Yop1p, the Yeast Homolog of the Polyposis Locus Protein 1, Interacts with Yip1p and Negatively Regulates Cell Growth*

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Rab proteins are small GTPases that are essential elements of the protein transport machinery of eukaryotic cells. Each round of membrane transport requires a cycle of Rab protein nucleotide binding and hydrolysis. We have recently characterized a protein, Yip1p, which appears to play a role in Rab-mediated membrane transport in Saccharomyces cerevisiae. In this study, we report the identification of a Yip1p-associated protein, Yop1p. Yop1p is a membrane protein with a hydrophilic region at its N terminus through which it interacts specifically with the cytosolic domain of Yip1p. Yop1p could also be coprecipitated with Rab proteins from total cellular lysates. The TB2 gene is the human homolog of Yop1p (Kinzler, K. W., Nilbert, M. C., Su, L.-K., Vogelstein, B., Bryan, T. M., Levey, D. B., Smith, K. J., Preisinger, A. C., Hedge, P., McKie, D., Finniear, R., Markham, A., Groffen, J., Boguski, M. S., Altschul, S. F., Horii, A., Ando, H. M., Y., Miki, Y., Nishisho, I., and Na-markham, A., Groffen, J., Boguski, M. S., Altschul, S. F., Horii, A., Ando, H. M., Y., Miki, Y., Nishisho, I., and Nakamura, Y. (1991) Science 253, 661–665). Our data demonstrate that Yop1p negatively regulates cell growth. Disruption of YOP1 has no apparent effect on cell viability, while overexpression results in cell death, accumulation of internal cell membranes, and a block in membrane traffic. These results suggest that Yop1p acts in conjunction with Yip1p to mediate a common step in membrane traffic.

The Rab family encompasses a conserved group of key molecules involved in membrane traffic and represents a distinct subgroup of the Ras superfamily (2). Each stage of membrane traffic through both the constitutive and regulated secretory pathways of all eukaryotic cells is associated with a distinct Rab protein that regulates the cascade of events that lead to SNARE-mediated membrane fusion (3). A hallmark of Rabs is their localization to specific compartments of the transport pathway. This distribution is consistent with the function of Rab proteins in distinct intracellular transport processes. In every case examined, the localization pattern of a Rab protein reflects the membrane transport step that it regulates. In keeping with this view, more than 30 members of the Rab family have been identified (2).

The specificity of Rab protein function, localization, and their presence on the surface of vesicles suggests the existence of a machinery that recruits Rab proteins to the proper target membrane. However, identification of such a machinery has proven elusive. To date, no factor mediating this process has been identified; however, several features of Rab membrane recruitment have been established: (i) Rab proteins are recruited to membranes in their inactive GDP-bound conformation bound to GDI (11); (ii) membrane recruitment is accompanied by the displacement of GDI (12); (iii) membrane recruitment is specific, and the C-terminal hypervariable region of the Rab protein mediates this specificity (13); (iv) prenylation of Rab proteins is crucial for membrane recruitment in addition to the C-terminal ~35 amino acid residues; (v) membrane recruitment is followed by nucleotide exchange, and the two processes can be distinguished kinetically (14, 15); and (vi) for Rab4, the existence of a membrane protein that acts as a specific Rab receptor has been demonstrated, although the precise identity of this receptor is unknown (16).

We have characterized a membrane protein in yeast, Yip1p, which appears to mediate the dissociation of the Rab heterodimer with GDI and facilitates recycling of the GDP-bound Rab back to the donor compartment (10). Consistent with this model, Rab proteins are complexed to GDI in the cytosol, and depletion of GDI in yeast causes loss of the soluble pool of Rabs and a concomitant inhibition of transport in the secretory pathway.

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1 The abbreviations used are: GDI, GDP dissociation inhibitor; GST, glutathione S-transferase; HA, influenza virus hemagglutinin epitope; PCR, polymerase chain reaction; ORF, open reading frame; PBS, phosphate-buffered saline; PNS, postnuclear supernatant; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; CPY, carboxypeptidase Y.

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TABLE I

| Strain | Genotype | Source |
|--------|----------|--------|
| NY605  | MATα ura3-52 leu2-3,112 | Novick laboratory |
| RCY376 | MATα ura3-52 leu2-3,112 - LEU2 GAL1-10 - HA-YOP1 | This study |
| RCY377 | MATα ura3-52 leu2-3,112 - LEU2 GAL1-10 - HA-YOP1 C terminus | This study |
| RCY404 | MATα leu2-3,112 ura3-52 YOP1 - URA3 GFP-YOP1 | This study |
| RCY407 | MATα ura3-52 leu2-3,112 - URAT1KAN<sup>2</sup> | This study |
| RCY423 | MATα ura3-52 leu2-3,112 - LEU2 GAL1-10 - GST YOP | This study |
| RCY425 | MATα ura3-52 leu2-3,112 - LEU2 GAL1-10 - GST YOP1 [URA3 2μ myc6-YIP1] | This study |
| RCY427 | MATα ura3-52 leu2-3,112 - LEU2 GAL1-10 - GST | This study |
| RCY428 | MATα ura3-52 leu2-3,112 - LEU2 GAL1-10 - GST [URA3 2μ myc6-YIP1] | This study |
| RCY429 | MATα ura3-52 leu2-3,112 - LEU2 GAL1-10 - GST YOP1 [URA3 2μ DS84/myc6] | This study |
| RCY450 | MATα ura3-52 leu2-3,112 - LEU2 GAL1-10 - GST YPT7 [pRS426] | This study |
| RCY455 | MATα ura3-52 his3Δ200 leu2-3,112 - LEU2 GAL1-10 - GST YPT7[2] [pRS426] | This study |
| RCY456 | MATα ura3-52 his3Δ200 leu2-3,112 - LEU2 GAL1-10 - GST SEC4 [pRS426] | This study |
| RCY457 | MATα ura3-52 his3Δ200 leu2-3,112 - LEU2 GAL1-10 - GST YPT1ΔC [pRS426] | This study |
| RCY465 | MATα ura3-52 his3Δ200 leu2-3,112 - LEU2 GAL1-10 - GST YPT6 [pRS316] | This study |
| RCY467 | MATα ura3-52 his3Δ200 leu2-3,112 - LEU2 GAL1-10 - GST YPT7 [pRS316] | This study |
| RCY468 | MATα ura3-52 his3Δ200 leu2-3,112 - LEU2 GAL1-10 - GST [pRS316 GAL1-10 - HA- YOP1 (pRC873)] | This study |
| Y190  | MATα gal4Δ ura3Δ trp1-901 ade2-101 leu2-3,112 URA3:: GAL10 - LacZ, LEU2:: GAL10 - HIS3 2 μ myc6 | Elledge laboratory |

