The Small GTP-binding Protein Rholp Is Localized on the Golgi Apparatus and Post-Golgi Vesicles in *Saccharomyces cerevisiae*

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Abstract. In *Saccharomyces cerevisiae* the ras-related protein Rholp is essentially the only target for ADP-ribosylation by exoenzyme C3 of *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 vesicles. Rholp comigrated with 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 *S. cerevisiae* Rholp resides primarily in the Golgi apparatus, and also in vesicles that are likely to be early post-Golgi vesicles.

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 oncoprotein.

The superfamily of ras-related proteins (Chardin, 1988) is composed of at least three subfamilies called Ras, Rho, and Ypt/Sec 4 (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 posttranslation 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 Yptlp, 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 *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). *S. cerevisiae* also contains rho genes; *RHO1* is an essential gene that codes for Rholp, the single yeast counterpart of the human rhoA, rhoB and rhoC proteins, *RHO2* codes for a nonessential protein 53% identical to Rholp (Madaule et al., 1987), and *CDC42* codes for an essential protein 41% identical to Rholp (Johnson and Prin-

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gle, 1990). Recently rhoA, rhoC, and presumably rhoB, were shown to be substrates of exoenzyme C3 from *Clostridium botulinum* (Kikuchi et al., 1988; Morii et al., 1988; Braun et al., 1989; Chardin et al., 1989; Quilliam et al., 1989), which catalyses the transfer of ADP-ribose from NAD^+ to a conserved asparagine residue at position 41 of rhoA (Sekine et al., 1989). Modification by C3 is expected to inactivate rho proteins, since Asn41 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 μg purified linear or circular plasmid DNA as described (Sherman et al., 1986).

Nucleic Acid Manipulations

Recombinant DNA manipulations were performed using standard procedures (Ausubel et al., 1989; Sambrook et al., 1989). Oligonucleotides were synthesized by the Iowa State University Nucleic Acid Facility (Ames, Iowa) using a Biosearch 8750EX automated DNA synthesizer. Oligonucleotide directed site-specific mutagenesis was by the method of Eckstein (Taylor et al., 1985), using a commercially supplied kit (Amersham Corp., Arlington Heights, IL). Nucleotide sequence analysis was by the chain termination method (Sanger et al., 1977). *E. coli* strains HB101 and TG-1 were used for amplification of plasmids and/or production of single stranded DNA.

Allele and Strain Construction

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'-GAATTCCAGAAA-GATG...-3). The EcoRI fragment was then ligated into the unique EcoRI site of the centromeric plasmid pBM150 (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, meiotic products were separated on YPGal, 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 JJ1 was constructed by integration of *GAL-RHO1* into the genome of wild-type strain aW303. 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 NcoI site within *URA3*, and used to transform strain aW303 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 phenylmethyl sulphonylfluoride, 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, P1, P2, and P3 were resuspended in lysis buffer so that equal volumes of all differential centrifugation fractions contained the extract from an 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 sucrose density gradients. NADPH cytochrome c reductase, vanadate sensitive Mg^{++} ATPase, cytochrome c oxidase, and α-mannosidase were assayed in the various fractions as described (Ruohola and Ferro-Novick, 1987; Walworth and Novick, 1987). Ca^{++}-dependent GDPase (cdGDPase) was assayed by the method of Abeijon et al. (1989). Kex-2 activity was measured as described by Cunningham and Wickner (1989).

[^{32}P]ADP-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 $MgCl_2$, 1 mM AMP, 2.5 mM [^{32}P]NAD [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 S1, S2, and S3, pellets P1, 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 [^{32}P]NAD followed by TLC on cellulose plates to identify the reaction products.

