Pan1p, Yeast eps15, Functions as a Multivalent Adaptor That Coordinates Protein–Protein Interactions Essential for Endocytosis

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Abstract. A genetic screen for factors required for endocytosis in the budding yeast *Saccharomyces cerevisiae* previously identified *PAN1*. Pan1p is a homologue of the mammalian protein eps15, which has been implicated in endocytosis by virtue of its association with the plasma membrane clathrin adaptor complex AP-2. Pan1p contains two eps15 homology (EH) domains, a protein–protein interaction motif also present in other proteins that function in membrane trafficking. To address the role of Pan1p and EH domains in endocytosis, a yeast two-hybrid screen was performed using the EH domain–containing region of Pan1p. This screen identified yAP180A, one of two yeast homologues of a class of clathrin assembly proteins (AP180) that exhibit in vitro clathrin cage assembly activity. In vitro binding studies using GST fusion proteins and yeast extracts defined distinct binding sites on yAP180A for Pan1p and clathrin. yAP180 proteins and Pan1p, like actin, localize to peripheral patches along the plasma membrane. Mammalian synaptojanin, a phosphatidylinositol polyphosphate-5-phosphatase, also has been implicated in endocytosis recently, and three synaptojanin-like genes have been identified in yeast. We observed genetic interactions between the yeast *SJL1* gene and *PAN1*, which suggest a role for phosphoinositide metabolites in Pan1p function. Together with other studies, these findings suggest that Pan1p coordinates regulatory interactions between proteins required for both endocytosis and actin-cytoskeleton organization; these proteins include the yAP180 proteins, clathrin, the ubiquitin–protein ligase Rsp5p, End3p, and synaptojanin. We suggest that Pan1p (and by extension eps15) serves as a multivalent adaptor around which dynamic interactions between structural and regulatory components of the endocytic pathway converge.

T HE composition of proteins and lipids in the plasma membrane is maintained by selective sorting of plasma membrane components and by regulation of the processes of exocytosis and endocytosis. This ensures proper control of many essential cellular processes such as nutrient uptake and receptor-mediated signal transduction. Newly synthesized plasma membrane proteins and lipids are delivered by exocytosis, whereas other proteins and lipids are removed by endocytosis. Both of these membrane trafficking events are mediated by transport vesicles that form at sites of cargo concentration, separate from the donor membrane, and target to an appropriate acceptor organelle (Rothman and Orci, 1992; Schekman and Orci, 1996; for review see Robinson, 1997).

The budding yeast *Saccharomyces cerevisiae* has proven to be a powerful model system for elucidating membrane-trafficking pathways; one such pathway is endocytosis. Traditionally, endocytosis in yeast has been monitored by following the internalization of the seven transmembrane domain pheromone receptors, Ste2p and Ste3p (Davis et al., 1993; Raths et al., 1993). In addition to numerous *end* mutants (e.g., Raths et al., 1993; Munn and Riezman, 1994; Munn et al., 1995), a temperature-sensitive clathrin heavy chain mutant (chc^{ts})[1] has been shown to exhibit kinetic defects in pheromone receptor internalization (Tan et al., 1993). Clathrin-dependent receptor–mediated endocytosis is, to date, the best characterized endocytic pathway in both yeast and mammalian cells (for review see Pearse and Robinson, 1990; Schmid, 1997). In this pathway, plasma membrane–localized receptors bind to their ligands and then associate with the heterotetrameric adaptor complex AP-2. AP-2 in turn recruits and polymerizes

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1. *Abbreviations used in this paper*: CALM, clathrin assembly lymphoid myeloid leukemia gene; *chc*, clathrin heavy chain; EH, eps15 homology; GFP, green fluorescent protein; IP, inositol polyphosphate; ORF, open reading frame; PI, polyphosphoinositide; YPD, yeast extract-peptone-dextrose.

clathrin, which ultimately leads to the formation of clathrin-coated vesicles. These vesicles then uncoat, the adaptors and clathrin are recycled, and the newly uncoated vesicles dock and fuse with an early endosome. Yeast genes encoding clathrin heavy and light chains, as well as subunits of a heterotetrameric adaptor AP-2-like complex, have been identified and characterized (Payne and Schekman, 1985; Silveira et al., 1990; Phan et al., 1994; Rad et al., 1995).

Ubiquitination and actin cytoskeleton dynamics have also been shown to play essential roles in mediating or regulating endocytosis. Ubiquitination of plasma membrane proteins functions as an endocytosis signal for plasma membrane proteins in yeast and is suggested to do so in mammalian cells as well (Galan et al., 1994; Hicke and Riezman, 1996; Roth and Davis, 1996; Strous et al., 1996). Actin is required for endocytosis in yeast (Kubler and Riezman, 1993; Munn et al., 1995), and the actin monomer sequestering drug latrunculin A depolymerizes actin filaments and inhibits receptor-mediated endocytosis in both yeast (Lappalainen and Drubin, 1997) and mammalian cells (Lamaze et al., 1997).

Studies on nerve terminals have implicated several interacting proteins in the endocytosis of synaptic vesicle membranes. Some of these include (*a*) the GTPase dynamin, which promotes fission of endocytic vesicles (Vallee et al., 1993), (*b*) amphiphysin, which has an SH3 domain that binds to the proline-rich tail of dynamin (Lichte et al., 1992; David et al., 1996), and (*c*) synaptojanin, a phosphatidylinositol polyphosphate-5-phosphatase that, like dynamin, also binds to the SH3 domain in amphiphysin via a proline-rich region (McPherson et al., 1996; de Heuvel et al., 1997). Whereas yeast as yet do not appear to have a dynamin-like protein that serves an analogous role in endocytosis, yeast do contain amphiphysin (Bauer et al., 1993; Sivadon et al., 1995) and synaptojanin homologues (Luo and Chang, 1997; Srinivasan et al., 1997). The yeast amphiphysin homologues Rvs161p/End6p and Rvs167p are required for the internalization step of endocytosis in yeast (Munn et al., 1995). There are three synaptojanin homologues in yeast, *SJL1*, *SJL2*, and *SJL3* (Srinivasan et al., 1997). Deletion of all three is lethal; however, double mutants are viable but, consistent with a role for *SJL* gene products in endocytosis, do exhibit marked defects in plasma membrane structure and actin cytoskeleton organization (Srinivasan et al., 1997).

Pan1p is another protein required for endocytosis and actin cytoskeleton organization in yeast (Tang and Cai, 1996; Wendland et al., 1996; Tang et al., 1997; Zoladek et al., 1997). Pan1p is a yeast homologue of eps15, and both proteins contain three distinct protein–protein interaction domains: (*a*) amino-terminal eps15 homology (EH) domains (Wong et al., 1995), (*b*) central coiled-coil domains, and (*c*) carboxy-terminal proline-rich regions. eps15, which contains three EH domains, has been implicated in endocytosis in mammalian cells due to its physical association with clathrin and the plasma membrane adaptor complex AP-2 (Benmerah et al., 1996; van Delft et al., 1997*b*). Pan1p contains two EH domains and interacts with another EH domain–containing protein, End3p (Tang et al., 1997), that is also required for endocytosis (Raths et al., 1993; Benedetti et al., 1994). Thus, EH domains are postulated to play a role in protein–protein interactions required for endocytosis.

Finally, clathrin assembly and adaptor proteins are required for clathrin-dependent membrane trafficking steps. Indeed, the heterotetrameric adaptor complex AP-2 is required for clathrin-dependent endocytosis in mammalian cells (Lin et al., 1991; Smythe et al., 1992). Interestingly, a distinct, nonheterotetrameric clathrin assembly protein called AP180 also binds to clathrin and promotes its assembly into cages (Ahle and Ungewickell, 1986; Keen and Black, 1986; Prasad and Lippoldt, 1988). AP180 has also been called AP-3, F1-20, pp155, and NP185 (Murphy et al., 1991; Zhou et al., 1993), and it is a neuronal-specific (Kohtz and Puszkin, 1988; Sousa et al., 1992) synaptic (Perry et al., 1991, 1992; Sousa et al., 1992) phosphoprotein (Keen and Black, 1986; Morris et al., 1990; Zhou et al., 1992) that localizes to clathrin-coated pits in synaptic nerve terminals (Takei et al., 1996). Until recently, AP180 was thought to serve a neuronal-specific function, perhaps in the endocytic recycling of synaptic vesicles (Zhou et al., 1993). Whereas there may be a neuron-specific role for AP180, the existence of yeast homologues, as well as a related and ubiquitously expressed human protein called <u>c</u>lathrin <u>a</u>ssembly <u>l</u>ymphoid <u>m</u>yeloid leukemia gene (CALM; Dreyling et al., 1996), suggests that these proteins may also participate more widely in membrane trafficking events in other cell types.

