Identification of the novel proteins Yip4p and Yip5p as Rab GTPase interacting factors

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Received 10 January 2002; revised 1 February 2002; accepted 1 February 2002

First published online 26 February 2002

Edited by Ulrike Kutay

Abstract The Rab GTPases are key regulators of membrane traffic. Yip1p is a membrane protein of unknown function that has been reported to interact with the Rabs Ypt1p and Ypt31p. In this study we identify Yif1p, and two unknown open reading frames, Ygl198p and Ygl161p, which we term Yip4p and Yip5p, as Yip1p-related sequences. We demonstrate that the Yip1p-related proteins possess several features: (i) they have a common overall domain topology, (ii) they are capable of biochemical interaction with a variety of Rab proteins in a manner dependent on C-terminal prenylation, and (iii) they share an ability to physically associate with other members of the YIP1 family.

Key words: Rab; YIP1; YIP4; YIP5; YGL198W; YGL161C

1. Introduction

Rab GTPases form the largest branch of small GTPases in the Ras superfamily and are found in all eukaryotic organisms [1]. Rab proteins perform essential functions in different membrane transport pathways of the cell such as vesicle biogenesis [2], targeting and fusion of membrane-bound containers [3], and the association of organelles with motor proteins [4].

Like other members of the Ras superfamily, the intrinsic interconversion rates between the GDP- and GTP-bound forms of the protein are regulated by accessory factors such as guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). In addition to their cycle of nucleotide binding and hydrolysis, Rab proteins also undergo cycles of membrane association and dissociation. Rab proteins stably attach to membranes by virtue of their post-translational prenylation modification: the attachment of two C20 geranylgeranyl groups onto C-terminal cysteines of the protein [5]. The Rab protein can be removed from the membrane through the action of Rab-GDP dissociation inhibitor (GDI). GDI is a soluble protein whose recognition site consists of both the GDP-bound Rab and its prenylation moiety [6]. The heterodimer of GDP/Rab-GDI enables the Rab protein to be recycled through the cytosol back onto membranes for subsequent rounds of transport. The membrane recruitment reaction of Rabs is highly specific, each organelle of the secretory and endocytic pathways is found to associate with a particular Rab protein(s).

To date, many of the Rab interacting proteins that have been identified are soluble factors whose activity can be assigned to defined classes such as effectors, GEFs, GAPs etc. based on their ability to modulate the Rab GTPase cycle. Recently, several Rab interacting membrane proteins have been identified. These include Yip1p, PRA1, rab5ip and Yop1p [7–10]. The existence of these proteins raises the exciting possibility that they are involved in regulating Rab function on membranes or perhaps modulate the association of Rab proteins with membranes. In this study, we have focused on one of this class of membrane proteins, Yip1p. Using Yip1p as a departure point we have identified YIP1-related sequences and demonstrate that the proteins encoded by these sequences have common characteristics and constitute a protein family. Because Yip1p is the founder member or prototype for this family we have termed it the YIP1 family. For small membrane proteins such as Yip1p, identification of homologs cannot be confidently predicted based on primary sequence comparison alone. This is due to the fact that large stretches of the protein consist of hydrophobic residues, reducing the complexity necessary for successful database mining. Our results define three additional criteria for a Yip1p-related protein. These criteria are a common domain topology, the ability to interact with Rab proteins in a manner dependent on C-terminal prenylation, and the ability to associate physically with other Yip1p family members. We demonstrate that Yif1p, and two unknown open reading frames (ORFs), YGL198W and YGL161C, share these features and qualify as YIP1 family members: we have termed these ORFs Yip4p and Yip5p respectively. The YIP1-related proteins are found across eukaryotes and YIP1 family members have both overlapping and distinct functions.

2. Materials and methods

2.1. Yeast strains and media

The *Saccharomyces cerevisiae* strains used in these studies are listed in Table 1. All yeast strains were manipulated as described in [11].

2.2. Yeast two-hybrid (Y2H) assay

The ORF sequences were subcloned into pAS1-CYH2 or pAS2-1 for 'bait' and and pACTII or pACT2 for 'prey' constructs respectively as listed in Table 2. pRC187 and pRC188 are two independent bait constructs which contain Yip1p. pRC1466 and pRC1467 are two in-

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Abbreviations: GDI, GDP dissociation inhibitor; GST, glutathione *S*-transferase; GAP, GTPase activating factor; GEF, guanine nucleotide exchange factor; Y2H, yeast two-hybrid; MBP, maltose binding protein; GFP, green fluorescent protein; 5-FOA, fluoroorotic acid

0014-5793/02/$22.00
PII: S0014-5793(02)02442-0

Table 1
S. cerevisiae strains used in this study

Strain	Genotype	Source
RCY427	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1-10}$*GST*	This laboratory [13]
RCY442	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT7*	This laboratory [13]
RCY539	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YIP1*	This laboratory [13]
RCY693	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT10*	This laboratory [13]
RCY694	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT11*	This laboratory [13]
RCY695	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT31*	This laboratory [13]
RCY696	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT32*	This laboratory [13]
RCY697	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT52*	This laboratory [13]
RCY698	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT6*	This laboratory [13]
RCY699	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-SEC4*	This laboratory [13]
RCY700	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT1ΔC*	This laboratory [13]
RCY701	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YPT1*	This laboratory [13]
RCY765	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST* [pRC1054]	This study
RCY850	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YIP1* [pRC1053]	This study
RCY780	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST* [pRC1047]	This study
RCY851	*MAT***a** *ura3-52 leu2-3,112::LEU2* $P_{GAL1/10}$*GST-YIP1* [pRC1047]	This study
RCY873	*MAT***a** *ura3-52 leu2-3,112* [$P_{GAL1/10}$GST-Yip4p CEN *LEU2* pRC1578] [MBP-Yip4p pRS426 pRC1053]	This study
RCY881	*MAT***a** *ura3-52 leu2-3,112* [$P_{GAL1/10}$GST-Yif1p CEN *LEU2* pRC1579] [MBP-Yip4p pRS426 pRC1053]	This study
RCY1354	*MAT***a** *ura3-52 leu2-Δ1 lys2-801 his3Δ200 ade2-101 trp1-Δ63 YIP1ΔHIS* [YCP50 *YIP1* pRC1245]	This study
Y190	*MAT***a** *gal4Δ gal80Δ trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL10→LacZ, LYS2::GAL10→HIS3* cyh^R	Elledge laboratory

dependent prey constructs which contain Ygl161p (Yip5p). The yeast strain Y190 was used to assay for interacting constructs [12]. Due to batch variability in Y2H assays each complete experiment was carried out in a complete set which included positive and negative controls. We also commonly observed variability in the Y2H system between two otherwise identical constructs and so two independently generated constructs were used to confirm interactions observed in our experiments. Pairs of plasmids were cotransformed into the yeast strain and