Yeast Strains and Media—The S. cerevisiae strains used in these studies are listed in Table I. All yeast strains were manipulated as described by Guthrie and Fink (18). YOP1 gene deletion was carried out using the KAN<sup>2</sup> module (19) as a selectable marker and the primers ATTCACTCTCCGTACGCTGCAGGTCGAC and GAGGATATAGGTGGATGATTCTCATGCAATGCAAAATCACTAGTATTCACTCTCAAATGAAAC). pRC695 expressing the hemagglutinin (HA)-tagged Yop1p protein expression vector pRC778 into RCY407. For immunofluorescence, YC469 was created by digesting pRC393 with EcoRV to direct integration of the plasmid at the URA3 locus of RCY407.

30 °C. Liquid media cultures were grown at room temperature. A single colony from each strain was inoculated into 5 ml of selective medium and grown to stationary phase. The day prior to the experiment, medium was inoculated with aliquots of stationary culture at room temperature to obtain cells in logarithmic phase growth. Turbidity measurements were made using a Beckman model DU-40 spectrophotometer at 600 nm.

Plasmids and DNA Constructs—The genomic YOP1 ORF contains a single intron. For convenience, this intron was removed for the majority of YOP1 constructs by overlap PCR with the primers RNS66 (GGAGGATAGTACCTAGTATTCACTCTCATGCAATGCAAAATCACTAGTATTCACTCTCAAATGAAAC) and RNS67 (GAGGATATAGGTGGATGATTCTCATGCAATGCAAAATCACTAGTATTCACTCTCAAATGAAAC) which physically interacts with Yip1p. Disruption of YOP1 has no apparent effect on cell viability, while overexpression results in cell death and accumulation of internal cell membranes. These results suggest that Yop1p acts in conjunction with Yip1p to mediate a common step in membrane traffic. Because of the essential nature of Rab recruitment for the activation and recycling of Rabs, characterization of Yop1p may provide crucial insight into the action of Rab proteins in mediating membrane transport.

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Yeast strains were streaked out on a selective plate and incubated at 30 °C. Liquid media cultures were grown at room temperature. A single colony from each strain was inoculated into 5 ml of selective medium and grown to stationary phase. The day prior to the experiment, medium was inoculated with aliquots of stationary culture at room temperature to obtain cells in logarithmic phase growth. Turbidity measurements were made using a Beckman model DU-40 spectrophotometer at 600 nm.

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2. R. Collins, unpublished data.
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**TABLE II**

| Bait construct | Fish construct | Yepl N-term | Yepl full length | Yepl N-term | Yepl full length |
|----------------|----------------|-------------|-----------------|-------------|-----------------|
| Yepl N terminus | --             | --          | ++              | --          | --              |
| Yepl full length| --             | +           | ++              | +           | ++              |
| Yepl N terminus | --             | --          | ++              | --          | ++              |
| Yepl C terminus | ++             | +           | ++              | +           | ++              |

β-Galactosidase activity was determined by filter assay. Pairs were contained in the reporter strain YC9. Plus represents a positive activity rate below 25 fmol of o-nitrophenyl-β-D-galactoside (ONPG) activity detected after 30 min; +, activity detected after 90 min; and −, activity detected after 5 h. −, a negative indication of activity. At least 30 independent transformants were tested for each pair. Yop1p N-terminal constructs contain amino acids 1–17, and Yop1p C-terminal constructs contain amino acids 18–180. Yop1p N-terminal constructs contain amino acids 1–117, ND, not detected.

**Electrophoresis and Western Blotting**—For electrophoresis, samples were boiled for 5 min in gel loading buffer (60 mM Tris, pH 6.8, 10% sucrose, 2% SDS, 5% β-mercaptoethanol, and 0.005% bromphenol blue), microcentrifuged for 5 min, and loaded onto 12 or 14% SDS-polyacrylamide gels (37.5:1 acrylamide:bisacrylamide). Prestained molecular weight markers were from Life Technologies, Inc. For Western blotting, gels were transferred to polyvinylidene difluoride membranes for 2 h at 200 mA. The membranes were stained with Ponceau S to observe the quality of the transfer. Antigens on the membrane were detected by incubating the filter with blocking buffer (5% nonfat dry milk in TBST; 150 mM NaCl, 50 mM Tris, pH 7.5, and 0.2% Tween 20). The HA-Yop1p was detected with mouse monoclonal 12CA5 antibody followed by anti-mouse alkaline phosphatase-conjugated secondary antibody. The membrane was developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (both from Bio-Rad) substrates in AP buffer (100 mM Tris, pH 9.5, 10 mM NaCl, and 0.1% Tween 20). Secondary alkaline phosphatase-conjugated antibodies were added in blocking buffer, followed by three washes and chromogenic blot development with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (both from Bio-Rad) substrates in AP buffer (100 mM Tris, pH 9.5, 10 mM NaCl, and 0.1% Tween 20). Secondary alkaline phosphatase-conjugated antibodies were added in blocking buffer, followed by three washes and chromogenic blot development with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (both from Bio-Rad) substrates in AP buffer (100 mM Tris, pH 9.5, 10 mM NaCl, and 0.1% Tween 20).