Table I. Yeast Strains

Strain	Genotype	Source
MM50	Mat α, *ura 3, leu 2, his 3, trp 1, ade 2, can 1, rho1::HIS3*	This study
aW303	*Mat a, leu2, his3, ura3, trp1, ade2 (URA 3, CEN 4, gal RHO 1)*	R. Rothstein (Columbia University, New York, NY)
JJ1	*Mat a, leu 2, his 3, trp1, ade 2, can 1, URA 3::gal RHO1**	This study
NY 13	*MAT a, ura 3-52*	P. Novick (Yale University, School of Medicine, New Haven, CT)
NY 3(*sec1-1*)‡	*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 17(*sec6-4*)‡	*MAT a, ura 3-52, sec6-4*	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 α, ura 3-52, sec18-1*	P. Novick

* Approximately 10 copies of GAL-RHO_1 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. Injections of antigen in incomplete Freund's adjuvant were repeated after 4, 6, and 11 wk. Blood samples were taken 1 and 2 wk after the latter two injections.

Antibodies specific for Rholp were purified from the crude supernatant by affinity chromatography using β-galactosidase-Rholp fusion protein produced in *E. coli*. To produce this fusion protein, a BglII site was created by site-specific mutagenesis immediately following the ATG initiation codon of *RHO1*. A DNA fragment extending from this site to a second BglII site downstream of *RHO1* (Madaule et al., 1987) was ligated in-frame to the BamHI site of plasmid vector pUR292 (Ruther and Muller-Hill, 1983). This latter site is located within the carboxy-terminal coding region of the *E. coli* gene *lacZ*. The *lacZRHO1* gene fusion was induced from the *lac* promoter and a total cell lysate was prepared (Carroll and Laughon, 1987). 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. These beads were used to bind β-galactosidase-Rholp fusion protein from the cell lysate, and washed extensively in 10 mM KPO_4 pH 7.2, 150 mM NaCl (PBS). Anti-β-galactosidase antibody and bound fusion protein were then chemically crosslinked (Carroll and Laughon, 1987). Crude antiserum from rabbits immunized with β-anthranilate (component I) synthase-Rholp was passed over this affinity matrix, the column was washed extensively with PBS, and bound antibodies were eluted in 4 M guanidine-HCl and dialysed against PBS. This antibody fraction is denoted anti-Rholp.

Immunoblotting

Antibodies against Sec4p and their use in Western blot analysis were as described by Goud et al. (1988) using [^{125}I]protein A (Amersham, Les Ulis, France). 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).

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 H_2O (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 20× 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 preadsorbed 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 total lysates from cells that express *RHO1* at various levels. Strain MM50 (Table I) contains only one functional *RHO1* coding sequence, which is under control of the *GAL10* promoter (*GAL-RHO1*). Growth in galactose medium (YPGal) is expected to cause induction of *GAL-RHO1*, resulting in accumulation of Rholp at high levels compared to wild-type cells. Conversely, growth of MM50 in glucose medium (YPD) should cause repression of *GAL-RHO1* and subsequent depletion of Rholp. Indeed, in YPD medium MM50