Table 2
Plasmids used in this study

Name	Relevant features	Source
pRC38	pAS1-CYH2 Gal4-DNA binding domain Ypt7p fusion	This study
pRC22	pAS1-CYH2 Gal4-DNA binding domain Yif1p fusion	This study
pRC27	pAS1-CYH2 Gal4-DNA binding domain Ypt11p fusion	This study
pRC34	pAS1-CYH2 Gal4-DNA binding domain Ypt52p fusion	This study
pRC33	pAS1-CYH2 Gal4-DNA binding domain Ypt53p fusion	This study
pRC804	pAS1-CYH2 Gal4-DNA binding domain Ypt1p fusion	This study
pRC805	pAS1-CYH2 Gal4-DNA binding domain Ypt51p fusion	This study
pRC966	pAS1-CYH2 Gal4-DNA binding domain Sec4p fusion	Novick laboratory [27]
pRC29	pAS1-CYH2 Gal4-DNA binding domain Ypt31p fusion	This study
pRC31	pAS1-CYH2 Gal4-DNA binding domain Ypt32p fusion	This study
pRC25	pAS1-CYH2 Gal4-DNA binding domain Ypt10p fusion	This study
pRC1253	pAS1-CYH2 Gal4-DNA binding domain Dss4p fusion	Novick laboratory [27]
pRC225	pAS2-1 Gal4-DNA binding domain human Yip1p fusion	This study
pRC181	pAS1-CYH2 Gal4-DNA binding domain Yip4p fusion	This study
pRC977	pAS1-CYH2 Gal4-DNA binding domain Sec4ΔCp (Sec4p lacking C-terminal cysteines) fusion	Novick laboratory [27]
pRC187/pRC188	pAS1-CYH2 Gal4-DNA binding domain Yip1p fusion	This study
pRC957	pACTII Gal4-DNA activation domain Yip1p fusion	This study
pRC42	pACTII Gal4-DNA activation domain Yif1p fusion	This study
pRC44	pACTII Gal4-DNA activation domain Yip4p fusion	This study
pRC1464	pACTII Gal4-DNA activation domain Gdi1p fusion	Novick laboratory [27]
pRC1466/pRC1477	pACTII Gal4-DNA activation domain Yip5p fusion	This study
pRC1047	MBP tagged Yif1p *URA3* 2μm (pRS426)	This study
pRC1049	MBP tagged Yip1p *URA3* 2μm (pRS426)	This study
pRC1053/pRC1054	MBP tagged Yip4p *URA3* 2μm (pRS426)	This study
pRC337	*LEU2* INT $GAL_{1/10}$ GST (pRS305)	This laboratory [13]
pRC696	*LEU2* INT $GAL_{1/10}$ GST-Ypt10p (pRS305)	This laboratory [13]
pRC697	*LEU2* INT $GAL_{1/10}$ GST-Ypt11p (pRS305)	This laboratory [13]
pRC698	*LEU2* INT $GAL_{1/10}$ GST-Ypt31p (pRS305)	This laboratory [13]
pRC699	*LEU2* INT $GAL_{1/10}$ GST-Ypt32p (pRS305)	This laboratory [13]
pRC700	*LEU2* INT $GAL_{1/10}$ GST-Ypt52p (pRS305)	This laboratory [13]
pRC701	*LEU2* INT $GAL_{1/10}$ GST-Ypt6p (pRS305)	This laboratory [13]
pRC702	*LEU2* INT $GAL_{1/10}$ GST-Sec4p (pRS305)	This laboratory [13]
pRC711	*LEU2* INT $GAL_{1/10}$ GST-Ypt1ΔC (pRS305)	This laboratory [13]
pRC1016	*LEU2* INT $GAL_{1/10}$ GST-Ypt1p (pRS305)	This laboratory [13]
pRC726	*LEU2* INT $GAL_{1/10}$ GST-Yip1p (pRS305)	This laboratory [13]
pRC1245	YCP50 containing *YIP1* with endogenous 5′ and 3′ UTR	This study
pRC1578	*LEU2* CEN $GAL_{1/10}$ GST-Yip4p (pRS315)	This study
pRC1579	*LEU2* CEN $GAL_{1/10}$ GST-Yif1p (pRS315)	This study

at least 30 independent colonies were assayed for β-galactosidase activity. β-Galactosidase activity was determined with the chromogenic substrate X-gal using a Macintosh computer-based imaging analysis with CanoScan N670U using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/)

2.3. Co-precipitation experiments

Rab proteins as indicated were expressed as glutathione *S*-transferase (GST) fusion proteins under the control of the $GAL_{1/10}$ promoter in yeast. These strains contain a plasmid expressing either maltose binding protein (MBP)-tagged Yip1p, Yif1p or Yip4p. The experimental protocol was as described in [13]. Strains used for pull-down experiments were grown overnight in 50 ml of selective medium containing galactose as carbon source (SGal) to an absorbance of ~0.7 A_{600}. Cells were harvested by centrifugation at 4°C and washed in 1 ml of ice-cold buffer (10 mM Tris pH 7.5, 10 mm NaN_3). Cell pellets were resuspended in 100 μl of ice cold lysis buffer (20 mM KPi pH 7.5, 80 mM KCl, 1 mM EGTA, 2% glycerol, 0.8% Tween 20) containing protease inhibitors (10 mM phenylmethylsulfonyl fluoride, 10μg/ml pepstatin A) before lysis with glass beads. A total detergent-solubilized extract was generated by incubating lysates