**Triton X-114 Phase Separation**—Triton X-114 (Roche Molecular Biochemicals) was purified by precondensation as described (25). 25 OD units of yeast strain RCY460 were harvested and washed in 1 ml of TAZ buffer. Postnuclear supernatants were generated as described above. 1.8 mg of PNS was added to the same volume of PBS containing 2% Triton X-114 with protease inhibitors (1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin). A postnuclear supernatant (PNS) was generated by two sequential centrifugation steps for 5 min at 50,000 x g. 2.7 mg of PNS was then spun sequentially at 10,000 x g for 15 min and at 100,000 x g for 12 min to generate P10 and P100 fractions. For Triton X-100 solubilization, the P100 membrane pellet was resuspended in fractionation buffer containing 1% Triton X-100. Samples were incubated for 10 min on ice and recentrifuged at 100,000 x g. For high salt treatment, the P100 membrane pellet was resuspended in fractionation buffer containing 1% NaCl. Samples were incubated for 10 min on ice and recentrifuged at 100,000 x g. Pellets and supernatants were resuspended in sample buffer and analyzed by SDS-PAGE and Western blot. The HA-Yop1p was detected with mouse monoclonal 12CA5 antibody followed by anti-mouse alkaline phosphatase-conjugated secondary antibody.

**Carboxypeptidase Y Analysis**—Yeast strains RCY376 and RCY377 containing HA-YOP1 full-length and C-terminal constructs (respectively) behind the galactose promoter, were grown in sucrose minimal medium to early log phase, before washing and resuspending in galactose minimal medium. At the indicated intervals, aliquots of 5 OD units were harvested for production of lysates. For the sec18 experiments, cells were grown at room temperature until log phase before shifting to the restrictive temperature (37°C) for 1 h. Lysates were then boiled with SDS-PAGE sample buffer for 5 min and analyzed by SDS-PAGE and Western blot. The membrane was probed with polyclonal anti-carboxypeptidase Y (CPY) (gift from P. Brenwald).
PBS/BSA (1 mg/ml BSA) and permeabilized for 5 min with either 0.1% SDS or 0.1% Triton X-100 in PBS/BSA. After washing five times in PBS/BSA, cells were blocked for 30 min in PBS/BSA. Polyclonal α-HA antibody (Y11; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to each well at a dilution of 1:5000 and incubated for 1 h at room temperature. Cells were washed 5 times in PBS/BSA and then incubated with Texas Red-labeled anti-rabbit secondary antibody (Molecular Probes, Inc.) at a dilution of 1:200 for 30 min at room temperature. Monoclonal 1.2.3 antibody was used to detect Sec4p (26), and a monoclonal anti-GFP antibody (3E6; Molecular Probes, Inc., Eugene, OR).

**Fig. 1.** A, schematic representation of Yop1p. Sequence data indicate a cytoplasmically oriented N terminus and a hydrophobic C-terminal domain that spans the membrane twice. B, Kyte-Doolittle hydrophobicity plot of Yop1p. This was generated using the program Proban (DNASTAR) with a 9-residue parameter average and shows the relative location of the two hydrophobic segments of the protein. C, alignment of Yop1p with database homologs. Shown is the sequence of Yop1p and a comparison with human and murine TB2 and full-length cDNAs from other organisms. T41634 is from Schizosaccharomyces pombe, CG4960 and CG8331 are from Drosophila melanogaster, AAF36016 is from Caenorhabditis elegans, and CAN11144 is from Plasmodium falciparum. Mammalian expressed sequence tag fragments are not included in this alignment. The sequences were aligned using MegAlign. Amino acid residues are numbered according to the protein sequence. The shaded residues exactly match the consensus sequence.
Identification of Yop1p

Two-hybrid Experiments—The ORF sequences were subcloned into pAS1-CYH2 or pAS2–1 for “bait” and pACTII for “fish” constructs, respectively. The yeast strain Y190 was used to screen the library for N-terminal Yip1p-interacting clones (27). The yeast reporter strain Y190, which contains the reporter genes lacZ and HIS3 downstream of the binding sequences for Gal4, was sequentially transformed with the pACT2 and pAS2–1 (CLONTECH) plasmids containing the genes of interest. Double transformants were plated on selective medium (lacking tryptophan and leucine) and incubated for 2–3 days at 30 °C. Tryp−:Leu+ colonies processed for the β-galactosidase filter assay as described (21).

RESULTS

The Cytosolic Domain of Yip1p Interacts with a Novel Membrane Protein—To explore the role of Yip1p in membrane traffic, we considered the possibility that it may exist in physical association with other proteins. Such a protein may perhaps act to provide a specificity component to the Rab membrane recruitment reaction. To identify such potential proteins, we performed a two-hybrid screen using the cytosolic domain of Yip1p as bait. For this interaction screen, we used two-hybrid libraries constructed from short fragments (0.5–1 kilobase pair) of yeast genomic DNA (28). Since the yeast genome is relatively compact with few intron-containing genes, such a library represents a collection of random protein fragments. The rationale for such a strategy was that a Yip1p-interacting protein may be a membrane protein interacting with Yip1p through exposed soluble loops. Interactions may not be revealed by expressing full-length cDNAs, but protein fragments of the isolated loops alone may demonstrate interaction in the two-hybrid system. Analogous strategies have been used successfully to explore interactions of multispanning membrane proteins using the two-hybrid system (29). Using this screen, we identified a previously uncharacterized membrane protein derived from ORF YPR028W. The interacting clone identified contained 17 amino acids derived from the extreme N terminus of the protein fused in frame with the GAL4 DNA activation domain. We have termed this gene YOP1 (YIP one partner). The interaction between the Yop1p fragment and Yip1p was recapitulated with a full-length Yip1p construct in the two-hybrid system. The interaction was also maintained whether or not the Yop1p fragment was a GAL4 DNA binding domain plasmid or a GAL4 DNA activation domain fusion; i.e. if the “bait” construct is swapped with the “fish” construct, the vast majority of false two-hybrid positives will not interact in such a test. However, Yop1p full-length constructs show no interactions with Yip1p in the two-hybrid system. These data are summarized in Table II.