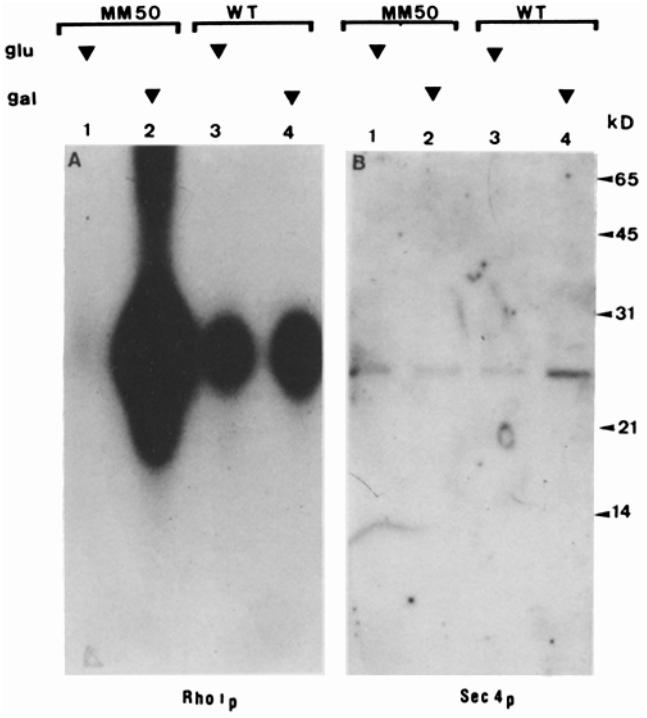

Figure 1. Rholp is the major substrate for C3 in yeast. MM50 or NY13 (*WT*) strains were grown on rich medium either with 2% glucose or 2% galactose on an OD_{600} nm = 1. Spheroplasts were prepared from 4 A_{600} units of culture, resuspended in 100 μl of lysis buffer. Lysates were obtained after four cycles of freeze–thawing. Aliquots (15 μl) were ADP-ribosylated by C3 as described in Materials and Methods and analyzed by 15% SDS-PAGE and autoradiography. 20-μl aliquots of the same preparations were also electrophoresed on 15% SDS-PAGE and transferred to nitrocellulose which was incubated with rabbit anti–Sec4p antibodies (diluted 1:1,000). The Sec4p was then detected by incubation of the Western blot with [^{125}I]protein A. (*Rholp*) ADP-ribosylation by C3. (*Sec4p*) Sec4p Western blot. Lanes *1* and *2* correspond to the MM50 strain and lanes *3* and *4* to the wild-type strain. Preparations in lanes *1* and *3* were grown in media with glucose as carbon source whereas those in lanes *2* and *4* were grown in media with galactose as carbon source. The autoradiogram was deliberately overexposed in order to detect other possible substrates for C3 in lysates. Molecular mass standard sizes are indicated in kilodaltons.

divides about five times and then ceases growth (data not shown), as expected from the finding that *RHO1* is an essential gene (Madaule et al., 1987). Addition of C3 to wild-type cell extracts in the presence of [^{32}P]NAD$^+$ resulted in transfer of ADP ribose to a protein that migrated as a single radioactive band of apparent molecular weight 23,000, the predicted size of Rholp (Fig. 1, *Rholp*, lanes *3* and *4*). MM50 cells grown in galactose displayed a 20-fold increase in labeling of the 23-kD substrate as compared to wild-type cells, while MM50 cells grown in glucose showed only a trace level of the C3 signal (Fig. 1, *Rholp*, lanes *1* and *2*). As a control, Western blot analysis showed that another ras-related protein, Sec4p (Salminen and Novick, 1987), is present at about the same levels in wild-type or MM50 cells grown either in galactose or glucose (Fig. 1, *Sec4p*). The data indicate that Rholp is the only detectable substrate of C3 in *S. cerevisiae*.

Rholp Is Located Primarily in the Golgi

The localization of Rholp in *S. cerevisiae* was studied initially by subcellular fractionation of wild-type strain NY13. Cells in late log phase (200 A_{600} units) were converted to spheroplasts and lysed osmotically. Lysates were spun at 450 *g* 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 II, 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. 2 *A*), indicating that the antibodies detect Rholp in total cell extracts. Differential centrifugation fractions from wild-type yeast were analyzed by Western blot (Fig. 2 *B*). 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 Rho1p and Marker Distribution in Wild-type Yeast NY13

Fraction	Rho1p	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 P2 + S2	97	98	104	123

Estimation of Rho1p 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.