In this study, we have identified two new members of the AP180 family, the homologous yeast proteins yAP180A and yAP180B. We show that these proteins bind to clathrin and to Pan1p to form a complex that is localized to punctate spots at the cell periphery. A genetic interaction between *PAN1* and the *SJL1* gene was also uncovered. Based on these and other observations, we propose that Pan1p coordinates the interactions of several structural and regulatory proteins required for endocytosis in yeast.

Materials and Methods

Strains, Media, and Materials

The strains used in these studies are listed in Table I. Yeast strains were grown in standard yeast extract-peptone-dextrose (YPD) or synthetic medium with dextrose supplemented with the appropriate amino acids as required for plasmid maintenance. Bacterial strains were grown on standard media supplemented with 100 μg/ml ampicillin or 30 μg/ml kanamycin, as appropriate, to maintain plasmids. Materials were purchased from Fisher Scientific Co. (Fairlawn, NJ) or Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Databases and Internet Sources

Searches and comparisons were conducted using the *Saccharomyces* Genome database (http://genome-www.stanford.edu/Saccharomyces/), the Yeast Proteome database (http://www.proteome.com/YPDhome.html), the XRef database (http://www.ncbi.nlm.nih.gov/XREFdb/), and ProSite (http://expasy.hcuge.ch/sprot/prosite.html).

Plasmid Construction

Standard recombinant DNA techniques were performed as previously described (Maniatas et al., 1982) with reagents obtained from Boehringer Mannheim Corp. (Indianapolis, IN) or New England Biolabs Inc. (Beverly, MA). The plasmids used in these studies are described in Table I.

The *YAP1801* and *YAP1802* genes were obtained by PCR amplification of chromosomal DNA to produce pYAP180A and pYAP180B, respectively. The *yap1801::HIS3* deletion construct removes 84% of the

Table I. Genotypes of Yeast Strains and Plasmid Descriptions

Strain	Genotype	Source
SEY6210	*MATα ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9*	Emr lab strain collection
SEY6210**a**	*MAT**a** ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9*	Emr lab strain collection
SEY6210**a**/α	*MATα ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9*	Emr lab strain collection
	*MAT**a** ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9*	
BWY20	*MAT**a** pan1-20 ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9*	This study
ΔSJL2/ΔSJL3	*MATα ura3-52 his3-Δ200 trp1-Δ63 leu2-Δ1 lys2-801 ade2-101 SJL2::HIS3 SJL3::TRP1*	Srinivasan et al., 1997
BWY236	*MAT**a** pan1-20 ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9*	This study
BWY249	*MAT**a** pan1-20 ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9 sjl1::HIS3 [pPAN1/416]*	This study
BWY250	*MATα ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9 yap1801::HIS3*	This study
BWY256	*MATα ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9 yap1802::LEU2*	This study
BWY255	*MATα ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9 yap1801::HIS3 yap1802::LEU2*	This study
HF7c	*MAT**a** ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 ade2-101 gal4-542 gal80-538*	Clonetech, Stonybrook, NY
	LYS2::GAL1-HIS3 URA3::(GAL4 17mers)₃ -CYC1-lacZ	
SFY526	*MAT**a** ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 ade2-101 can^r gal4-542 gal80-538*	Clonetech
	URA3::GAL1-lacZ	
TVY614	*MAT**a** ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9 pep4::LEU2 prb1::HIS3 prc1::HIS3*	Emr lab strain collection
PSY15C	*MATα end1-1 ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9*	Emr lab strain collection
BWY237	*MATα ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112 lys2-801 suc2-Δ9 pan1-like(YBL047c)::HIS3*	This study
TGY413-6D	*MATα erg6(ise1) ura3-52 his3-Δ200 leu2-3, 112 lys2-801 suc2-Δ9*	Graham et al., 1993

Plasmid	Description	Construction	
pPAN1	*PAN1*	XmnI-DraI fragment in pRS415	Wendland et al., 1996
pPAN1.1	Pan1p EH1-2 $_{(96–713)}$	MscI-ClaI fragment of pPAN1 in SmaI of pGBT9	This study
pPAN1.2	Pan1p EH1 $_{(96–403)}$	Drop out SpeI-SalI fragment of pPAN1.1	This study
pPAN1.3	Pan1p EH2 $_{(96–145/403–713)}$	Drop out StyI-SalI fragment of pPAN1.1	This study
pPAN1.4	Pan1p EH2 $_{(544–713)}$	NciI-ClaI of pPAN1 in BamHI of pGBT9	This study
pPAN1.5	Pan1p $_{(96-1480)}$	MscI-SalI (polylinker site) fragment of pPAN1 in SmaI of pGBT9	This study
pBS(-RV-HII)	cloning vector	pBS(KS)II- cut with EcoRV and HindII and religated	This study
pYAP180A	YAP1801	EcoRI fragment in pBS(-RV-HII)	This study
pYAP180A.TA	YAP1801	EcoRI fragment in pCR2.1 (TA cloning vector)	This study
pYAP180A.1	yAP180A $_{(432–637)}$	Isolated in two-hybrid screen, pGADGH vector	This study
pYAP180A.2	yAP180A $_{(439–637)}$	Isolated in two-hybrid screen, pGADGH vector	This study
pYAP180A.3	yAP180A $_{(24–637)}$	Blunt BamHI fragment of pYAP180A.TA in blunt BamHI of pGADGH	This study
pYAP180AΔ	YAP1801::*HIS3*	*HIS3* replacement of EcoRV-ClaI fragment of pYAP180A	This study
pYAP180B	YAP1802	HindIII fragment in pBS(KS)II-	This study
pYAP180B.1	yAP180B $_{(429-568)}$	DraI fragment of pYAP180B in SmaI of pGADGH	This study
pYAP180BΔ	YAP1802::*LEU2*	3′ HindII fragment of yAP180B in pBS(KS)II-, 5′ AvaI fragment into SmaI site. *LEU2* inserted between	This study
pGEX-KG-KAN	cloning vector	pGEX-KG vector with kanamycin resistance gene inserted	This study
pYAP180A.4	GST-yAP180A $_{(24–637)}$	Blunt BamHI fragment of pYAP180A.TA in blunt EcoRI of pGEX-KG-KAN	This study
pYAP180A.5	GST-yAP180A N1	pYAP180A.4 cut with NsiI and XhoI, blunted and religated	This study
pYAP180A.6	GST-yAP180A C1	pYAP180A.4 cut with SmaI and NsiI, blunted and religated	This study
pYAP180A.7	GST-yAP180A C2	pYAP180A.6 cut with SacI and religated	This study
pYAP180A.8	GST-yAP180A C3	Blunt SacI fragment of pYAP180A.7 into blunt XbaI of pGEX-KG-KAN	This study
pYAP180A.9	GST-yAP180A C4	pYAP180A.8 cut with SacI and PstI, blunted and religated	This study
pYAP180A.10	GST-yAP180A C4	Blunt SacI=PstI fragment of pYAP180A.8 into blunt EcoRI of pGEX-KG-KAN	This study
pYAP180B.2	GST-yAP180B C1	DraI fragment of pYAP180B in SmaI of pGEX-KG-KAN	This study
pGOGFP/416	cloning vector	GFP fusion cassette in pRS416	C. G. Odorizzi, unpublished
pGOGFP/426	cloning vector	GFP fusion cassette in pRS426	Cowles et al., 1997
pYAP180A.11	GFP-yAP180A	PCR-generated SalI-KpnI fragment in pGOGFP/416	This study
pYAP180A.12	GFP-yAP180A	PCR-generated SalI-KpnI fragment in pGOGFP/426	This study
pYAP180B.3	GFP-yAP180B	PCR-generated SalI-KpnI fragment in pGOGFP/416	This study
pYAP180B.4	GFP-yAP180B	PCR-generated SalI-KpnI fragment in pGOGFP/426	This study
pMS1	*SJL1::HIS3*	PCR-generated 5′ and 3′ fragments in pBS(KS)II- with *HIS3* inserted between	M.N.J. Seaman, unpublished

open reading frame (ORF), corresponding to deletion from 39 nucleotides upstream of the start ATG to amino acid 537. This plasmid was linearized with XhoI and ScaI to allow homologous recombination and gene replacement. The *yap1802::LEU2* deletion construct results in deletion of amino acids 25–516, or 86% of the *YAP1802* ORF. To delete the *YAP1802* locus, this *LEU2* deletion construct was linearized with SnaBI. The deletion strains were generated by sequential deletion of *YAP1801* and *YAP1802* in an SEY6210a/α diploid, followed by sporulation and dissection. GFP–

yAP180 fusion constructs were made using oligonucleotides and PCR to introduce an in-frame SalI site upstream of the start ATG.