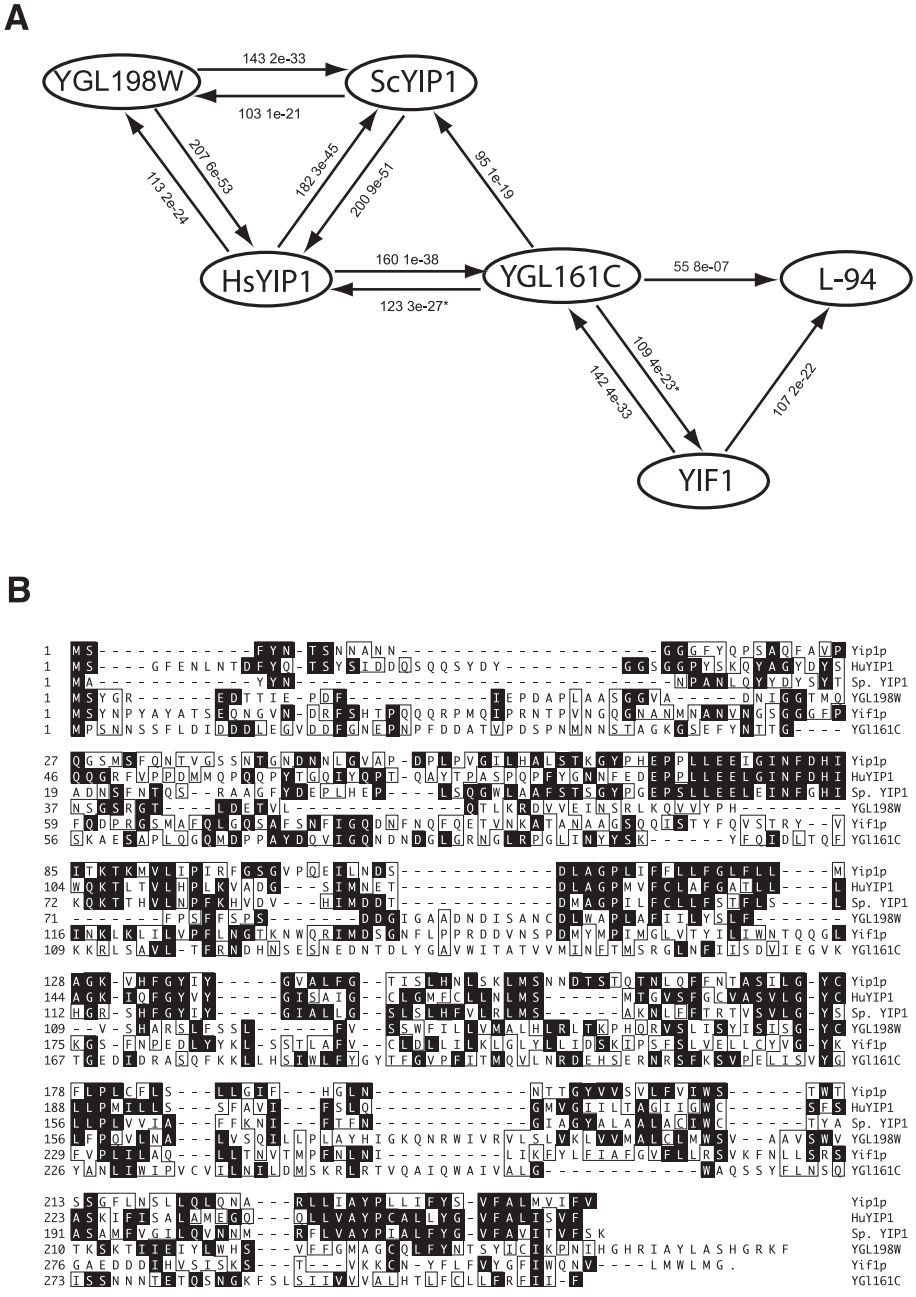


Fig. 1. A: PSI-BLAST identification of related YIP1 sequences. PSI-BLASTP 2.2.1 was performed on each protein sequence indicated. Relationships identified are indicated using lines whose directionality points from the query sequence towards the identified sequence. Analysis was carried out using a threshold value of $p = 0.01$ (p value = 0.1 indicated with asterisk) and BLOSUM 62 matrix against the non-redundant protein database consisting of 772 993 sequences. The complete set of statistical values for these sequence relationships is given in Table 3. B: Alignment of Yip1p with *S. cerevisiae* and human homologs. Sequence of Yip1p and comparison with full length cDNAs from *S. pombe* (SpYIP1), human YIP1 (HsYIP1), Yif1p and the novel *S. cerevisiae* ORFs YGL198W and YGL161C. The sequences were aligned in MegAlign (DNASTAR) using Clustal analysis [25] with a gap length penalty of 10. Amino acid residues are numbered according to the protein sequence. The shaded residues exactly match the consensus sequence, the boxed residues are standard functional groupings [26] of acidic (DE), basic (HKR), hydrophobic (AFILMPVW), and polar (CGNQSTY) residues. Sequence identity values are given in Table 4.

with an additional 1 ml of lysis buffer for 10 min at 4°C. Detergent-solubilized lysates were cleared by two sequential centrifugation steps in a microfuge for 5 min at 13 000 rpm. Samples were incubated with rocking for 30 min at 4°C with 20 μl of amylose resin (New England Biolabs). The bead-bound material was washed four times with lysis buffer. Similar procedures were followed for GST pull-downs except glutathione *S*-Sepharose resin (Pharmacia) was used to isolate the GST-tagged proteins. Proteins were eluted from the beads by boiling in SDS sample buffer. The proteins were analyzed by 10% SDS–PAGE gel electrophoresis and Western blotting with anti-GST antibody to detect the presence of the GST-tagged Rab proteins (for these purposes the anti-green fluorescent protein (GFP) antibody Santa Cruz Cat. No. SC-8334, lot G030 was used, this antibody recognized GST in Western blots with far higher avidity than GFP). Anti-MBP antibody (gift of G.R. Whittaker) was used at 1:6000 to detect MBP-tagged proteins. Secondary alkaline phosphatase-conjugated goat anti-rabbit antibodies (Kirkegaard and Perry Laboratories) were added in blocking buffer, followed by washing and chromogenic blot development with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (both from Bio-Rad) substrates in AP buffer (100 mM Tris pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$).

Protein expression under the control of the $GAL_{1/10}$ promoter was achieved by subcloning the ORF containing the Rab protein in frame with GST into the vector pRC337. These constructs (Table 2) were linearized with a restriction enzyme and integrated into the genome at the *LEU2* locus. Expression of a GST fusion protein of the correct molecular weight was determined by growing the cells in media containing 2% galactose as a carbon source. The plasmid containing MBP-tagged Yip1p (pRC1047) was constructed using polymerase chain reaction to insert a MBP tag cassette immediately after the initiating methionine in order to express the fusion protein under the control of the endogenous promoter and terminator in the yeast vector pRS426.

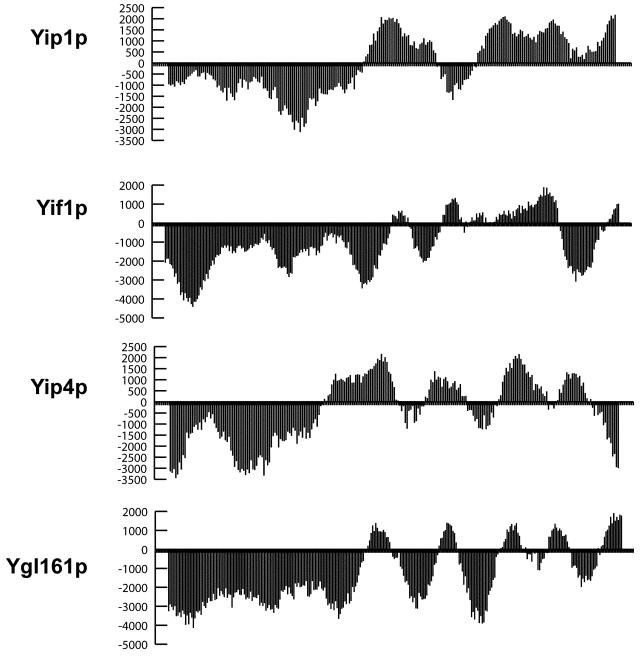


Fig. 2. TMpred plot of Yip1p, Yif1p, Ygl198p and Ygl161p. The TMpred plots for Yip1p, Yif1p, Ygl198p (Yip4p), and Ygl161p (Yip5p) were generated using the program TMpred with a 17 residue minimal and 33 residue maximal length of the hydrophobic part of the transmembrane helix. The TMpred plot shows the relative location of the hydrophobic/hydrophilic segments of the protein. Sequence data indicate a cytoplasmically oriented N-terminus and a hydrophobic C-terminal domain with several potential membrane-spanning/insertion segments.