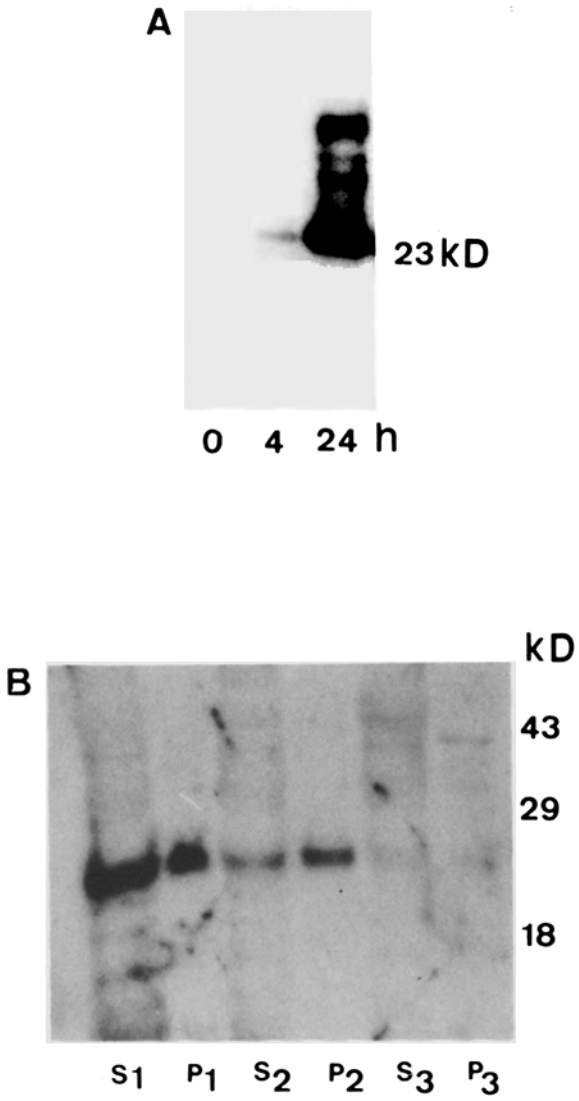

Figure 2. Detection of Rholp by Western blot analysis. (*A*) JJ1 cells were grown to mid-log phase (0.5–1.0 × 10^8 cells/ml) in liquid YPD medium. Cells were then pelleted from the medium, washed once in YPGal, resuspended in YPGal at the original cell concentration, and returned to a shaker at 30°C. 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 (10 μg protein/lane). The proteins were transferred to nitrocellulose filters, and probed with anti-Rholp. (*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.

tected in the S3 fraction, although recovery of Rholp from S2 into P3 and S3 was not quantitative. Thus, the observed distribution of Rholp in differential centrifugation fractions was similar in wild-type yeast by either immunodetection or C3 labeling.

The organelles in the P2 pellet were fractionated further by equilibrium density gradient centrifugation as described by Goud et al. (1988). Gradient fractions were assayed for Rholp using C3, and also for various enzymatic markers specific for the different organelles of the P2 fraction. These markers are NADPH cytochrome c reductase (Kreibich et al., 1973; Kubota et al., 1976) for the ER, vanadate sensitive Mg^{++} ATPase for the plasma membrane, cytochrome c oxidase (Mason et al., 1973) for the mitochondrial inner membrane, α-mannosidase (Van der Wilden et al., 1973) for the vacuole membrane, Ca^{++}-dependent GDPase for the Golgi apparatus, and Kex-2 for the late Golgi apparatus. Fig. 3, *A* and *B* show the results of typical gradient analysis; these are similar to the profiles of Goud et al. (1988), except that one major ER peak was detected instead of the two peaks found in the previous study. This discrepancy may reflect slight differences in strains or lysis procedures. Migration of Rholp in the gradients roughly followed the cdGDPase Golgi marker (Fig. 3, *A* and *B*) and followed very closely the Golgi marker Kex-2. Clearly, Rholp migration was distinct from any of the other organelle markers (Fig. 3 *A*). Thus, most of the particulate Rholp appeared to be located in the late Golgi apparatus.

Rholp 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 Rholp 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 nonpermissive 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 Rholp 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 Rholp abundance in each fraction was determined by C3 labeling. In all three mutants the total amount of Rholp in the particulate fractions (ie., P2 + P3) was about the same as that found in wild-type cells (Table III). Migration of Rholp 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 Rholp between P2 and P3 was similar to wild-type (Table III). However, in the *sec6* mutant a large increase in the abundance of Rholp was found in the P3 fraction, with a concomitant decrease in P2 (Table III). This result suggests that Rholp 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*) Rholp, (*b*) invertase, which accumulates in post-Golgi vesicles in the thermosensitive mutant strain *sec6* (Novick et al.,