The primary sequence of Yop1p is predicted to have at least two membrane-spanning domains (Fig. 1, A and B). A BLAST search of GenBank indicated homology to the human TBP2 protein in addition to several other proteins present in data bases (Fig. 1C). Yop1p and human TBP2 share 25.6% identity at the amino acid level, and there is 22.4% identity between Yop1p and murine TB2. It is notable that the overall structure of the mammalian and yeast protein is conserved. Both proteins contain extensive hydrophobic domains with the N terminus predicted to be exposed to the cytoplasmic face of the membrane. No other Yop1p homologs could be identified in S. cerevisiae.

Yop1p Is an Integral Membrane Protein—Sequence information predicts Yop1p to be a 20-kDa protein with two membrane-spanning segments that is likely oriented with its N terminus toward the cytoplasm (Fig. 1). We examined whether Yop1p has the expected properties of an integral membrane protein. First, Yop1p fractionated exclusively in the pellet of a total
postnuclear supernatant centrifuged at 100,000 \times g, indicating that it is either membrane-associated or present in a large pelletable aggregate (Fig. 2A). Second, we tested whether Yop1p was a peripheral membrane protein and could be removed by washing membranes in high salt-containing buffers. Yop1p could not be extracted from membranes by incubation in buffer containing 1 M NaCl; however, Yop1p was quantifiably extracted in Triton X-100 detergent-containing buffers.

Identification of Yop1p
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(FIG. 2B). Third, we performed Triton X-114 phase extraction experiments to determine whether Yop1p has the physicochemical properties of an integral hydrophobic membrane protein. In this technique, total cellular proteins are first detergent solubilized at 0 °C. The mixture is then warmed to 30 °C, exploiting the cloud point of Triton X-114 to create two phases that can be separated by gentle centrifugation: a detergent-rich phase containing membrane proteins and an aqueous phase containing hydrophilic proteins. Yop1p partitioned exclusively into the detergent-rich phase (FIG. 2C), indicating that it contains hydrophobic domains that anchor it in the lipid bilayer. As a control, fractions were also probed for a known integral membrane protein, Snc1/2p (30), which partitioned into the detergent phase as expected. Taken together, these data show that Yop1p is an integral membrane protein.

Physical Association of Yip1p and Yop1p—To confirm the two-hybrid data, we performed biochemical studies of the Yip1p/Yop1p interaction. For this purpose, we created the strain RCY425, which expresses GST-Yop1p fusion protein under the control of the regulatable GAL1/10 promoter and contains a multi-copy plasmid expressing Myc9-Yip1p. We also created an isogenic control strain, RCY428, which expresses GST alone together with Myc9-Yip1p. Tween 20 detergent-solubilized total lysates were produced from mid-log phase cells grown in galactose and GST fusion proteins were isolated on gluthathione-agarose beads followed by SDS-PAGE and Western blotting to detect any associated Myc-tagged proteins. The results of this experiment are shown in FIG. 3A. Myc9-Yip1p was detected in the GST-Yop1p pull-down but was not detected in the pull-down of GST alone, showing that Myc9-Yip1p exists in physical association with Yop1p. To rule out any possibility of GST-Yop1p interacting with the Myc epitopes of Myc9-Yip1p, we repeated the experiment with RCY429, which expresses GST-Yop1p together with Dss4p-Myc9. In this experiment, the Western blot was first probed with anti-Myc antibody and then reprobed with anti-GST antibody. The results are shown in FIG. 3B. Dss4p-Myc9, Myc9-Yip1p, and GST-Yop1p are expressed at equivalent levels in the detergent-solubilized lysates. The GST pull-downs reveal that Myc9-Yip1p associated with GST-Yop1p but Dss4p-Myc9 did not associate and could not be detected in the pull-down, demonstrating that the biochemical association of Yip1p and Yop1p is specific.

We also repeated the experiment with Yip1p expressed at wild-type levels on a single-copy centromeric plasmid under the control of its own promoter and terminator. For these experiments, Yip1p was tagged with GFP, and lysates were produced with Triton X-100 detergent solubilization. Western blot analysis of the glutathione resin pull-downs (FIG. 3C) showed that GFP-Yip1p (RCY509) was specifically isolated with GST-Yop1p, while a control protein, GFP-Ypt7p (RCY508), was not. Western blots of the detergent-solubilized lysates confirmed that GST-Yop1p and the GFP fusion proteins were expressed at equivalent levels in both cases.

To further investigate the relationship between Yip1p and Yop1p, we asked whether the interaction in our pull-down experiments occurred in vivo prior to cell lysis or postlysis in vitro. For these experiments, we performed the glutathione resin pull-downs on lysates derived from cells coexpressing GST-Yop1p and Myc9-Yip1p (RCY425) or by combining lysates from individual strains RCY429 (containing GST-Yop1p and Dss4p-Myc9) or RCY462, which contains Myc9-Yip1p only. These results can be seen in FIG. 3D. We were only able to detect the interaction of Yip1p and Yop1p from cells expressing both proteins simultaneously. These results indicate that Yip1p and Yop1p interact in vivo, in a complex that is formed prior to cell lysis.