Table 3
PSI-BLAST score values amongst YIP1-related proteins

Query	Subject sequence													
	ScYIP1		YGL198W		YGL161C		YIF1		HsYIP1		SpYIP1		L-94	
	Score (bits)	*E* value	Score (bits)	*E* value	Score (bits)	*E* value	Score (bits)	*E* value	Score (bits)	*E* value	Score (bits)	*E* value	Score (bits)	*E* value
ScYIP1	221	3e-57	103	1e-21	34	1.8	36	0.24	200	9e-51	184	4e-46	NF	
YGL198W	142	2e-33	300	7e-81	NF		NF		207	6e-53	160	1e-38	NF	
YGL161C	98	1e-19	NF		272	2e-72	109	4e-23	131	7e-30	91	1e-17	55	8e-07
YIF1	NF		NF		NF		320	7e-87	NF		NF		106	3e-22
HsYIP1	182	3e-45	113	2e-24	160	1e-38	33	2.2	296	1e-79	201	6e-51	NF	
SpYIP1	172	2e-42	114	5e-25	44	0.001	NF		216	9e-56	218	2e-56	NF	

PSI-BLASTP 2.2.1 was used to identify YIP1-related proteins. Analysis was carried out using a threshold value of $p = 0.01$ (except for YGL161C for which a p value = 0.1) and BLOSUM 62 matrix against the non-redundant protein database consisting of 772 993 sequences. Identified sequences converged after five iterations (YGL198W), six iterations (HsYIP1, ScYIP1), seven iterations (YIF1, YGL161C, SpYIP1). NF, not found.

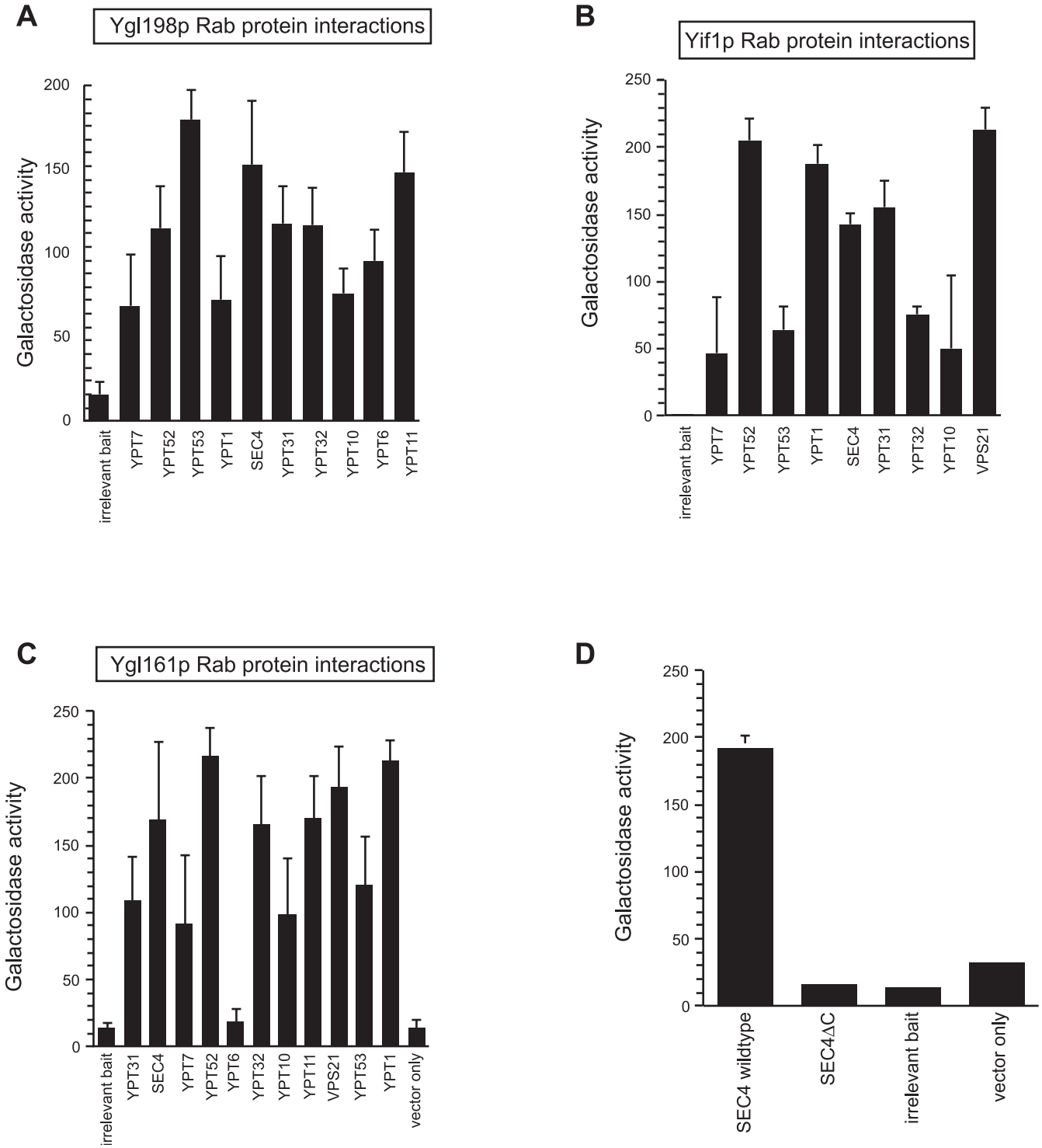


Fig. 3. Y2H interactions of Rab proteins with Yip1p family members. Pairs of constructs were coexpressed in the reporter strain Y190 and β-galactosidase activity (arbitrary units) in the resulting transformants was measured. At least 30 independent transformants were tested for each pair. The Rab protein bait constructs as indicated on the *x*-axis were tested against prey constructs of Ygl198p (A), Yif1p (B), and Ygl161p (C). D: Ygl161p prey construct tested against the Rab protein Sec4p with and without the C-terminal cysteines. A construct expressing Dss4p (pRC1253) was used as an irrelevant bait control.