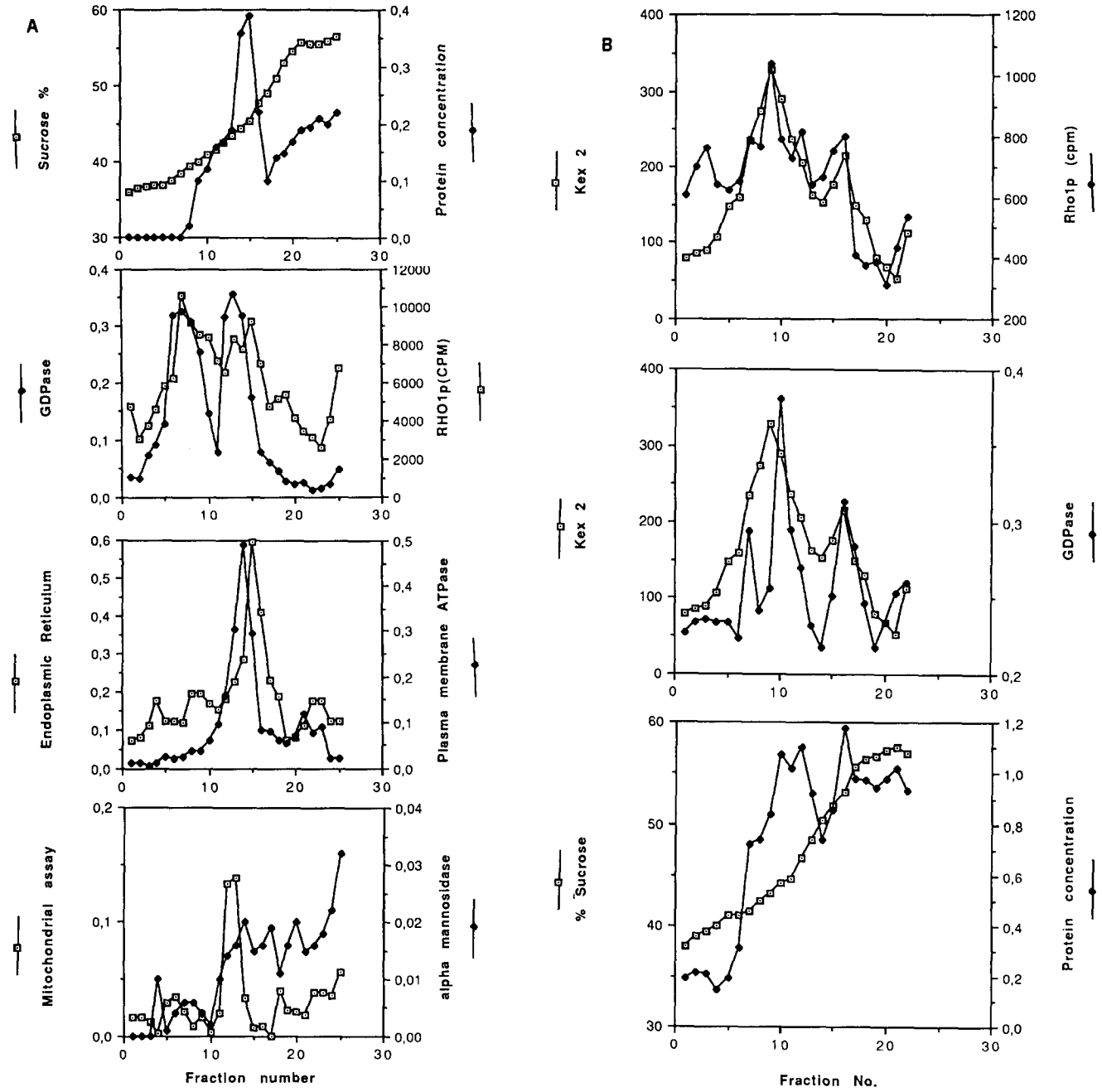

Figure 3. Rholp comigrates with the Kex 2 marker in P2 gradients. The P2 pellet was resuspended in 60% sucrose (2 ml) and loaded on the bottom of a 35–60% sucrose gradient. After centrifugation to equilibrium, gradients were fractionated from the top in 500-μl fractions and labeled 1–25. For each fraction, the different enzyme markers were assayed as well as Rholp by C3-mediated specific ADP-ribosylation. For Rholp estimation, 15-μl aliquots of each fraction were incubated with C3 under the conditions described in Materials and Methods followed by 15% SDS-PAGE and autoradiography. Radioactive spots were cut out and counted for quantitative estimation and expressed in total cpm. Sucrose density is expressed in grams percent. Protein concentrations were estimated by the standard Bradford assay and results are expressed as absorbance at $_{595}$nm. NADPH cytochrome c reductase(endoplasmic reticulum) absorbance at $_{550}$nm. cdGDPase (Golgi) absorbance at $_{660}$nm (*A* and *B*). ATPase (plasma membrane) absorbance at $_{710}$nm. Cytochrome c reductase (mitochondria) absorbance at $_{650}$nm. α-mannosidase (vacuole) absorbance at $_{400}$nm. Kex 2 activity (late Golgi) fluorescence emission at $_{460}$nm; *A* and *B* represent two independent experiments.

1981), 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 *NY13* (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,

Table III. Percent Rho1p Distribution in Mutants of the Secretory Pathway

Strain	Secretory 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

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-

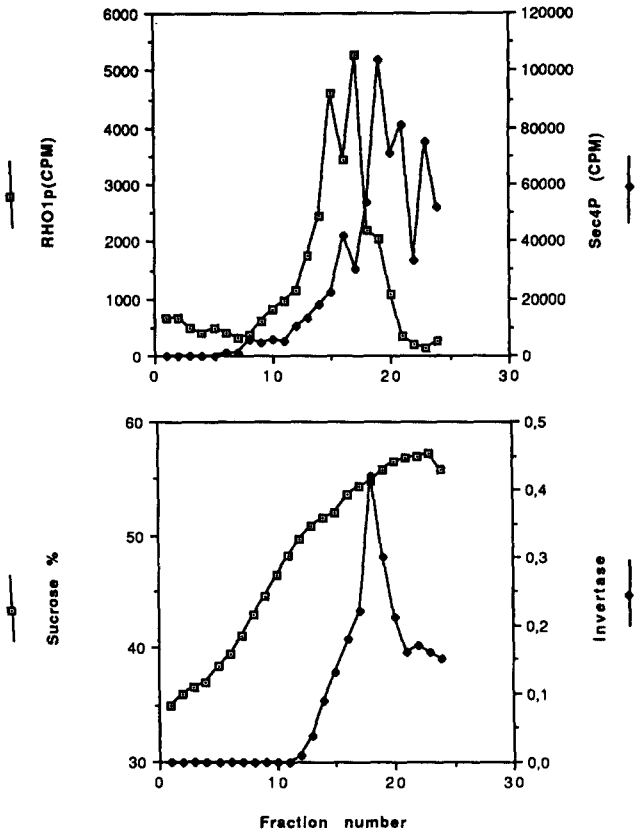

Figure 4. Analysis of Rholp and Sec4p in P3 sucrose density gradients of *sec6*. P3 pellets were prepared by differential centrifugation from *sec6* strain (shifted to 37°C in 0.1% glucose) and resuspended in 2 ml of 60% sucrose and loaded on the bottom of a 35–60% sucrose gradient. Rholp was assayed 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.