Yeast Two-Hybrid Assays

The strain HF7c was first transformed with pPAN1.1 followed by transformation with a yeast cDNA library constructed in the GAL4 activator

domain plasmid pGADGH (*LEU2*). Colonies that arose on Trp$^-$/Leu$^-$/His$^-$ selective plates were replica plated and one set transferred to filter disks, (Whatman Inc., Clifton, NJ) lysed by freezing in liquid N$_2$, and incubated at 30°C with 0.1 M NaPO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 0.27% β-mercaptoethanol, 0.33 mg/ml XGal, pH 7.0. Blue colonies were selected, retested, and *LEU2* plasmids isolated and sequenced as previously described (Wendland et al., 1996). Quantitative β-gal assays were performed by transforming the strain SFY526 with the appropriate plasmids, selecting three colonies from each transformation, and performing the assay in triplicate for each colony as described in the Clonetech Matchmaker II manual (Stonybrook, NY).

GST Fusion Protein/Yeast Extract–binding Experiments

Lysates from bacteria expressing GST fusion proteins were produced and incubated with glutathione–agarose beads to purify the GST fusion proteins. For this, glutathione–agarose beads were equilibrated with PBS, incubated with lysate for 30 min at 4°C, washed five times with PBS, and resuspended with a volume of PBS to yield ~50% beads/vol. 20 µl of beads containing a total of 5–10 µg fusion protein were used for each binding experiment.

Yeast extracts were generated by growing TVY614, PSY15C, and BWY237 in YPD to OD$_{600}$ 2–8. P16 membranes (for Pan1p binding) or P100 membranes (for clathrin binding) were isolated as described (Mueller and Branton, 1984). The pellets were extracted with 1% TX-100 in 100 mM MES, 0.5 mM MgCl$_2$, 1 mM EGTA, 0.2 mM DTT, 0.1 mM AEBSF (Calbiochem-Novabiochem Corp., La Jolla, CA), 0.02% NaN$_3$, pH 6.5 at 4°C for 1–3 h, followed by centrifugation at 15,000 *g* for 5 min at 4°C. The protein concentration of the resulting extracts ranged from 1–3 mg/ml. Approximately 150 µg of extract was incubated with 20 µl 50% GST fusion protein–bound beads at 4°C for 1 h, followed by washing three to five times with 1 ml PBS + 0.1 mM AEBSF. Liquid was removed from the beads, 40 µl protein sample buffer added, and 5–10 µl loaded per lane on SDS-PAGE minigels. The gels were then stained with either silver or Coomassie blue, or transferred to nitrocellulose for immunoblotting with affinity-purified rabbit polyclonal antibodies, recognizing Pan1p (Nos. 91 or 92; provided by Sachs, A., University of California, Berkeley, CA; Boeck et al., 1996), or yeast clathrin light chain (a gift from Payne, G., UCLA, Los Angeles, CA), or a mouse monoclonal recognizing yeast clathrin heavy chain (SKL1; Lemmon et al., 1988).

Coimmunoprecipitation Experiments

Appropriate yeast strains were grown in YPD to midlog and 20 OD$_{600}$ were harvested. The cells were spheroplasted as previously described (Rieder et al., 1996), and homogenized at 5 OD/ml in a Kontes glass dounce (Kimble/Kontes Glass, Vineland, NJ) with 10 up-and-down strokes in 1% Triton X-100, 0.2 M sorbitol, 50 mM KOAc, 20 mM Hepes, 2 mM EDTA, pH 6.8. The homogenate was extracted on ice for 15 min, and then centrifuged at 13,000 *g* for 5 min. Equal aliquots of 5 OD equivalents of lysate were distributed to eppendorf tubes and either no antibodies or 4 µl of rabbit polyclonal α-Pan1p was added and incubated at 4°C for 2 h with agitation. 100 µl protein A–Sepharose beads were next added and incubated for an additional hour, followed by washing twice with 1 ml PBS. The last traces of liquid were removed with a Hamilton syringe, and the bound proteins eluted with SDS-PAGE protein sample buffer. An 8% acrylamide gel was run with 2 OD equivalents loaded, with 0.1 OD equivalent of total lysate loaded for comparison. After transfer to nitrocellulose, the paper was incubated with Ponceau S to stain the rabbit IgG heavy and light chains, and this portion of the blot was excised. The remainder of the blot was processed for labeling with the yAP180 antiserum and developed using ECL reagents. Immunoprecipitation of Pan1p was confirmed by immunoblotting.

Subcellular Fractionation

Appropriate yeast strains were grown in YPD to midlog and 20 OD$_{600}$ harvested. The cells were spheroplasted as previously described (Rieder et al., 1996), and homogenized in a Kontes glass dounce with 10 up-and-down strokes. Sequential differential centrifugation at 300, 13,000, and 100,000 *g* generated P13, P100, and S100 fractions that were then resolved by SDS-PAGE and transferred to nitrocellulose for immunoblotting.

Generation of Antibodies

To produce a polyclonal antiserum that recognizes the yAP180 proteins, rabbits were immunized with the GST–yAP180A fusion protein encoding amino acids 24–637 (Scantibodies Inc., Ramona, CA). An affinity column was prepared by covalently cross-linking the antigen to cyanogen bromide–activated beads for affinity purification of anti-yAP180 immunoglobulins. The peak fractions were pooled and diluted 1:500 for immunoblots.

GFP Fusion Protein Microscopy

Yeast cells expressing GFP fusion proteins were grown in selective medium to midlog phase and examined using a Delta Vision deconvoluting light microscope on a Silicon Graphics workstation. Optical sections were collected at 200–300 nm steps in the z-axis. Extracts from GFP fusion protein–expressing cells also were examined by immunoblotting with an affinity-purified antiserum directed against GFP (a gift provided by Zuker, C., and R. Tsien, UCSD, La Jolla, CA).

pan1–20 and Synaptojanin-like Genetic Interactions

To test for lethality of *pan1–20 sjl1Δ*, BWY249 was mated to SEY6210 followed by sporulation and tetrad dissection. To test for lethality of *pan1–20* in combination with *sjl2Δ* or *sjl3Δ*, a diploid strain was produced by mating BWY20 with ΔSJL2 ΔSJL3, followed by sporulation and dissection. The genotype of the viable spores was determined by replica plating to appropriate selective media, and the genotypes of inviable spores deduced from the viable counterparts in the same tetrad. *pan1–20 sjl2Δ* and *pan1–20 sjl3Δ* spores were viable, but grew more slowly than wild-type or single mutants. The only *pan1-20 sjl1Δ* spores recovered also contained the wild-type *PAN1* gene on a plasmid; inviable spores that did not grow beyond eight cell colonies were deduced to have a *pan1–20 sjl1Δ* genotype, and did not contain the pPAN1 plasmid. The corresponding open reading frame (ORF) designations for the yeast synaptojanin-like genes are: *SJL1*, YIL002c; *SJL2*, YNL106c; *SJL3*, YOR109w.

Results

Yeast Two-Hybrid Screen with Pan1p EH Domains Identifies yAP180A

Pan1p, a protein required for endocytosis in yeast, contains two EH domains (Tang and Cai, 1996; Wendland et al., 1996). EH domains are found in several proteins implicated in endocytosis, including Pan1p, End3p, and eps15. To understand the role of EH domains and how these domains influence Pan1p function, a two-hybrid screen (Fields and Song, 1989) was used to identify proteins that interact with the EH domains of Pan1p. The bait was comprised of the GAL4 DNA–binding domain fused to amino acids 96–713 of Pan1p, which includes both EH domains (Fig. 1 *A*), and was screened against a yeast cDNA library fused to the prey plasmid encoding the GAL4-activator domain. 250,000 colonies were tested for positive interactions by selecting for His$^+$, followed by examining the expression of β-galactosidase using a standard blue/white screen (see Materials and Methods). Of 50 blue colonies selected, only two turned blue very rapidly (blue color developed in 15 min), indicating a high level of β-galactosidase expression. The other 48 colonies were a much weaker blue (blue color developed in ≥ 2 h), and retesting allowed for selection of the 12 strongest blue colonies for further analysis. These colonies were isolated, and the prey plasmids recovered and sequenced. The plasmids from the very blue colonies encoded in-frame fusions between the GAL4 activator domain and the carboxy terminus of the yeast ORF YHR161c: one fusion began at amino acid 432, whereas the other began at residue 439.