3. Results and discussion

3.1. A family of Yip1p-related proteins

We used PSI-BLAST [14] with $p = 0.01$ and the BLOSUM62 matrix to identify Yip1p- and HsYip1p-related proteins. This analysis revealed one known ORF (*YIF1*) and one unknown ORF in *S. cerevisiae* (*YGL198W*), unknown ORF SPCC61.04c in *Schizosaccharomyces pombe*, together with numerous expressed sequence tag (EST) fragments from different species, indicating that *YIP1* is part of a gene family conserved among eukaryotes (Fig. 1A and Table 3). Using

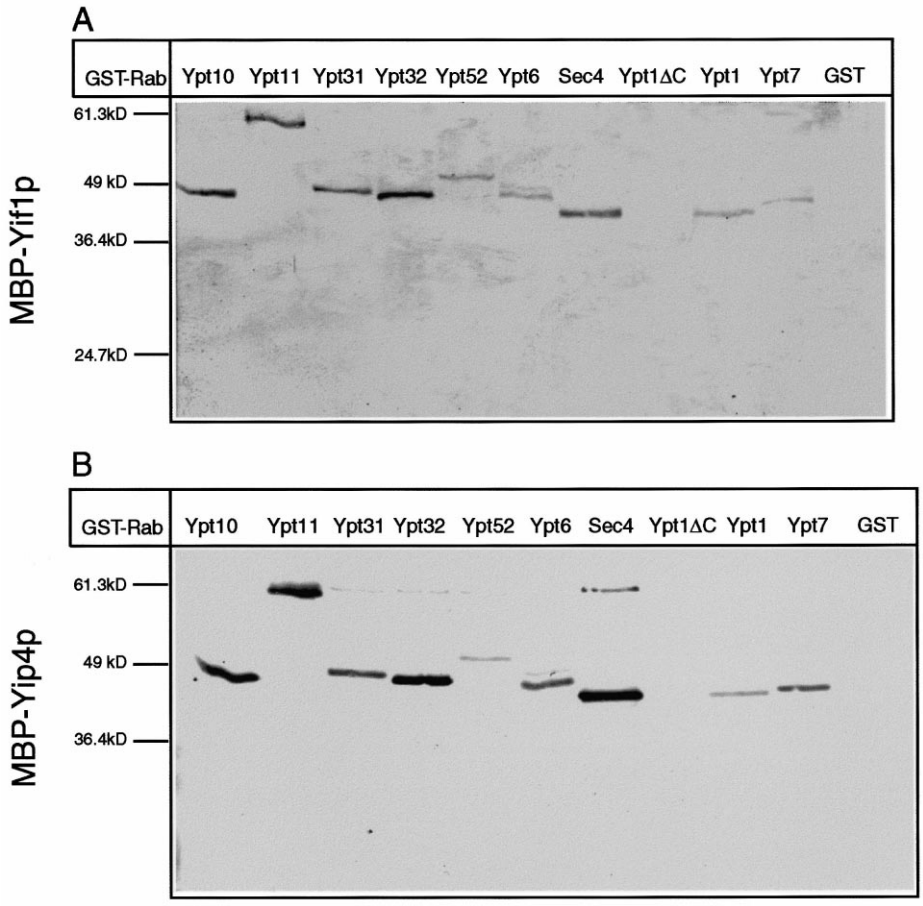


Fig. 4. Co-precipitation of Yip1p-related proteins with Rabs. The panel shows glutathione-resin pull-downs from yeast cells expressing various GST-Rab constructs as indicated. Although the level of expression of proteins in this system is not as high as recombinant expression, it was necessary to use a eukaryotic system due to the dependence of the interaction on correct C-terminal prenylation of the Rab protein. Lysates were prepared from cells expressing either GST alone or various GST-Rab constructs as indicated, together with MBP-tagged Yif1p, or Yip4p. Detergent-solubilized lysates containing 0.5% Tween 20 were incubated with amylose resin for 30 min at 4°C as described in Section 2. After washing, the bead-bound material was subjected to SDS–PAGE electrophoresis and analyzed by Western blotting. Membranes were probed with polyclonal anti-GST (dilution 1:800) to detect the bead-bound GST Rab fusion proteins. Relevant protein marker sizes are indicated. All Rab constructs were under the control of the $GAL_{1/10}$ promoter and were expressed by inducing with galactose for ~8 h.

Yif1p as the query for a PSI-BLAST search with the same parameters yielded the sequences L1–94 and an unknown ORF, YGL161C, with a convergence after seven iterations. We used the identified ESTs to generate a full length clone for human YIP1 which sequencing revealed was 38.3% identical to that of yeast *YIP1*. This sequence is identical to YIP1A, a human protein that has been reported to localize to endoplasmic reticulum exit sites [15] and also to the smooth muscle cell-associated protein-5 (accession number BAB20270). L1–94 is a partial sequence identified as a putative Rab5-interacting protein from human HeLa cells [16]. Yif1p is a protein previously isolated as a Yip1p interacting factor [17], although its homology to Yip1p was not identified. YGL198W and YGL161C are novel ORFs of unknown function in the *S. cerevisiae* database. The PSI-BLAST score (bits) and *E* values showing the relationships amongst these proteins are shown in Table 3 and a family alignment of the YIP1-related proteins is shown in Fig. 1B. This alignment includes only complete ORFs, L1–94 is not included in the alignment as it is only a partial sequence.

The YIP1-related ORFs identified in our analysis contain significant stretches of hydrophobic residues. We used the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html) to make a prediction of membrane-spanning regions and their orientation for YIP1, YIF1, YGL198W and YGL161C. The TMpred algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins using a combination of several weight matrices for scoring [18]. The results of this analysis are shown in Fig. 2. All of these proteins are small (Yip1p 27.1 kDa, Yif1p 35.5 kDa, Ygl198p 29.1 kDa, and Ygl161p 34.8 kDa) with significant hydrophobic segments which potentially span or are inserted into the membrane. All the Yip1p-related proteins share a predicted topology suggesting that they contain two domains. The N-terminus contains the only significant soluble portion of the protein and constitutes one putative domain. The remainder of the protein constitutes the C-terminal domain and contains several potential membrane-spanning segments. The N-terminal domain is oriented towards the cytosol and the C-terminal domain where the hydrophobic segments are located is largely buried in the membrane. Such a topology has been verified experimentally for Yip1p and Yif1p [9,10,17,19]; the results of our sequence analysis would suggest that this topology is also shared by Ygl198p and Ygl161p.

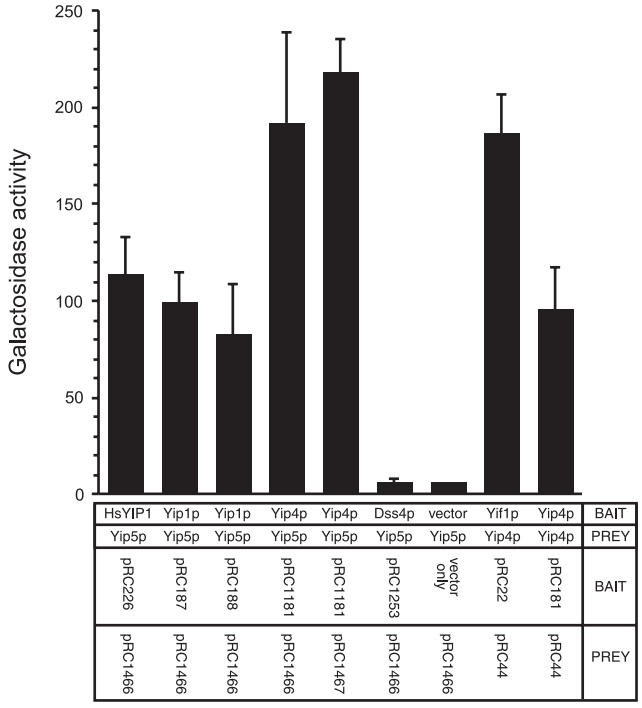


Fig. 5. Yip5p can interact with other YIP1 family members. Pairs of constructs were co-expressed in the reporter strain Y190 and β-galactosidase activity in the resulting transformants was measured. At least 30 independent transformants were tested for each pair. The construct pairs are indicated on the *x*-axis; pRC1466 and pRC1467 are two independent prey constructs which express Ygl161p, and pRC44 is a prey construct expressing Yip4p. pRC187 and pRC188 are two independent bait constructs which express Yip1p; pRC226, pRC181, pRC1253 and pRC22 are bait constructs expressing HsYIP1, Yip4p, Dss4p, and Yif1p respectively. Note the slight variability between two independent constructs expressing identical genes, a common feature of this Y2H system.