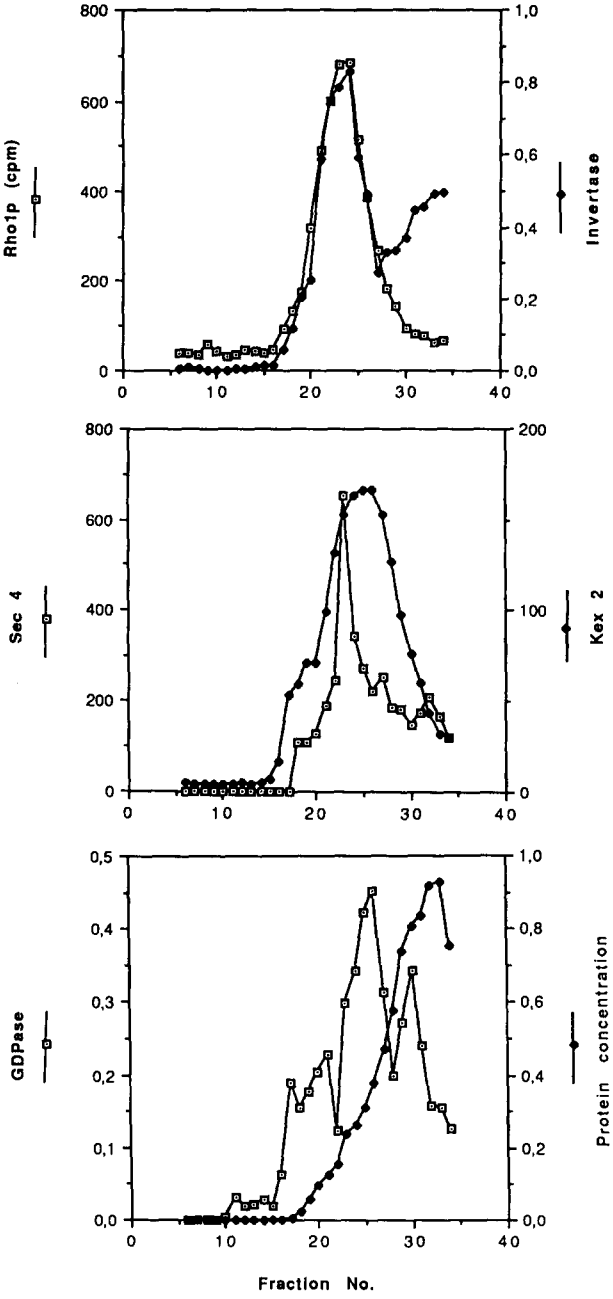

Figure 5. Rholp bearing vesicles are of the same size as invertase containing vesicles from *sec6* strain. 35 4-ml fractions were collected from Sephacryl S-1000 column as described in Materials and Methods. Each fraction was assayed for Rholp, Ca^{++}-dependent GDPase, Sec4p, invertase, and protein content (Bradford). The results are expressed as cpm or OD.

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 *sec 1* mutants. The secretion

Table IV. Percent Rho1p Distribution in Post-Golgi Secretory Mutants

Strains	S1	S2	P2	S3	P3
NY 13(WT)	100	21	76	9	16
sec6	100	47	47	9	43
sec2	100	51	49	10	50
sec3	100	25	72	8	20
sec4	100	41	70	9	42
sec8	100	30	70	10	26
sec15	100	33	67	9	30
sec1	100	31	69	10	28

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. 6 *D*). However, preadsorption with the same concentration of galactose-grown JJ1 cell material completely eliminated the dotted staining pattern (Fig. 6 *C*). 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 (Chardin et al., 1989). Amongst the three rho proteins known in *S. cerevisiae*, Rholp, Rho2p, 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 (Chardin 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.