Database searches with the predicted protein sequence of YHR161c revealed significant homology to four other proteins. The most similar protein was another yeast ORF designated YGR241c, which is predicted to encode a protein 43% identical to YHR161c. The other homologous proteins were the mammalian neuronal-specific protein AP180 (Murphy et al., 1991; Zhou et al., 1992, 1993), a ubiquitously expressed human protein termed CALM (Dreyling et al., 1996), and C32E8.10, a *Caenorhabditis elegans* gene (Wilson et al., 1994). The primary region of homology between the yeast, mammalian, and *C. elegans* proteins resides in the amino-terminal 300 residues (Fig. 1 *B*). Because of the significant similarity between the yeast proteins and the previously described mammalian proteins, the yeast proteins corresponding to YHR161c and YGR241c have been designated yAP180A and yAP180B, and the genes have been named *YAP1801* and *YAP1802*, respectively. Of the 12 weaker blue prey plasmids sequenced, three corresponded to fusions within the uncharacterized ORFs YOL101c (beginning at amino acid 254), YDL161w (beginning at amino acid 248), and YOR105w (beginning at amino acid 14), whereas the other nine encoded either out-of-frame fusions or fusions with untranslated regions of chromosomal DNA.
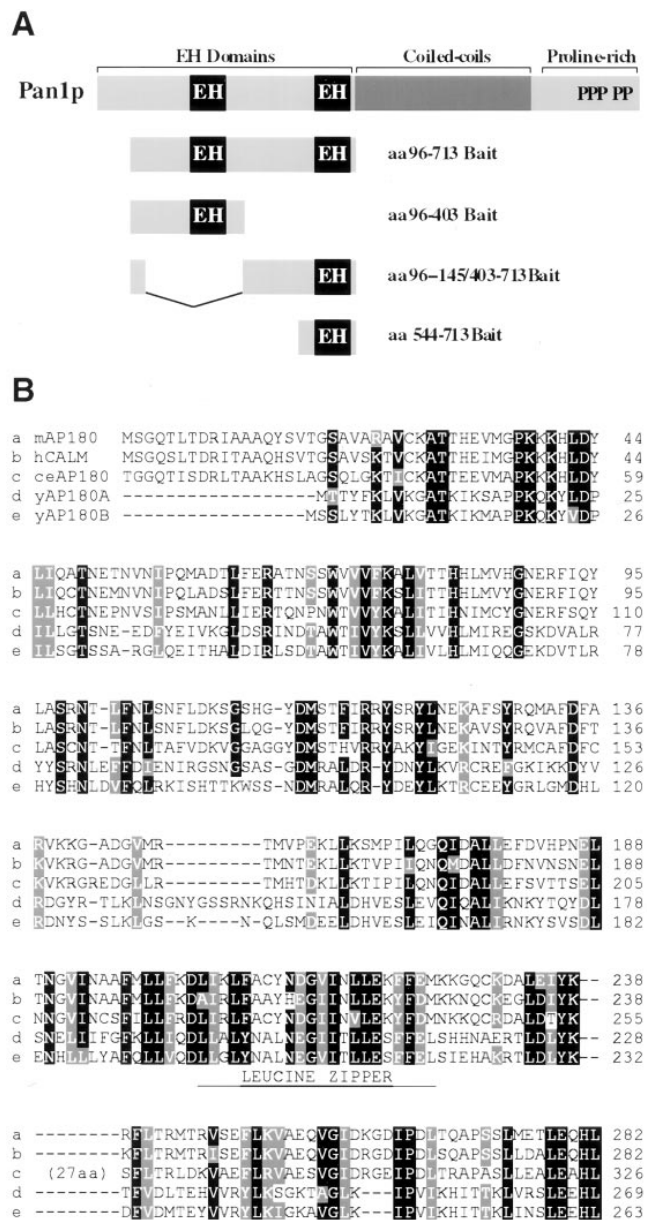
Bait plasmids expressing truncated versions of Pan1p were constructed to define further the region of Pan1p that interacts with yAP180A. Prey plasmids corresponding to nearly full-length yAP180A and to the carboxy terminus of yAP180B were also generated. Pairwise combinations of bait and prey plasmids were introduced into the strain SFY526, that was used for quantitative β-galactosidase assays on cells expressing the various pairs of fusion proteins. The results of these experiments are shown in Table II. Both Pan1p EH domains, either together or individually, were found to interact to varying degrees with the yAP180 proteins. In control experiments, empty bait plasmid encoding the GAL4 DNA-binding domain alone, when combined with the prey plasmids, did not induce high levels of expression of the β-galactosidase enzyme.

Amino Acid Sequence Features of yAP180 Proteins

yAP180A and yAP180B display 43% identity over their entire lengths, including a glutamine-rich (Q) carboxy-terminal region. Both are hydrophilic proteins with no amino-terminal signal sequence and are therefore predicted to reside in the cytoplasm. The yAP180 proteins contain a motif (residues 15–35 in yAP180A) similar to one implicated in the binding of phosphatidylinositol 3,4,5 trisphosphate (PI[3,4,5]P_3) that is also shared with mammalian AP180, centaurin α, and synaptotagmin (Ham-

Figure 1. Two-hybrid screen with Pan1p EH domains identifies yeast members of the AP180 protein family. (*A*) Schematic of the yeast two-hybrid Pan1p bait plasmids used for the screen, which identified the prey plasmids encoding fusion proteins containing the yAP180A protein. (*B*) An alignment of the conserved amino-terminal 300 residues of the members of the AP180 protein family, shown in single letter code. The corresponding accession numbers are as follows: (*a*) mAP180, A44825 (PIR); (*b*) hCALM, U45976 (Genbank/EMBL/DDBJ); (*c*) ceAP180, U88308 (Genbank/EMBL/DDBJ); (*d*) yAP180A, P38856 (Swiss-Prot); (*e*) yAP180B, P53309 (Swiss-Prot). Amino acids identical in at least four of the proteins are indicated in black, similarities are shown in grey. A predicted leucine zipper in the yAP180 proteins is underlined.

Table II. Two-Hybrid Interactions between Pan1p and yAP180 Proteins

Bait	Prey pGADGH (vector)	yAP180A 24–637	yAP180A 432–637	yAP180B 429–568
pGBT9 (vector)	<1	<1	<1	<1
Pan1p EH1–2 (96–713)	8.3 ± 2.0	78 ± 2	117 ± 75	362 ± 67
Pan1p EH1 (96–403)	<1	18 ± 7	6 ± 1	70 ± 9
Pan1p EH2 (96–145/ 403–713)	<1	23 ± 4	33 ± 4	342 ± 15
Pan1p EH2 (544–713)	<1	2 ± 0.4	4 ± 1	158 ± 15
Pan1p 96–1480	<1	2 ± 0.1	2 ± 0.2	16 ± 9
(Units β-galactosidase activity)				

Units of β-galactosidase activity were determined in SFY526 cells transformed with the indicated pair of plasmids as described in Materials and Methods.

monds-Odie et al., 1996; Hao et al., 1997). The sequence of this motif in centaurin α is $P(X_{13-14})R/K(X_4)R/K(X)K(X_5)$ $F(X_6)E$. In all AP180 proteins the $P(X_{13-14})$ is absent, whereas in both yAP180 proteins, the F is replaced by L, and in yAP180B, the E is replaced by R. Detailed mapping of the inositol polyphosphate (IP)- and polyphosphoinositide (PI)-binding sites within mAP180 has not yet been performed; however, mAP180 contains this motif within the region of the protein that binds to IP (Norris et al., 1995; Ye et al., 1995) and to PI (Hao et al., 1997). It is currently unknown whether the yAP180 proteins bind to IP or PI. Another domain found in the yAP180 proteins is a putative leucine zipper that begins at residue 194 (yAP180A) or at residue 188 (yAP180B), in a region particularly well conserved between the yAP180 proteins and their mammalian/ *C. elegans* homologues (underlined in Fig. 1 *B*). All of these motifs are candidates for mediating regulation of the yAP180 proteins and their interactions with other proteins.

yAP180 Proteins Bind to Pan1p and Clathrin

To confirm the interactions between Pan1p and yAP180A and to investigate further the possible functional role of distinct domains of yAP180 proteins, glutathione-S-transferase (GST) fusion proteins (Smith and Johnson, 1988) corresponding to various domains of the yAP180A protein were constructed (Fig. 2 *A*). The expression and stability of the fusion proteins were confirmed by isolation of GST fusion proteins on glutathione–agarose beads and analysis by SDS-PAGE. The GST–yAP180A fusion proteins were tested for interactions with Pan1p from yeast extracts because expression of any part of the Pan1 protein was toxic to bacteria (Sachs and Deardorff, 1992; our unpublished observations). Approximately equal amounts of the GST fusion proteins (5–10 μg) were immobilized to glutathione–agarose beads and incubated with 150 μg of protein derived from wild-type yeast extracts. After binding and washing, the bound proteins from the extract were separated by SDS-PAGE, and analyzed by immunoblotting with a Pan1p-specific antiserum (Fig. 2 *B*). Consistent with the interaction observed by two-hybrid analysis, Pan1p bound to the carboxy-terminal yAP180A C1 fragment but not to the amino-terminal N1 fragment. When the C1 fragment was further subdivided into the C2 and C3 fragments, Pan1p-binding activity was contained within the C2 region of yAP180A. Analysis of this C2 region that interacted with Pan1p EH domains revealed a tripeptide sequence asparagine-proline-phenylalanine (NPF) that was repeated five times in both yAP180 proteins (denoted by ● in Fig. 2 *A*; see Discussion). A GST fusion protein corresponding to the carboxy-terminal half of yAP180B was also capable of binding to Pan1p from yeast extracts (data not shown). GST alone showed no binding to Pan1p.