Overexpression of YOP1 Is Dominant Negative and Can Be Suppressed by Co-overexpression of YIP1—We deleted the entire YOP1 ORF in a diploid cell that was sporulated and dissected into tetrad to study the phenotype of the haploid-disrupted strain. The YOP1 haploids were viable, indicating that YOP1 is dispensable for vegetative growth. Furthermore, a strain carrying the null allele has no apparent growth defect under several conditions commonly used to detect phenotypes in S. cerevisiae (31): high temperature (37 °C), low temperature (15 °C), 2 mM caffeine, 2% formamide, high salt (1M NaCl), and glycerol as carbon source (data not shown). We next examined the phenotype of Yop1p overexpression. For this experiment, we expressed both full-length Yop1p (Yop1p full-length,
RCY376) and Yop1p that lacks 17 amino acids at the N terminus identified as the Yip1p-interacting region (Yop1p C terminus, RCY377). Both constructs were expressed in yeast as HA-tagged proteins under the control of the GAL1/10 promoter. Immunoblot analysis of the lysates verified that both proteins were expressed in equivalent amounts upon shift of the growth medium to galactose (Fig. 4A). Both of these constructs were dominant negative for growth upon overexpression (Fig. 4B) but resulted in different morphologies. Overexpression of full-length Yop1p resulted in huge swollen cells of aberrant shape, while overexpression of the Yop1p C-terminal construct gave rise to much smaller cells, similar in shape but considerably smaller than cells harboring a control construct (Fig. 4C). To quantitate the observed effect, measurement of cell size was performed (Fig. 4D). Wild-type cells have an average width of 5.52 ± 0.90 μm; cells expressing full-length Yop1p have an average width of 6.85 ± 0.97 μm; and cells expressing Yop1p C-terminal construct have an average width of 3.37 ± 0.367 μm.

The dominant negative phenotype of full-length Yop1p overexpression was suppressed by co-overexpression of Yip1p from a multicopy plasmid (Fig. 5). However, multicopy YIP1 had no effect on the dominant negative phenotype of the Yop1p C-terminal construct, which lacked the Yip1p interaction region. These data further demonstrate that the interaction of Yip1p and Yop1p is a bona fide physiological interaction and support the identification of the Yop1p N terminus as the site of Yip1p interaction.

**Dominant Negative YOP1 Results in Alteration of Membrane Structures and a Block in Membrane Traffic**—To investigate morphological alterations in dominant negative YOP1 cells in detail, we performed electron microscopy. Cells containing the full-length dominant negative YOP1 construct and isogenic wild-type cells were grown in galactose before being fixed with and processed for electron microscopy. These results are shown in Fig. 6. Expression of the full-length YOP1 dominant negative construct resulted in the disappearance of large vacuoles normally seen in wild-type cells and the appearance of smaller and aberrantly shaped compartments filled with darkly stained material (Fig. 6, A–C). These cells also contained numerous discontinuous ring-shaped structures; some membrane structures resembled the cup-shaped “Berkeley bodies” known to represent normal Golgi structures, while others had pleiomorphic, clublike shapes. In some cells, an accumulation of ER membranes, as judged by their connection to the nuclear envelope, was also observed. Such aberrant membrane structures are not observed in wild-type cells (Fig. 6D) and represent...
a gross distortion of the normal pathways of membrane traffic in the YOP1 dominant negative cells.

To investigate the effect of YOP1 overexpression on membrane traffic, we monitored the steady state level of newly synthesized precursors of the vacuolar protease CPY, the product of the PRCl gene. CPY is a soluble vacuole protein that undergoes processing from a core-glycosylated ER form (p1, 67 kDa) to a modified Golgi form (p2, 69 kDa) before being proteolytically cleaved in the vacuole to mature CPY (61 kDa). Using an anti-CPY antibody, we analyzed total cell lysates for the relative levels of the precursor and mature CPY forms and to provide a positive reference for the accumulation of p1 CPY, marked with an asterisk.

| sec18 | RCY376 | RCY458 |
|-------|--------|--------|
| RT 37°C | 7h | 11h | 15h |
| p2 | p1 | mCPY |

Fig. 7 A, CPY immunoblot analysis of cells expressing dominant negative YOP1 constructs. Shown is immunoblot analysis of total cell lysates for the relative level CPY processing in cells expressing dominant negative constructs containing full-length YOP1 (RCY376), C-terminal YOP1 (RCY377), and an isogenic control strain (RCY458). At the time points indicated after the switch from sucrose- to galactose-containing medium, samples were taken and processed for total cell lysates. The arrows indicate the relative migration of the p1 (core-glycosylated ER), p2 (Golgi-modified), and m (mature vacuolar) forms of CPY. sec18 cells are shown at room temperature (permissive temperature) and after shift to 37 °C (restrictive temperature) for 1 h as a control for the migration of the various CPY forms and to provide a positive reference for the accumulation of p1 CPY, marked with an asterisk. B, cell growth of dominant negative YOP1. Growth of cells expressing full-length YOP1 dominant negative construct (RCY376) relative to isogenic wild-type strain (RCY458). Cells were grown to log phase in sucrose-containing selective medium before being switched to galactose-containing medium to induce expression of construct. At various times, as indicated, a turbidity measurement was made as a record of cell growth. Cell concentration was maintained in log phase for the duration of the experiment.