3.2. *Yip1p family members can interact with Rab proteins*

To investigate the Yip1p-related proteins further, we examined them for potential Rab protein interactions by both Y2H and biochemical pull-down experiments. We constructed a panel of Y2H constructs containing every Rab protein present in *S. cerevisiae* and tested them against the YIP1-related ORFs identified in Fig. 1A. Y2H analysis (Fig. 3A–C) showed that Yif1p, Ygl198p and Ygl161p are capable of interaction with several Rab proteins. In general we found weaker interactions with the Rab proteins Ypt6p and Ypt7p although these constructs still retained the ability to interact with yeast Rab-GDI in this system (data not shown). These data reveal that YIP1-related proteins are capable of binding to determinants shared by many Rab proteins. We have demonstrated this for Yif1p and two novel ORFs, YGL198W and YGL161C. In addition to Rab interactions, our analysis suggests these proteins share a common overall domain topology with a significant hydrophilic N-terminal segment that is cytoplasmically oriented and a largely hydrophobic C-terminal domain (Fig. 2). ORFs named *YIP2* (also termed *YOP1* [10]) and *YIP3* (also termed *PRA1* [20]) are already present in databases, however it is important to note that these ORFs are unrelated in primary sequence to Yip1p. By analogy with Yip1p and to avoid confusion, we suggest that the ORF YGL198W be named Yip4p (Ypt-interacting protein 4) and YGL161C be named Yip5p (Ypt-interacting protein 5).

A common feature of Rab proteins is the prenylation on two C-terminal cysteine residues by the enzyme geranylgeranyl transferase II [5]. To assess the contribution of this post-translational modification to YIP1 family member interaction we generated a Rab construct lacking its C-terminal cysteines. We chose Sec4p as the representative Rab protein as it interacts well with all the YIP1 family members tested. Y2H experiments, shown in Fig. 3D, demonstrated that interaction of Sec4p with Yip5p was completely dependent on its C-terminal cysteines and presumably on correct post-translational modification of the protein. Biochemical experiments (see below) demonstrated that Rab proteins also require prenylation for stable association with Yif1p and Yip4p.

3.3. *Interaction of Yif1p and Ygl198p with Rab proteins in cellular lysates*

To verify the Y2H interactions of YIP1 family members with Rab proteins with an independent technique, we made GST fusions of all yeast Rab proteins. These proteins were expressed under the control of the galactose promoter in yeast, where they would be expected to be correctly post-translationally modified and expressed in cells grown in media with galactose as a carbon source. Expression of a GST fusion of the expected size could be observed for each Rab protein (data not shown). We tested the GST-Rab protein fusions for biochemical interaction by co-precipitation with Yif1p and Yip4p. Yif1p and Yip4p were tagged with an N-terminal MBP fusion and expressed from endogenous promoters. The cellular lysates were incubated with amylose resin for 30 min at 4°C to pull down the MBP-Yif1p or MBP-Yip4p protein. After extensive washing, the bead-bound material was analyzed by SDS–PAGE and Western blotting. The Western blots were probed with anti-GST polyclonal antibody to detect any associated Rab proteins. The results of this analysis are shown in Fig. 4. MBP-Yif1p and Yip4p did not co-precipitate with GST alone, and neither with a Ypt1p construct lacking its C-terminal cysteines which are the sites of prenylation. Both MBP-Yif1p and Yip4p were able to interact with several different Rab proteins. These results parallel the data obtained in the two-hybrid assay and show that Yip1p-related proteins interact with diverse Rab proteins in cellular lysates. Do Rab proteins show different affinities for YIP1 proteins? A precise answer is beyond the scope of this study, however our data (Fig. 4) show that the amount of protein that is precipitated varies between individual Rab proteins. As the expression level of the Rab proteins does not vary significantly this suggests that Rab proteins may have preferences for the YIP1 family member with which they associate. This suggestion must be taken with caution however, as these experiments have utilized tagged proteins which may also influence the observed strength of interaction. If YIP1 family members display differential affinities for each Rab protein this would imply that prenylation, although necessary, is not the sole determinant for interaction.

3.4. *Interactions amongst Yip1p family members*

Yif1p was originally identified as a Yip1p binding partner although its identity as a YIP1-related sequence has not previously been identified [17]. In addition, several Y2H high-throughput screens have identified a plethora of Yip1p-interacting factors amongst which are included YGL198W (*YIP4*) and YGL161C (*YIP5*) [21–23]. These data suggest that Yip1p