The mammalian AP180 protein has been shown to bind to clathrin in vitro (Murphy et al., 1991; Morris et al., 1993; Ye and Lafer, 1995). When the C1, C3, or C5 GST fusion proteins were incubated with yeast extracts and washed, the bound fraction contained one major band of ~170 kD that was detected by silver staining (Fig. 2 *C*). This band migrated more rapidly than Pan1p, and its size was consistent with the mobility of yeast clathrin. Therefore, the ability of the yAP180A fusion proteins to interact with

yeast clathrin was also examined. The experiments were performed as above, and the clathrin interaction was detected using antibodies recognizing the clathrin heavy or light chains. Results obtained using a monoclonal antibody that specifically recognizes the clathrin heavy chain (Chc1p) or a polyclonal antiserum directed against the clathrin light chain (Clc1p) are shown in Fig. 2 *D*. As observed for the Pan1p interaction, yeast clathrin bound to the C1 yAP180A fusion protein. However, the clathrin-binding activity resides in a domain of yAP180A distinct from the domain that binds to Pan1p. The region of yAP180A that
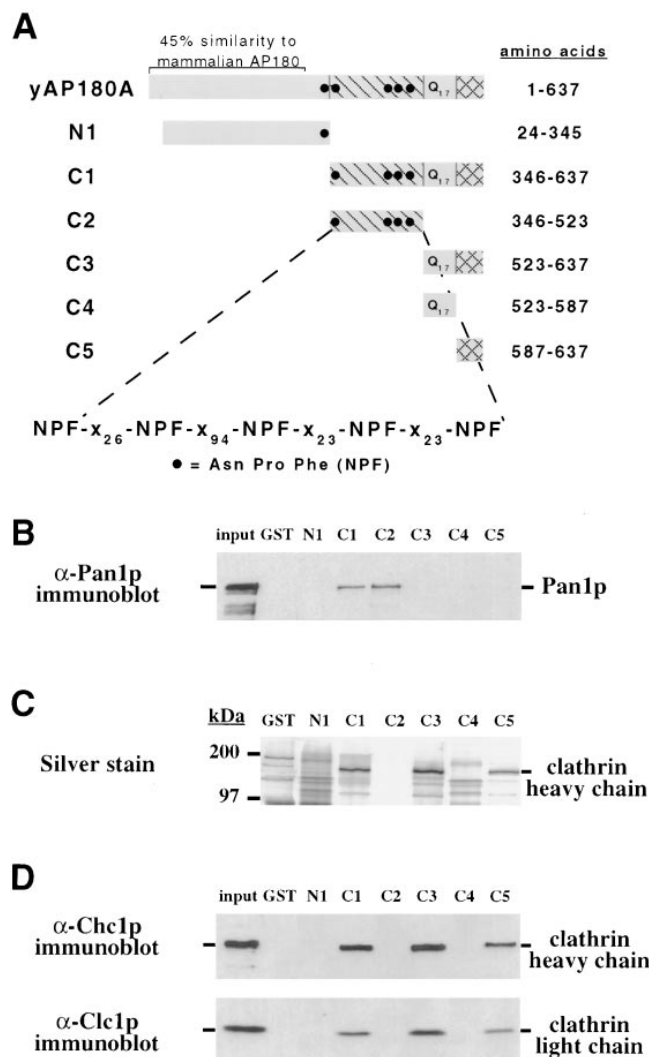

Figure 2. GST–yAP180A fusion protein binding to Pan1p and clathrin in vitro. (*A*) Schematic of the yAP180A protein with fragments used for GST fusion proteins shown below. ●, indicates the location of the tripeptide "NPF," hatched region delineates the Pan1p-binding domain, cross-hatched region shows the clathrin-binding domain, and Q_{17} indicates 17 consecutive glutamine residues. (*B*) Extracts from wild-type yeast cells were incubated with immobilized GST fusion proteins, washed, and bound proteins resolved by SDS-PAGE followed by immunoblotting with a Pan1p-specific antiserum. (*C*) Bound proteins visualized by silver staining a 7.5% acrylamide gel. (*D*) A similar experiment as in *B*, but using a monoclonal antibody specific for yeast clathrin heavy chain and a polyclonal antibody specific for yeast clathrin light chain.

interacts with clathrin was further defined to lie within the C5 fusion protein, which encodes the extreme carboxy-terminal 50 residues of yAP180A, and not within the glutamine-rich (Q_{17}) region directly preceding the C5 segment. When the unbound fractions were examined by immunoblotting for Chc1p, all of the Chc1p had been depleted by the C1 and C3 fusions and 50% depleted by the C5 fusion protein (data not shown). Yeast clathrin also interacted with the carboxy-terminal half of yAP180B (data not shown). When compared to the database and to the domains of other proteins known to bind clathrin, no previously identified clathrin-binding motifs were found within this sequence. The yAP180 proteins thus appear to have a three domain structure: (*a*) an amino-terminal AP180 homology domain, (*b*) a central Pan1p-binding domain with NPF repeats, and (*c*) a carboxy-terminal clathrin association domain. Together, these data suggest the existence of a complex between Pan1p, yAP180 proteins, and clathrin.

Additional independent evidence for in vivo interactions between Pan1p and yAP180A was obtained by native coimmunoprecipitation experiments. Wild-type and *yap1801Δ yap1802Δ* cells were spheroplasted, homogenized, and solubilized by incubation with 1% Triton X-100. Parallel samples of extracts were prepared to which either no antibodies were added or a rabbit polyclonal antiserum recognizing Pan1p was added followed by the addition of protein A–Sepharose beads. The protein A–Sepharose beads were washed, and the bound proteins eluted and analyzed by Western blotting. The portion of the Western blot with the rabbit IgG heavy and light chains was excised (omitting proteins that migrate faster than ~55 kD), and the remainder of the blot probed with the polyclonal antiserum recognizing yAP180 proteins (the specificity of antiserum is demonstrated below). For comparison, separate, parallel samples of the extracts that were used for the coimmunoprecipitations were run on the same gels to determine the total amount of yAP180 protein in the starting material. A distinct band which comigrated with yAP180A was clearly apparent in the lane where wild-type extracts were used. Comparison with the total starting amount of yAP180A indicated that ~3–5% of the yAP180A protein from wild-type cells could be coimmunoprecipitated with Pan1p (Fig. 3). This band was absent from coimmunoprecipitations performed on extracts of cells lacking the yAP180A and yAP180B proteins and from extracts lacking added antibodies. Furthermore, an increased signal was observed using extracts from cells overproducing yAP180A
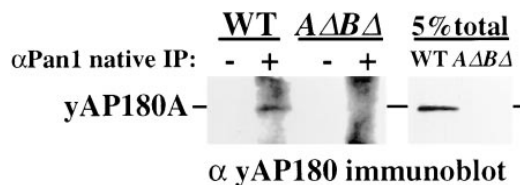
(data not shown). The background smear in the lanes where antibody was added is due to cross-reactivity of the rabbit α-Pan1p IgGs with the secondary antibody used to develop the Western blot. Although only a small percentage of yAP180A was complexed with Pan1p by native coimmunoprecipitation, these data nevertheless support a specific physical interaction between Pan1p and yAP180A. Together, these three independent experimental techniques (two-hybrid, GST binding, and native coimmunoprecipitation) all reveal interactions between Pan1p and yAP180A.