Identification of Yop1p

We examined the localization of Yop1p by subcellular fractionation and immunofluorescence. For this purpose, we constructed the strain RCY460, which contains wild-type levels of HA-tagged Yop1p as the sole cellular source of Yop1p. Separation of postnuclear supernatants into P10 (after 10,000 × g centrifugation) and P100 and S100 (after 100,000 × g centrifugation) followed by immunoblotting with monoclonal anti-HA antibody (12CA5) is shown in Fig. 8A. HA-Yop1p fractions with both light and heavy membranes (P10, P100) but not with cytosol (S100).

By immunofluorescence microscopy, HA-Yop1p appears as a punctate pattern that appears to be at, or near, the periphery of the cell, roughly proportionally distributed between the mother and bud with a greater concentration in the more actively growing region of the cell (Fig. 8B). To identify the cellular location of Yop1p, we performed double label immunofluorescence with Sec4p and with GFP-Ypt6p. HA-Yop1p does not localize to the bud tip or at the neck during cytokinesis and can be clearly distinguished from Sec4p immunofluorescence, which is solely concentrated at the leading edge of the cell (Fig. 9A). The Yop1p signal partially overlapped with the Ypt6p fluorescence, especially toward the leading edge of the cell, indicating the presence of Yop1p on Golgi membranes (Fig. 9B).

The HA-Yop1p pattern of expression is identical whether or not the construct is integrated into the genome or maintained as a centromeric plasmid; the expression pattern is also identical in diploids and haploids and on cells grown in glucose, galactose, or glycerol carbon sources (data not shown). To further examine the subcellular localization of Yop1p and its interactions with Yip1p, we performed confocal microscopy. Cells coexpressing HA-Yop1p and GFP-Yip1p are shown in Fig. 9C. Substantial overlap of the Yop1p and Yip1p signal was observed toward the growing edge of the cell, confirming our biochemical data indicating that a physical interaction between Yop1p and Yip1p occurs in vivo.

Interaction of Yop1p with Rab Proteins—Since Yip1p is required for secretory pathway function, presumably through its
effects on Rab proteins, we sought to investigate any possible interaction of Yop1p with Rab proteins. There are 11 Rab protein family members in *S. cerevisiae*; however, some of these represent closely related isoforms (e.g. Vps21p, Ypt52p, and Ypt53p). To gain as complete an insight as possible, we made GST fusions with several Rab proteins encompassing representatives from each subset. These proteins were expressed in cells behind the galactose promoter and were tested for interaction by coprecipitation with Yop1p, which was tagged with a single HA epitope and also expressed behind the galactose promoter. The results of this analysis are shown in Fig. 10A. HA-Yop1p did not coprecipitate with GST alone but was able to precipitate with GST fused to the Rab proteins Ypt52p, Sec4p, Ypt6p, and Ypt7p. The observed interaction was stable in buffers containing 0.5% Tween 20; however, it could not be observed in 0.5% Triton X-100 containing buffers (data not shown). No interaction was observed with a Ypt1p construct lacking its C-terminal cysteines, which are the sites of prenylation. The interactions between Yop1p and Rab proteins are unlikely to be real in *vivo* interactions; otherwise, the steady-state localization of Yop1p would probably be more universally distributed among subcellular membranes. It is more likely that the observed interactions reflect a generalized biochemical ability of Yop1p to interact with a common determinant of fully post-translationally modified Rab proteins, an interaction that can be revealed by overexpressing both proteins and performing coprecipitation assays as shown in Fig. 10A.

To reveal which Rab protein may be important for Yop1p action *in vivo*, we performed suppression analysis of the *YOP1* dominant negative constructs with multicopy plasmids encoding all 11 Rab proteins of *S. cerevisiae*. The full-length *YOP1* dominant negative construct, while able to be suppressed by multicopy *YIP1* (Fig. 5), could not be suppressed by overexpression of any of the genes encoding the yeast Rab proteins (data not shown). However, multicopy *YPT6* was able to suppress the dominant negative phenotype of the *YOP1* C-terminal construct. The suppression of the *YOP1* C-terminal construct (RCY377) by 2μ *YPT6* together with *YPT7* and *DSS4* as a comparison is shown in Fig. 10B. *YPT6* was the only Rab gene capable of causing *in vivo* suppression of Rcy377; no other Rab gene tested (*SEC4*, *YPT1*, *YPT31*, *YPT32*, *VPS21*, *YPT52*, *YPT53*, *YPT5*, *YPT10*, *YPT11*, and *YPT7*; data not shown except for *YPT7*, Fig. 10B), was able to restore growth.

**DISCUSSION**

We have isolated *YOP1* as a novel *YIP1*-interacting clone in a yeast two-hybrid screen of a yeast genomic library. Yop1p and Yip1p are both integral membrane proteins. The interaction of a membrane protein in the two-hybrid system is perhaps surprising and worthy of comment. Yip1p is not alone in this regard; other membrane proteins have also been shown to functionally interact in such a system (29). Although some membrane proteins clearly cannot maintain their native structure and functional interactions in the two-hybrid system, there are at least two factors that might indicate whether or not the two-hybrid system will be useful for any given protein. (i) The GAL4 system contains a strong nuclear localization signal and so may dominate over other localization signals...
Identification of Yop1p

Fig. 9. A–B, double label immunofluorescence microscopy of Yop1p with Sec4p and Ypt6p. RCY489 cells expressing wild-type levels of HA-tagged Yop1p as the only source of Yop1p together with wild-type levels of either GFP-tagged Sec4p or Ypt6p were examined by double label immunofluorescence microscopy. Cells were labeled with the anti-HA tag antibody Y11 to visualize HA-Yop1p (A and B (i)) and with anti-Sec4p (A (ii)) or anti-GFP (B (ii)). Nuclei were counterstained with the DNA stain Hoechst 33258 (A and B (ii)). A merge of all three channels is shown in A and B (ii). Note that under the processing conditions for immunofluorescence, there was no interference from the intrinsic GFP fluorescence. C, double label confocal immunofluorescence microscopy of Yop1p with Yip1p. RCY496 cells expressing wild-type levels of HA-tagged Yop1p as the only source of Yop1p together with wild-type levels of GFP-tagged Yip1p were examined by double label confocal microscopy. Cells were labeled with anti-HA tag antibody (i) and with anti-GFP antibody (ii). A merge of both channels is shown in iii.

present in the two-hybrid construct and be a better system for this purpose than a system that relies on passive diffusion to enter the nucleus (32). (ii) S. cerevisiae is probably more capable of correctly folding endogenous yeast proteins rather than proteins from other organisms. In addition, membrane channels have been observed in the nucleus (33), and some viruses acquire membranes in the nucleus (34), indicating that the ultrastructure of the nucleus may be more complex than originally thought.