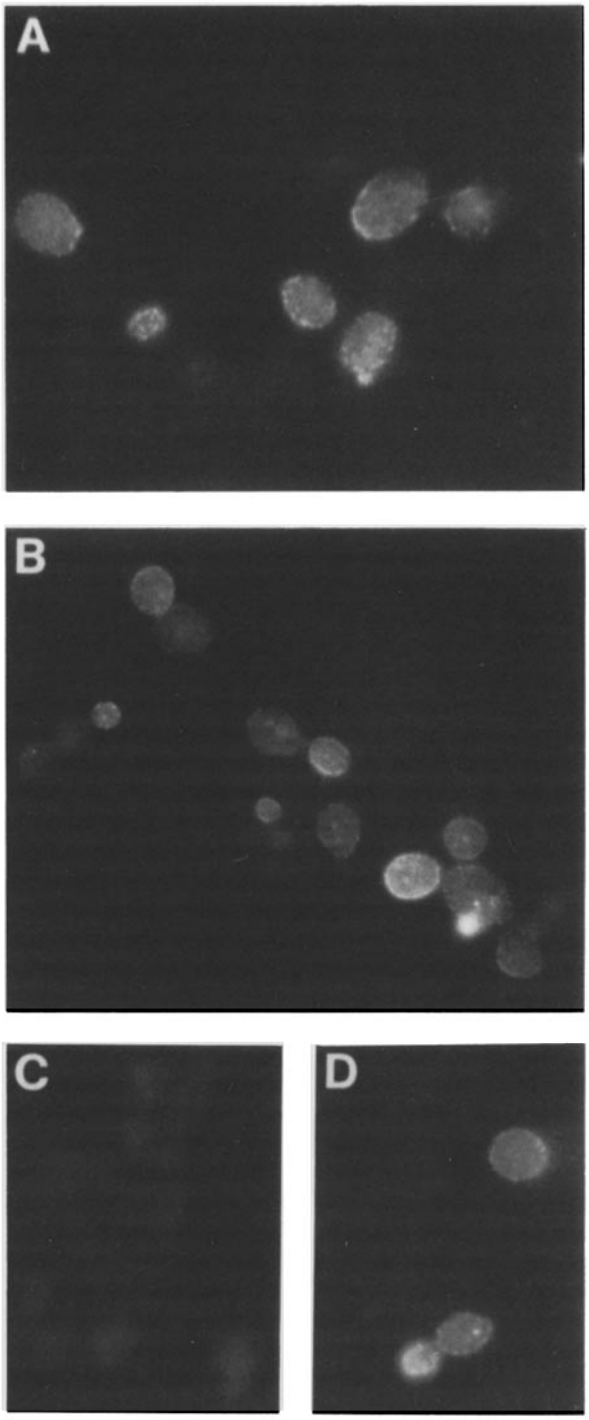

Figure 6. Localization of Rholp by immunofluorescence microscopy. Wild-type cells were grown in YPD to early log phase, and processed for immunofluorescence microscopy using anti-Rholp at a concentration of 40 μg/ml. (*A*) NY13; (*B*) aW303; (*C*) aW303, anti-Rholp preadsorbed with a lysate from JJ1 cells grown in galactose (depleted of Rholp antibodies); (*D*) aW303, anti-Rholp preadsorbed with a lysate from MM50 cells grown in glucose (not depleted of Rholp antibodies).

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, artifactual 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.

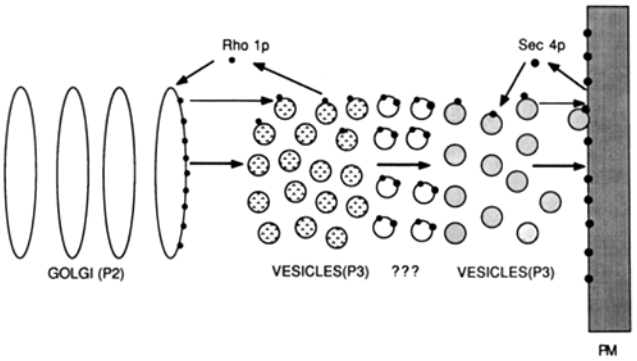

Figure 7. Model for Rholp localization.

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*,

cdc43, and the *rho*-related gene *cdc42* (Pringle and Hartwell, 1981, Sloat 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 Rholp 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.

We thank P. Novick for the gift of wild-type and secretory mutant strains of yeast.

This work was supported by Institut National de la Santé et de la Recherche Medical grant 871009 to P. Madaule, ARC grant 6620 to P. Madaule, and National Institutes of Health grant GM-39254 to A. Myers. M. McCaffrey is a recipient of a post-Doctoral Fellowship from the European Community.

Received for publication 2 November 1990 and in revised form 6 June 1991.

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