Phenotypes of YAP1801 and YAP1802 Deletion Mutants

To investigate the function of the genes encoding the yAP180 proteins, *YAP1801* and *YAP1802* were deleted either individually or together. Strains that lack both yAP180 proteins were viable and grew under all conditions tested, including high temperature (38°C) and 3% glycerol (data not shown). Under the conditions tested thus far, the mutant strains have revealed no major defects in secretion, endocytosis, or structure of the actin cytoskeleton. When the *YAP180* genes were deleted in combination with the *pan1–20* mutation, no synthetic growth defects were observed. Only slightly slower growth was observed in strains with the *YAP180* genes deleted in combination with a *chc*[ts] mutation. A lack of major synthetic growth defects using these alleles of *PAN1* and *CHC1* does not, of course, rule out genetic interactions between these genes (e.g., Zoladek et al., 1997). Together, these data indicate that *YAP180* genes are nonessential and that the proteins may have a regulatory function and/or that there is an additional protein with redundant functions (e.g., the ORF YJR125c, which also displays a low degree of homology to the amino terminus of AP180 proteins; Fig. 4 *D*). Although the precise function(s) of the yAP180 proteins may not mirror the roles of the AP-1 and AP-2 adaptor complexes, deletions in yeast of genes encoding either the yAP180A or yAP180B proteins or subunits of the AP-1 and AP-2 adaptor complexes are all notable for their absence of phenotypes.

yAP180 Protein Characterization by Subcellular Fractionation

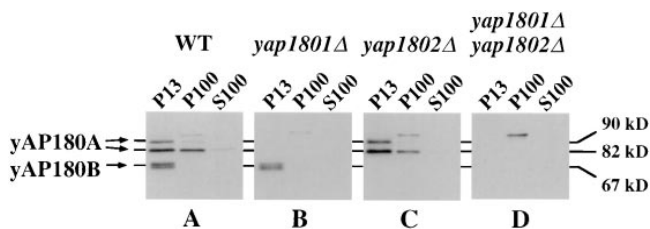A polyclonal rabbit antiserum was generated against a GST fusion protein corresponding to amino acids 24–637

Figure 3. Native coimmunoprecipitation of yA180A with Pan1p. 1% Triton X-100 extracts of wild-type or *yap1801Δ yap1802Δ* cells were generated and no antibodies (−) or Pan1p antiserum (+) were added. Native immune complexes were isolated, separated on SDS-PAGE, and analyzed by immunoblotting for yAP180 proteins. For comparison, 5% of total extract was loaded (*right*).

Figure 4. Fractionation of yAP180A and yAP180B. (*A*) Wild type, (*B*) *yap1801Δ*, (*C*) *yap1802Δ*, and (*D*) *yap1801Δ yap1802Δ* cells were spheroplasted, homogenized, and subjected to differential centrifugation fractionation. The fractions were resolved by SDS-PAGE, and yAP180 proteins were detected by immunoblotting with affinity-purified antibodies that recognize both yAP180A and yAP180B proteins.

of yAP180A. The specificity of this antiserum, its cross-reactivity with the homologous yAP180B protein, and subcellular fractionation of the yAP180 proteins (described in further detail below) are shown in Fig. 4. Yeast spheroplasts were homogenized to generate a lysate that was subjected to differential centrifugation to produce low speed 13,000 g pellet (P13) and supernatant (S13) fractions. The S13 was then spun at 100,000 g to generate high speed pellet (P100) and supernatant (S100) fractions. Differential centrifugation was performed on extracts from wild-type, *yap1801Δ*, *yap1802Δ*, and *yap1801Δ yap1802Δ* strains, followed by analysis of the fractions by immunoblotting (Fig. 4). Similar results were obtained using metabolically labeled cells followed by fractionation, immunoprecipitation, and fluorography (data not shown).

A strain in which both *YAP180* genes were deleted revealed one low abundance cross-reactive band in the high speed pellet (Fig. 4 *D*). The identity of this antigenically related protein is currently unknown but may in part explain the absence of major phenotypes in the double deletion strain (see above). The yAP180A and yAP180B protein bands in the wild-type cell fractionation could be clearly identified by comparing the wild-type fractions to those of *yap1801Δ* and *yap1802Δ* cells. The top two 90- and 82-kD bands correspond to yAP180A, as this signal is also observed in *yap1802Δ* cells (Fig. 4 *C*). Furthermore, this signal is intensified ∼10–20 fold in a strain overexpressing the yAP180A protein (data not shown). The predicted molecular mass for yAP180A is 72 kD; therefore, it is possible that the protein is subject to posttranslational modification(s) such as phosphorylation and/or ubiquitination. Alternatively, the aberrant migration of yAP180A may be due to primary sequence and/or secondary structure as observed for mAP180, which is predicted to be 91 kD yet migrates at 180 kD (Murphy et al., 1991). Pulse/chase analysis indicated that the 82- and 90-kD yAP180A proteins are stable and showed no indication of a precursor/product relationship, suggesting that the multiple bands are not due to proteolysis of yAP180A protein (data not shown). The lower pair of 67-kD bands represent yAP180B, as this signal is also observed in *yap1801Δ* cells (Fig. 4 *B*), and a strain overexpressing yAP180B yields an ∼10–20 fold increase in this signal (data not shown). These bands correlate well with the predicted molecular mass of 64 kD for yAP180B, but the doublet of bands observed for yAP180B suggest that this protein also may be subject to posttranslational modification(s).

The yAP180 proteins associated primarily with the P13 fraction (Fig. 4); this fraction is enriched in the plasma membrane marker protein PM-ATPase (data not shown). This fraction is also enriched in ER, vacuolar, and mitochondrial membranes (Marcusson et al., 1994); however, given the putative role(s) of yAP180 proteins and localization of GFP–yAP180 fusion proteins (see below), their localization to these compartments seems unlikely. The majority of Pan1p was also found in the P13 fraction (Tang and Cai, 1996, our observations). Both Pan1p and the yAP180 proteins associated with the P13 fraction were observed to float when loaded at the bottom of a sucrose gradient (data not shown), consistent with P13 membrane association. Interestingly, a small amount of yAP180A was also found in the P100 fraction (Fig. 4). Specifically, the

82-kD form of yAP180A was approximately equally distributed between the P13 and P100 fractions, whereas the 90-kD form was observed only in the P13 fraction. Typically, 100,000 g pellets are enriched in Golgi membranes, transport vesicles, secretory vesicles, and endosomal membranes (Becherer et al., 1996); thus, one cannot assign the precise subcellular localization of this pool of yAP180A using this technique. Nevertheless, this is in contrast to yAP180B, which is found almost entirely in the P13 fraction and suggests a differential localization of the yAP180 proteins.

yAP180 Proteins Localize to Peripheral Punctate Spots

To visualize the yAP180 proteins and determine more clearly their subcellular localization, green fluorescent protein (GFP) fusion proteins (Chalfie et al., 1994) were generated and expressed in wild-type and *yap1801Δ yap1802Δ* cells. When examined by fluorescence microscopy, each GFP fusion protein was observed in bright spots near the cell surface that were observed in various focal planes at the periphery of the cell. This was true for GFP fusion proteins expressed using either single copy (CEN) or 2 μ overexpression plasmids (GFP–yAP180A, Fig. 5; GFP–yAP180B, data not shown). Similar results were obtained with either wild-type or *yap1801Δ yap1802Δ*
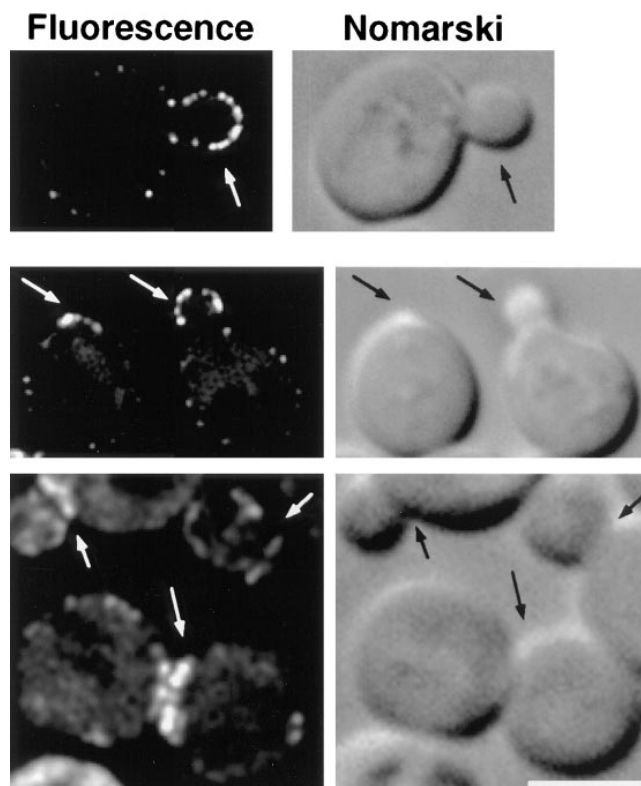