We have identified two functional domains of Yop1p that act in a dominant manner to inhibit cell growth upon overexpression. The first domain consists of the cytosolic N terminus of Yop1p that corresponds to the first exon of the YOP1 gene. The second domain comprises the C terminus of the molecule that is mainly hydrophobic and corresponds to the second genomic exon. Overexpression of full-length Yop1p leads to inhibition of cell growth and a phenotype of enlarged cells that accumulate internal membrane structures. This phenotype can be suppressed by co-overexpression of Yip1p. Presumably, Yip1p is able to suppress the toxic effect of Yop1p by sequestration. These data would suggest that YOP1 overexpression inhibits cell growth by inhibiting the function of Yip1p, since Yip1p is an essential gene required for secretion. Consistent with this interpretation is the phenotype of YOP1 full-length overexpression, which results in the accumulation of membrane structures and an accumulation of the ER core-glycosylated form of CPY, indicating a block in ER to Golgi traffic. Recently, a Yip1p homolog, Yi1p, has been identified (35) that appears to act similarly to Yip1p in blocking ER to Golgi transport. Although nothing is known about the precise function of Yi1p, the identification of Yi1p and Yop1p as Yip1p binding partners suggests that Yip1p may be involved in several different Rab-mediated events through a combinatorial assortment with different binding partners.

The phenotype of the YOP1 C-terminal construct is distinct from that of the full-length construct. Yip1p co-overexpression cannot suppress the dominant negative effect of Yop1p C terminus overexpression. The Yop1p C-terminal construct lacks the domain that is both necessary and sufficient for Yip1p interaction by two-hybrid analysis. The mechanism by which this construct inhibits growth cannot be directly via an inhibition of Yip1p function. One clue may be provided by the fact that YPT6 can suppress the dominant negative YOP1 C-terminal construct but not that of the full-length construct, indicating that the action of Yop1p is intimately connected to Rab function. This finding further underscores our results, demonstrating that Yop1p can be specifically coprecipitated with Rab proteins in cellular lysates. Since Yop1p shows a restricted sub-cellular localization, we hypothesize that the biochemical interaction of Yop1p with Rab proteins is limited in vivo, possibly only to YPT6, which our suppression analysis demonstrates to interact genetically with YOP1. Consistent with this interpretation are our data demonstrating that the steady-state immunofluorescent localization of Yop1p and Ypt6p shows overlap in vivo.

The two domains of Yop1p may act antagonistically, or perhaps the exposed Yop1p N terminus may constitute a signaling domain that acts in a dose-dependent manner to negatively regulate membrane traffic. There is a growing appreciation that many proteins involved in the regulation of intracellular membrane traffic may act as signal transducers that coordinate membrane traffic with other cellular events (36–38). Different branches of the Ras superfamily are ideally placed to coordinate such cross-talk, and our data indicate that YOP1 and possibly its human homolog TB2 may also play a role in the regulation of cell growth through its facilitation of membrane traffic. Our genetic data suggest that YOP1 is a recessive gene that negatively regulates cell growth. Deletion of YOP1 has no apparent effect on cell viability, and full-length and C-terminal YOP1 constructs
possess a dose-dependent growth inhibitory effect.

Sequence comparison revealed that Yop1p is homologous to the human TB2 gene with sequence similarities throughout the protein. The amino acid sequence conservation of Yop1p across species clearly points to its functional importance, and an interesting finding is that TB2 is a human familial adenomatous polyposis locus (39) gene (40), adjacent to the tumor suppressor genes MCC and APC (41). TB2 encodes a 197 amino acid polypeptide (1). The deduced amino acid sequence predicts that Yop1p contains at least two extensive membrane-spanning segments. The human TB2 gene also contains a similar size and type of membrane-spanning segments and would be predicted to have the same topology. This similarity raises the possibility that Yop1p and TB2 may share a common function in mediating vesicular transport. It is now clear that the machinery and mechanisms of membrane traffic share much in common between yeast and higher eukaryotes (42). For example, the complex observed between Sec9p, Sso1/2p, and Snc1/2p, which is required for exocytosis in yeast, is the structural and functional counterpart of the neuronal SNARE complex (30). Rabs are also extremely well conserved over evolution. In some cases, yeast and mammalian Rab proteins are functionally interchangeable. For example, Vps21p/Ypt51p, a homolog of mammalian Rab5, is also required at an early step in endocytic traffic (43). Remarkably, Ypt51p expression in animal cells not only localizes to Rab5-positive early endosomes but also stimulates endocytosis (44). This latter fact indicates that the machinery involved in mediating Rab protein function is probably conserved across diverse species. Our data indicate that Yop1p, probably in conjunction with Yip1p, acts to facilitate a Rab-mediated event in membrane traffic. It remains to be demonstrated whether TB2 has a role in membrane transport.