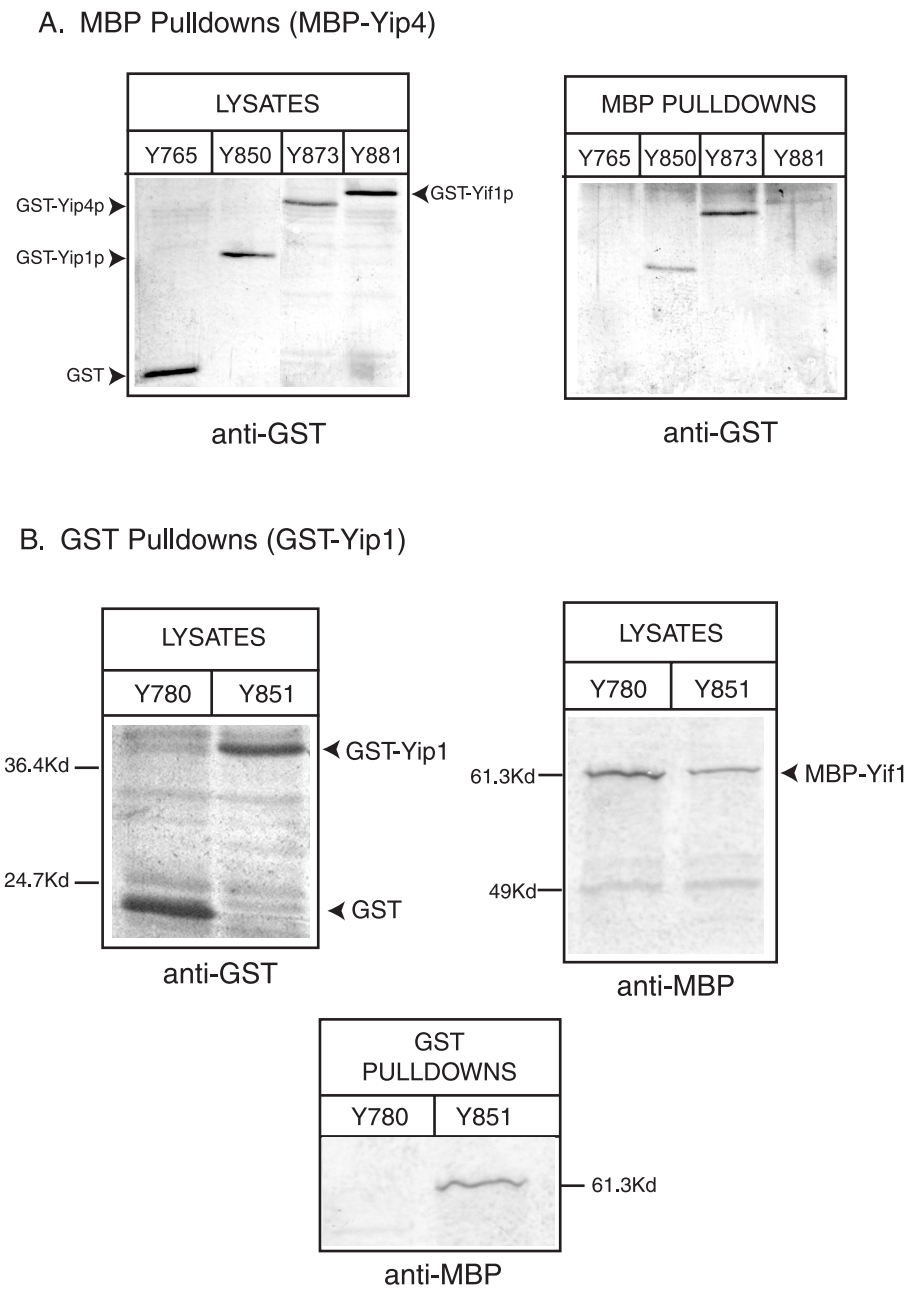
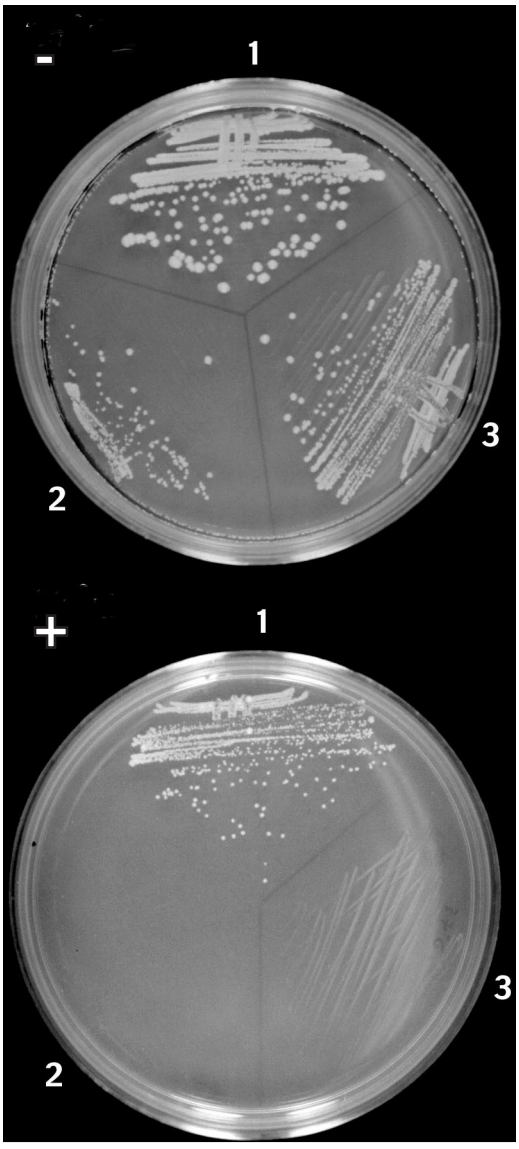


Fig. 6. Biochemical analysis of Yip1p interactions with the Yip1p family members Yif1p and Yip4p. Lysates were prepared from yeast cells expressing (A) GST alone, GST-Yip1p, GST-Yip4p or GST-Yif1p together with MBP-Yip4p (B) GST alone or GST-Yip1p together with MBP-Yif1p (B). Detergent-solubilized total cell lysates were incubated with GST beads (A) or amylose resin (B) for 30 min at 4°C as described in Section 2. After washing, the bead-bound material was subjected to SDS–PAGE electrophoresis and analyzed by Western blotting. Membranes were probed with polyclonal anti-GST (1:800) to detect GST-Yip1p (A) and polyclonal anti-MBP (1:6000) to detect MBP-Yif1p (B). Relevant protein marker sizes are indicated. GST-Yip1p, GST-Yip4p and GST-Yif1p but not GST alone could be detected after MBP-Yip4p pull-downs. MBP-Yif1p could be detected in RCY851 but not RCY780 after glutathione resin pull-downs.

has an ability to physically associate with other YIP1-related sequences. We wished to examine whether YIP1 family members in general share the ability to physically associate amongst themselves. We decided to test these interactions biochemically in deliberate pairwise combinations in both Y2H and biochemical co-precipitation experiments. We chose *YIP5* to test interactions in the Y2H system. The results of this analysis are shown in Fig. 5. Yip5p interacted very strongly with Yip4p and less strongly with Yip1p or the mammalian sequence HsYIP1. Yip4p was also able to self-associate with an interaction level comparable to its interaction with Yip1p. No interactions were observed with an irrelevant plasmid and the Yip5p plasmid showed no autoactivation. As expected, Yip1p and Yif1p also showed strong interactions in the Y2H system.