Figure 5. Localization of GFP–yAP180A fusion protein. *yap1801Δ yap1802Δ* cells expressing GFP–yAP180A from either single copy (CEN; *top*) or multi-copy (2 μ; *bottom*) vectors were examined using fluorescence and differential interference (*Nomarski*) light microscopy. The images were processed using deconvoluting software (Delta Vision) on a Silcon Graphics workstation. Buds and cytokinesis necks containing concentrated spots of signal are indicated by arrows. Bar, 5 μm.

cells; however, brighter signals were seen with the deletion strain (data not shown). Extracts from these cells were examined for expression of the fusion proteins by immunoblotting with GFP and yAP180 antisera, which confirmed the production of full-length fusion proteins, and also revealed a small amount of proteolysis near the fusion junction that resulted in free, soluble GFP (data not shown). These experiments also showed that higher levels of the GFP fusion proteins were produced in the deletion strain, consistent with the brighter fluorescence signal observed. The bright peripheral spots may correspond to actin cortical dots because they were concentrated in small buds of budding cells and at the necks of cells undergoing cytokinesis (Fig. 5, *arrows*); this is similar to the distribution of actin cortical spots observed as cells progress through the cell cycle (Kilmartin and Adams, 1984). Attempts to observe both GFP and actin simultaneously have not been possible because the fixation procedure required to visualize actin (phalloidin staining) interfered with detection of GFP. However, the yAP180-interacting protein Pan1p has previously been localized to actin cortical spots (Tang and Cai, 1996), which are proposed to be the site of secretion and endocytosis in yeast (Mulholland et al., 1994). These data suggest that a complex of Pan1p and the yAP180 proteins is found at or near actin cortical spots where several other proteins that mediate endocytosis are concentrated (e.g., Ayscough et al., 1997).

pan1–20 Exhibits Synthetic Lethality with a Deletion of the Yeast Synaptojanin Homologue SJL1

A complex of dynamin, amphiphysin, and synaptojanin has been implicated in the endocytosis of synaptic vesicle membranes in nerve terminals (David et al., 1996; de Heuvel et al., 1997). Synaptojanin is a phosphatidylinositol polyphosphate-5-phosphatase that hydrolyzes $PI(1,4,5)P_3$ and $PI(4,5)P_2$ and may promote down regulation of signaling pathways that are stimulated by these important second messengers (De Camilli et al., 1996; McPherson et al., 1996). $PI(4,5)P_2$ levels affect many important cellular systems. Among these is the actin cytoskeleton, as several actin regulatory proteins are modulated by $PI(4,5)P_2$ binding (Janmey et al., 1987; Yonezawa et al., 1990). Pan1p contains a consensus sequence for binding to $PI(4,5)P_2$ (Tang and Cai, 1996). It also regulates the structure of the actin cytoskeleton (Tang and Cai, 1996; Wendland et al., 1996) and is localized to actin cortical spots (Tang and Cai, 1996). Consistent with these observations, yeast strains, in which two of the three synaptojanin-like genes were deleted (*sjl1Δ sjl2Δ*), exhibited defects in actin cytoskeleton organization and exaggerated membrane invaginations (Srinivasan et al., 1997) similar to those observed in *pan1* mutants (Wendland et al., 1996).

To investigate potential genetic interactions between *PAN1* and the three yeast synaptojanin-like genes *SJL1*, *SJL2*, and *SJL3/SOP2* (Luo and Chang, 1997; Srinivasan et al., 1997), a heterozygous diploid strain was generated by mating a *pan1–20* strain with an *sjl2Δ sjl3Δ* strain. This was followed by sporulation and tetrad dissection. Haploid spores that were deduced to have the double mutant phenotypes *pan1–20 sjl2Δ* or *pan1–20 sjl3Δ* exhibited only minor synthetic growth defects. The *SJL1* gene is adjacent to

the *PAN1* gene on chromosome IX. To produce a double mutant for this pair of genes, a *pan1–20* haploid strain harboring a plasmid encoding the wild-type *PAN1* gene was used for targeted disruption of the *SJL1* gene. After disruption of the *SJL1* locus was confirmed by PCR, two independent strains were mated with a wild-type strain, sporulated, and tetrads were dissected. *pan1–20 sjl1Δ* spores germinated but were unable to grow beyond an eight cell colony unless they also contained the *PAN1* plasmid (Table III), indicating that the combination of the *pan1–20* and *sjl1Δ* mutations is synthetically lethal. These genetic interactions suggest that phosphoinositide metabolites regulate a Pan1p-dependent aspect of endocytosis in yeast.

Discussion

Pan1p is an essential protein required for normal endocytosis in yeast. Its three structural domains—two EH domains, a coiled-coil region, and proline-rich motifs—are each likely to participate in distinct protein–protein interactions. This multivalent structure implicates Pan1p as a central regulator or adaptor that may transiently recruit and activate multiple components of an endocytic and/or actin cytoskeletal regulatory network, perhaps in an analogous manner to the signal transduction adaptor proteins IRS-1 and GRB2 (for review see Myers et al., 1994; Carraway and Carraway, 1995).

Here, using the EH domains of Pan1p in a yeast two-hybrid screen, we demonstrate interactions with two new members of the AP180 clathrin assembly protein family, yAP180A and yAP180B. The EH domain interaction site was mapped to a domain in the yAP180 proteins that contains a repeated tripeptide sequence, NPF, that is likely to contribute to the specificity of the EH–yAP180 interaction. yAP180A also interacts with clathrin via its 50 carboxy-terminal residues, a region distinct from the NPF-containing domain that binds to Pan1p. Subcellular fractionation studies suggested that the yAP180 proteins localize to the plasma membrane. Consistent with this finding, GFP–yAP180 fusion proteins were observed in punctate spots at the cell periphery. These data, together with previously published data on Pan1p (Tang and Cai, 1996), suggest that complexes between Pan1p and yAP180 proteins primarily localize to sites at or near actin cortical dots at the plasma membrane. A small pool of yAP180A was differentially localized to the P100 fraction, whereas the GFP analysis suggested similar localizations of the yAP180 proteins. This discrepancy could be due to the yAP180A protein also localizing to small transport vesicles or to pelletable protein complexes that are not visible by fluorescence. Lastly, a genetic interaction between *PAN1* and *SJL1*, a yeast

Table III. pan1-20 sjl1::HIS3 Synthetic Lethality

	Wild type		pan1-20 sjl1Δ	
	ura3	pPAN1URA3	ura3	pPAN1URA3
Live spores	23	37	—	23
Dead spores	—	—	37*	—

*PAN1 and SJL1 loci are linked; pan1-20 sjl1::HIS3 without the pPAN1 plasmid formed microcolonies of one to eight cells.

homologue of the phosphatidylinositol polyphosphate-5-phosphatase synaptojanin, implies a regulatory role for inositol phospholipids in a Pan1p-dependent step of endoytosis. Together, these data suggest that Pan1p and yAP180 form a plasma membrane–localized complex, which is important for regulating endocytosis in yeast.

The NPF-containing Domain of yAP180 Proteins Interacts with the EH Domains of Pan1p

Two-hybrid, GST-binding, and native coimmunoprecipitation analyses (Table II, Figs. 2 and 3) demonstrated that Pan1p interacts with yAP180 proteins. These analyses also revealed that this interaction occurs via specific domains, with the NH$_2$-terminal EH domains of Pan1p binding to NPF motif–containing domains of the yAP180 proteins. This interaction is consistent with recent data demonstrating EH domain–NPF motif interactions by a random peptide phage display screen (Salcini et al., 1997). These data suggest a model in which the specificity of pairing between distinct EH domains and NPF motifs is determined by nonconserved residues both surrounding the NPF motifs (Salcini et al., 1997) and within the EH domains, as only one-third of the residues are highly conserved in EH domains (Wong et al., 1995). Indeed, varying levels of β-galactosidase activity were observed in two-hybrid studies using the two alternative EH domains of Pan1p and the NPF-containing regions of both yAP180 proteins. Furthermore, one of the GST–yAP180A fusion proteins that contained a single NPF motif (N1 fusion, Fig. 2) did not bind to Pan1p. This suggests that, in addition to the importance of the NPF motif context, the presence of multiple NPF motifs may enhance binding to EH domains. The in vitro data can also be interpreted to mean that the interaction between Pan1p and yAP180A may be transient, as only a small amount of complex was coimmunoprecipitated under the experimental conditions used. Further studies will be necessary to determine if there are specific cofactors or posttranslational modifications that regulate the affinity of association or the stability of this complex.