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REFERENCES
1. Kinzler, K. W., Nilbert, M. C., Su, L.-K., Vogelstein, B., Bryan, T. M., Levey, D. B., Smith, K. J., Preisinger, A. C., Hedge, P., McKeehinie, D., Finnie, R., Markham, A., Groffen, J., Boguski, M. S., Altschul, S. F., Hori, A., Ands, H. M., Miki, Y., Nishisho, I., and Nakamura, Y. (1991) Science 253, 661–665
2. Collins, R. N., and Brennwald, P. (1999) Front. Mol. Biol. 24, 137–175
3. Pfeffer, S. (1999) Nat. Cell Biol. 1, E17–E22
4. Casey, P. J., and Seabra, M. C. (1999) J. Biol. Chem. 274, 5289–5292
5. Seabra, M. C., Brown, M. S., Slaughter, C. A., Sudhof, T. C., and Goldstein, J. L. (1992) Cell 70, 1049–1057
6. Wu, S. K., Zeng, K., Wilson, I. A., and Balch, W. E. (1996) Trends Biochem. Sci. 21, 472–476
7. Araki, S., Kikuchi, A., Hata, Y., Isomura, M., and Takai, Y. (1990) J. Biol. Chem. 265, 13007–13015
8. Musha, T., Kawata, M., and Takai, Y. (1992) J. Biol. Chem. 267, 9821–9825
9. Garrett, M. D., Zahner, J. E., Cheney, C. M., and Novick, P. J. (1994) EMBO J. 13, 1718–1728
10. Soldati, T., Riederer, M. A., and Pfeffer, S. R. (1993) Mol. Cell Biol. 4, 425–434
11. Pfeffer, S. R., Dirac-Svejstrup, A. B., and Soldati, T. (1995) J. Biol. Chem. 270, 17057–17059
12. Dirac-Svejstrup, A. B., Sumizawa, T., and Pfeffer, S. R. (1997) EMBO J. 16, 465–472
13. Chavrier, P., Gorvel, J. P., Stelzer, E., Simons, K., Gruenberg, J., and Zerial, M. (1991) Nature 353, 769–772
14. Soldati, T., Shapira, A. D., Svejstrup, A. B., and Pfeffer, S. R. (1994) Nature 369, 76–78
15. Ullrich, O., Horisuchi, H., Bucci, C., and Zerial, M. (1994) Nature 368, 157–160
16. Ayad, N., Hull, M., and Mellman, I. (1997) EMBO J. 16, 4497–4507
17. Yang, X., Matern, H. T., and Gallwitz, D. (1998) EMBO J. 17, 4954–4963
18. Guthrie, C., and Fink, G. R. (1991) Methods Enzymol. 194, 77–93
19. Wach, A., Brachut, A., Pohlmann, R., and Philipp, P. (1994) Yeast 10, 1793–1808
20. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
21. Collins, R. N., Brennwald, P., Garrett, M., Lauring, A., and Novick, P. (1997) J. Biol. Chem. 272, 18281–18289
22. Crompton, B. B., Bertram, G., Egerton, M., Gow, N. A. R., Falkow, S., and Brown, A. J. P. (1997) Microbiology 143, 303–311
23. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) Mol. Cell. Biol. 5, 3610–3616
24. Seedorf, M., Damelin, M., Kahana, J., Taura, T., and Silver, P. (1999) Mol. Cell. Biol. 19, 1547–1557
25. Bordier, C. (1981) J. Biol. Chem. 256, 1604–1607
26. Brennwald, P., and Novick, P. (1993) Nature 362, 560–563
27. Fields, S., and Sterngranz, R. (1994) Trends Genet. 10, 286–292
28. James, P., Halladay, J., and Craig, E. (1998) Genetics 144, 1425–1436
29. Young, K. H. (1998) Biol. Regul. 36, 202–311
30. Rossi, G., Salminen, A., Rice, L. M., Brunger, A. T., and Brennwald, P. (1997) J. Biol. Chem. 272, 16610–16617
31. Hampsey, M. (1997) Yeast 13, 1099–1133
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32. Drees, B. L. (1999) *Curr. Opin. Chem. Biol.* 3, 64–70
33. Fricker, M., Hollinshead, M., White, N., and Vaux, D. (1997) *J. Cell Biol.* 135, 531–544
34. Whittaker, G. R., and Helenius, A. (1998) *Virology* 246, 1–23
35. Matern, H., Yang, X., Andruulis, E., Sternglanz, R., Trepte, H.-H., and Gallwitz, D. (2000) *EMBO J.* 19, 4485–4492
36. Floyd, S., and De Camilli, P. (1998) *Trends Cell Biol.* 8, 299–301
37. Lehman, K., Rossi, G., Adamo, J. E., and Brennwald, P. (1999) *J. Cell Biol.* 146, 125–140
38. Wu, W.-J., Erickson, J. W., Lin, R., and Cerione, R. A. (2000) *Nature* 405, 800–804
39. Utsunomiya, J., and Lynch, H. T. (1990) *Hereditary Colorectal Cancer*, Springer-Verlag, Tokyo
40. Knudson, A. G., Jr. (1971) *Proc. Natl. Acad. Sci. U. S. A.* 68, 820–823
41. Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., Hedge, P., Markham, A., Kruch, A. J., Petersen, G., Hamilton, S. R., Nilbert, M. C., Levy, D. B., Bryan, T. M., Preisinger, A. C., Smith, K. J., Su, L.-K., Kinzler, K. W., and Vogelstein, B. (1991) *Science* 253, 665–669
42. Ferro-Novick, S., and Jahn, R. (1994) *Nature* 370, 191–193
43. Singer-Kruger, B., Stenmark, H., Dusterhoft, A., Philippsen, P., Yoo, J. S., Gallwitz, D., and Zerial, M. (1994) *J. Cell Biol.* 125, 283–298
44. Singer-Kruger, B., Stenmark, H., and Zerial, M. (1995) *J. Cell Sci.* 108, 3509–3521