For the co-precipitation experiments, Yip1p, Yip4p and Yif1p were tagged with GST, Yip4p and Yif1p were tagged with MBP. GST alone was used as a control. An amylose resin pull-down from detergent-solubilized lysates of cells expressing MBP-Yip4p together with either GST alone, GST-



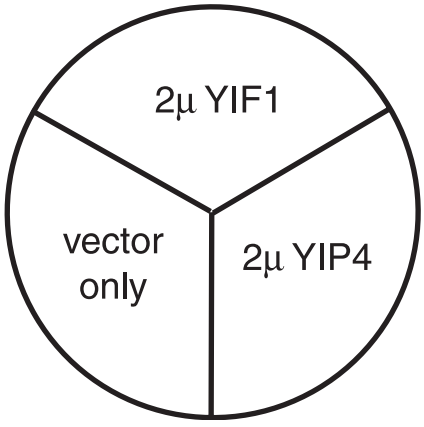


Fig. 7. High-copy plasmids containing the YIP1-related sequence *YIF1* can bypass the requirement for *YIP1*. Cells bearing their only copy of *YIP1* on plasmid containing the counter-selectable marker *URA3* were tested for ability to grow on 5-FOA after transformation with the *YIP1*-related ORFs *YIF1* and *YIP4*. Colonies transformed with multi-copy vectors containing (1) *YIF1*, (2) no insert control, or (3) *YIP4* (YGL198W) were tested for growth on synthetic media with and without 5-FOA to select against retention of the *YIP1* plasmid. Only cells containing multi-copy *YIF1* can survive the loss of the *YIP1*-containing plasmid on 5FOA.
←

Yip1p, GST-Yip4p or GST-tagged Yif1p revealed that Yip1p, Yif1p and Yip4p could be specifically co-precipitated with Yip4p (Fig. 6A). For Yif1p, we performed the reverse experiment, the GST alone or GST-Yip1p constructs were expressed in cells together with MBP-Yif1p and isolated from detergent solubilized extracts with glutathione agarose. The bead-bound material was probed for associated MBP-Yif1p with an anti-MBP antibody (Fig. 6B). This experiment demonstrated that Yif1p can physically interact with Yip1p, a result which confirms previous findings [17] and demonstrates that the tag used for our experiments does not interfere with protein–protein interactions. Our data confirm and extend the Y2H observations identified in high-throughput screens for Yip1p and suggest that the ability for YIP1 family members to interact amongst themselves is a common feature. Clearly, further experiments are required to ascertain the precise oligomeric nature of these YIP1 family member complexes and determine if the family members have particular preferences for association amongst themselves.

3.5. Overlapping functions of Yip1p family members

Our results demonstrate that YIP1 family members share a common domain topology, bind to Rab proteins in a prenylation-dependent manner and can physically associate amongst themselves. To what extent do the YIP1-related proteins have distinct and overlapping functions? We can begin to answer some of these questions through manipulation of the relevant genes in a genetically tractable organism such as yeast. One of the most stringent tests of function is to ask if one gene can functionally substitute for the deletion of the other. *YIP1* is an essential gene [9] so we tested *YIP4* and *YIF1* for the ability to complement *YIP1* function by asking if these genes could overcome the loss of *YIP1* when expressed from a multicopy plasmid. For this experiment, a strain was generated where the genomic copy of *YIP1* was deleted and viability was maintained by the inclusion of an episomal plasmid containing *YIP1* with a counter-selectable marker, *URA3*. The strain was transformed with a multi-copy plasmid encoding either *YIF1* or *YIP4* and plated on media containing fluoroorotic acid (5-FOA) to select against the *YIP1* gene. Remarkably, *YIF1* overexpression can overcome the loss of *YIP1*; however, *YIP4* was unable to do so (Fig. 7). There are several possible explanations for this result. The overexpression of a gene can suppress defects in other gene products by providing a similar function to that of the absent gene, by providing an alternative pathway or by bypassing the requirement for the absent gene if the suppressor gene lies downstream in the pathway. The fact that *YIF1* can substitute for the absence of *YIP1* indicates that it performs a similar function, further strengthening the suggestion that the YIP1 family may have shared functions and interacting partners. *YIP4* cannot substitute for the loss of *YIP1* indicating that this gene may function upstream of *YIP1* or may act on a different pathway even though these two genes share several potential interacting partners.

Groupings of small membrane proteins with significant hydrophobic segments such as those of the YIP1 family are difficult to establish by conventional means such as BLAST

Table 4
Sequence distances amongst YIP1-related proteins

	1	2	3	4	5	6	
1		30.2	38.3	12.9	14.9	13.3	Yip1p
2	64.3		38.8	12.1	13.2	12.5	HsYIP1
3	59.3	58.2		14.5	13.2	12.8	SpYIP1
4	82.8	83.9	82.5		13.8	11.5	YGL198W
5	84.7	84.5	85.9	79.1		11.9	Yif1p
6	84.2	84.5	85.0	88.2	86.9		YGL161C

The sequence distance table shows the calculated divergence and similarity of each pair of sequences aligned by the Clustal method as outlined in Fig. 1

algorithm searches and must be supported by additional experimental criteria. We propose that for the YIP1-related family, these criteria are: (i) a topology that includes a significant N-terminal hydrophilic domain that faces the cytosol with an hydrophobic C-terminal domain, (ii) the ability to interact with Rab proteins in a manner dependent on C-terminal prenylation, and (iii) the ability to associate with other members of the YIP1 family. We have demonstrated the unknown ORFs YGL198W (*YIP4*) and YGL161C (*YIP5*) are also Rab-interacting factors and bona fide Yip1p homologs even though they share very little sequence similarity (Table 4). The putative Rab5-interacting protein L1–94 shares two of these criteria [16] and we predict it also to be a member of the YIP1 protein family.

What is the cellular role played by Yip1p-related proteins? One possibility is that they serve as membrane proteins which aid in the recruitment of Rab proteins from the cytosol onto membranes, enabling Rab proteins to be correctly localized and used for many rounds of vesicle transport. Our data suggest that YIP1-related proteins are potential membrane counterparts to Rab-GDI. Similarly to Rab-GDI, they are biochemically capable of interacting with different Rab proteins in a manner dependent on the C-terminal prenylation, perhaps indicating that they can compete with Rab-GDI for Rab protein association. Although there is a plethora of evidence indicating that Rab proteins act downstream of vesicle budding, it is becoming apparent that Rab proteins may also play critical roles in vesicle biogenesis [2]. One rationalization for this may be that a functional vesicle must be equipped with the membrane components required for tasks at a later stage. V-SNAREs, for example, are required for fusion with the acceptor membrane, so these proteins must be included into nascent vesicles with high fidelity. Rab proteins too must be incorporated into the transport vesicle, implying a link between the Rab recruitment machinery and vesicle biogenesis. In support of this idea, Yip1p has been observed to interact with the SNARE protein *TLG1* [23] and we have recently obtained information that Yip1p will interact with the v-SNARE *SNC2* in the Y2H system (unpublished data). Although these data are preliminary and we do not know how far this extends to other YIP1 family members, it is tempting to speculate that there is a functional significance to this interaction. Further strengthening this suggestion is the finding that Yip1p and Yif1p have been observed to be selectively packaged into COPII vesicles in vitro [24], perhaps providing a link between YIP1 family members, Rab proteins and the vesicle biogenesis machinery. Clearly much remains to be understood about these important and intriguing membrane proteins.

Acknowledgements: Many thanks to Gary Whittaker for critical reading of the manuscript and his generous gift of anti-MBP antibody and to Wenyan Zhu for excellent technical assistance. M.C. is the recipient of Army Predoctoral Fellowship DAMD17-00-1-0218. This work was supported in part by the USDA Animal Health and Disease Research Program, American Heart Association Grant 0030316T, and NSF Grant MCB-0079045 (to R.C.).

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