent with the fact that Rholp is located both in the Golgi apparatus and in post-Golgi vesicles, while Sec4p resides both in post-Golgi vesicles and the plasma membrane (Goud et al., 1988). This model also explains the slightly different physical characteristics observed for the Rholp- and Sec4p-bearing vesicles. The possibility that vesicles leaving the trans-Golgi are different from those arriving at the plasma membrane has already been hypothesized (Payne and Schekman, 1989). According to one model proposed by Payne and Schekman (1989) clathrin coated vesicles leave the trans-Golgi and form a retrieval compartment for Golgi enzymes. Secretory vesicles could be generated from this retrieval compartment and sent to the plasma membrane. The data does not distinguish between the possibilities that Rholp and Sec4p are located on entirely distinct populations of vesicles, or, alternatively, whether some intermediate vesicles contain both proteins.

Immunofluorescence of wild-type yeast was performed using affinity-purified polyclonal antibodies directed against Rholp. A pattern of small dots was observed at the periphery of the cell, with more intense labeling in the bud. Since wild-type cells were used in this analysis, most of the Rholp is expected to be in the late Golgi apparatus. Very little information is available from the literature about the structure of the Golgi apparatus in S. cerevisiae. Immunofluorescence detection of the Golgi protein Yptlp (Segev et al., 1988) revealed a pattern similar to that of Rholp, except that the Yptlp dots are larger, and are located further inside the cell. However, Yptlp is likely to reside on the cis-Golgi (Bacon et al., 1989; Baker et al., 1990). The anti-Rholp immunofluorescence result, taken together with the subcellular fractionation data, suggest that the Golgi of S. cerevisiae consists of small saccules located close to the periphery of the cell.

An important question is to relate the subcellular location of Rholp to its function. Secretion of invertase was assayed in a rhol mutant strain, and no striking differences were observed either in the internal or external pools of enzyme (our unpublished observations). Therefore, Rholp appears to function in a process other than enzyme export. Previous studies suggested that the mammalian counterparts of Rholp are needed for assembly of actin filaments (Chardin et al., 1989; Paterson et al., 1990). In S. cerevisiae, rhol mutants (Johnson, J. S., A. M. Myers, M. McCaffrey, P. Bouquet, and P. Madaule, manuscript in preparation) appeared very similar to a group of cell division cycle mutants, including cdc24.
cd43, and the rho-related gene cd42 (Pringle and Hartwell, 1981; Sloot et al., 1981). All of these conditional mutants are unable to form buds, develop into greatly enlarged cells, and display abnormalities in the cytoskeleton typical of some actin mutants. Location of Rho1 in the Golgi apparatus is not surprising, given that it functions in bud formation. Most secretory vesicles are directed toward the site of bud formation, to build additional plasma membrane. The Golgi and post-Golgi vesicles are likely organelles to regulate the structure of newly synthesized plasma membrane, which should develop into a bud only when required. Bud formation could be regulated by modifying the structure and/or composition of integral membrane proteins in future plasma membrane, which already are present in post-Golgi vesicles. This process could include a modification of the nascent bud site required for attachment of actin microfilaments, thus explaining the observed effect of rho proteins on actin structure.

This work is dedicated to the memory of our friend D. Michael Gill.

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