The yeast genome contains five ORFs that are predicted to contain EH domains: Pan1p, End3p, YBL047c (Pan1-like), YJL083w, and YKR019c (Wendland et al., 1996). End3p, Pan1p, and the Pan1-like protein contain one, two, or three amino-terminal EH domains, respectively, whereas YJL083w and YKR019c are homologous proteins of unknown function with single carboxy-terminal EH domains. End3p also contains two internal repeats at the carboxy terminus which, by two-hybrid analysis, interact with the amino terminus of Pan1p (Tang et al., 1997). This interaction requires both the second EH domain of Pan1p and the Sla1p homology domain directly preceding the second EH domain (Sla1p is an actin-binding protein; Holtzman et al., 1993). However, this interaction is distinct from the Pan1p–yAP180 interaction because (*a*) End3p does not contain an NPF motif, and (*b*) the yAP180 proteins interacted with both the first and second EH domains of Pan1p, and these interactions did not require the Sla1p homology domain (Table II). It is not yet known if the yAP180 proteins bind to EH domains in other yeast proteins; however, because yAP180A interacted with Pan1p from both *end3Δ* and *pan1-likeΔ* extracts (our unpublished results),

the End3 and Pan1-like proteins are not likely to be required for formation of the Pan1p–yAP180 complex. Functional relevance of the NPF motif is also supported by its presence in the cytoplasmic tail domains of Kex2p and Ste3p (NPFXD), where it functions as an endocytosis signal (Tan et al., 1996). It is intriguing to speculate that endocytosis of these and other plasma membrane proteins harboring NPF motifs (e.g., Axl2p, Cch1p, and Drs2p) may be mediated by direct interactions with Pan1p or the other EH domain–containing proteins in yeast.

Similarities and Differences among AP180 Protein Family Members

The mammalian neuronal–specific AP180 protein has been biochemically well characterized. The amino-terminal 300 residues, which are conserved in the AP180 family of proteins, bind to IP, PI, and to clathrin triskelions (Norris et al., 1995; Ye et al., 1995; Hao et al., 1997). The remaining carboxy terminus interacts with clathrin triskelions and cages, and contains in vitro clathrin cage assembly activity (Ye and Lafer, 1995). Interestingly, mAP180 clathrin assembly activity is inhibited in the presence of IP (Norris et al., 1995; Ye et al., 1995), consistent with a role for IP or PI as direct regulators of mAP180 activity. The carboxy termini of both mAP180 and the yAP180 proteins bind to clathrin, although the primary sequence is not highly conserved. We are currently testing the yAP180 proteins for in vitro clathrin cage assembly activity. The potential binding and regulatory interactions of the yAP180 proteins with IP and/or PI also remain to be determined.

The mammalian AP180 proteins are 896–915 amino acids in length and migrate with an aberrantly high molecular mass, which is due to a highly charged central region of the protein (Murphy et al., 1991). In contrast, the yAP180 proteins, CALM, and *C. elegans* AP180 are all smaller proteins of 586–684 amino acids which lack the highly charged region. The yeast proteins do not display a large disparity in apparent molecular mass. Another difference is that the amino-terminal domain of neuronal AP180 binds to clathrin, whereas the yAP180A amino-terminal fusion (N1) does not. However, the lack of clathrin-binding activity in the N1 fusion might be explained by the truncation of the first 24 residues. In addition, some potential regulatory interactions may be affected, as the putative IP/PI binding site is also interrupted in the N1 fusion protein. Interestingly, although neuronal AP180 lacks the NPF repeats, the more divergent carboxy termini of the smaller AP180 protein family members (CALM, yAP180s, *C. elegans* AP180) all contain multiple NPF motifs.

Pan1p as a Multivalent Adaptor That Coordinates Both Endocytosis and Organization of the Actin Cytoskeleton

The structure of Pan1p suggests that it is a protein with the capacity to interact with multiple proteins through distinct binding domains. The data presented in this paper, together with findings from other labs, have allowed us to propose a model in which Pan1p dynamically coordinates the recruitment and possibly the regulation of several proteins required both for endocytosis and for organization of the actin cytoskeleton (Fig. 6). The proteins that interact

with each of the domains of Pan1p (EH domains, coiled-coil, and proline-rich motifs) as well as the implications of these interactions on the control of endocytosis and the actin cytoskeleton are described below.

Pan1p EH Domains. The EH domains of Pan1p associate with the NPF-containing domain of the yAP180 proteins. Because yAP180A has distinct binding domains for Pan1p and clathrin, yAP180A may serve as a bridging protein that links Pan1p to clathrin and presumably regulates clathrin function and/or cage assembly (Fig. 6). Although the methods used for these studies cannot definitively rule out indirect interactions between yAP180A, Pan1p, and clathrin, our in vitro interaction and subcellular fractionation data suggest that these proteins undergo at least transient interactions as part of a multimeric protein complex. One model is that Pan1p/End3p/yAP180 complexes localize to endocytic sites, present EH domains for interaction with NPF motifs in the tails of plasma membrane proteins destined for endocytosis, and the yAP180 proteins recruit and polymerize clathrin triskelions. Given the genetic interactions between *PAN1* and *SJL1* (Table III), we are currently testing for physical interactions between the carboxy-terminal NPF of Sjl1p and the EH domains of Pan1p. By analogy, the NPF-containing domain of mammalian synaptojanin recently has been shown to bind to the EH domains of eps15 (Haffner et al., 1997).

Pan1p Coiled-Coil. The central coiled-coil domain of eps15 mediates the formation of dimers, which in turn form higher molecular weight oligomeric complexes (Tebar et al., 1997). Pan1p may similarly dimerize/oligomerize through coiled-coil interactions either with itself and/or with other coiled-coil containing proteins. Gel filtration of yeast cytosol has indicated that soluble Pan1p

fractionates in a size range consistent with the formation of a large complex (our unpublished results).

Pan1p Proline-rich Domain. The proline-rich domain of Pan1p contains binding motifs for both SH3 and WW domains. WW motifs are 33–amino acid blocks (Sudol et al., 1995) that bind to the proline-rich sequences PPLP and PPXY (Chan et al., 1996; Bedford et al., 1997; Linn et al., 1997). Interestingly, genetic interactions have been identified between Pan1p and the WW domain–containing Rsp5p (Zoladek et al., 1997; Fig. 6). Rsp5p, a ubiquitin-protein ligase required for endocytosis (Hein et al., 1995; Galan et al., 1996; Zoladek et al., 1997), also contains a C2 domain predicted to bind Ca^{2+} and acidic phospholipids and a carboxy terminal hect (homologous to the E6-AP carboxy terminus) domain, the signature motif of ubiquitin-protein ligase E3 enzymes. Whether physical interaction between Pan1p and Rsp5p occurs via one or more of the three WW motifs in Rsp5p binding to the three proline-rich WW–binding consensus sequences present at the carboxy terminus of Pan1p is currently being investigated. Rsp5p also contains one NPF motif that could bind to Pan1p via the EH domains. Pan1p may act as a connector, bringing Rsp5p close to potential ubiquitination substrates like the tail domains of certain plasma membrane proteins destined for endocytosis. Ubiquitination is required for the endocytosis of several plasma membrane proteins both in yeast and mammalian cells. These plasma membrane proteins are subject to ubiquitination, and endocytosis of these proteins does not occur when ubiquitination is blocked (Kolling and Hollenberg, 1994; Hicke and Riezman, 1996; Roth and Davis, 1996; Strous et al., 1996). Alternatively, Pan1p may itself be ubiquitinated by Rsp5p, as the Pan1p homologue eps15 is ubiquitinated in mammalian cells (van Delft et al., 1997*a*).

SH3 domains are ∼60 residues in size and bind to proline-rich consensus sequences (Ren et al., 1993; Yu et al., 1994) that are distinct from those that interact with WW domains. Recent evidence suggests that some proteins contain overlapping SH3- and WW-binding motifs that compete for binding partners (Sudol, 1996; Bedford et al., 1997). The proline-rich domain of Pan1p contains both adjacent and overlapping SH3- and WW-binding motifs. SH3-domain–containing proteins that are good candidates for binding to Pan1p include the yeast amphiphysin homologue Rvs167p and the type I myosins Myo3p and Myo5p that are required for endocytosis (Bauer et al., 1993; Geli and Riezman, 1996) and the actin-binding proteins Sla1p and Abp1p (Holtzman et al., 1993). Like Rsp5p, Sla1p also contains a single NPF motif. Additional studies will be required to determine which, if any, of these proteins binds to Pan1p.

Endocytosis and the Actin Cytoskeleton. As our understanding of endocytosis increases, unanticipated parallels in the molecular mechanisms of endocytosis in yeast and mammalian cells are being uncovered. Studies of endocytosis in *S. cerevisiae* have long pointed to a central role for the actin cytoskeleton in this ubiquitous and essential process (Kubler and Riezman, 1993). The structure of the yeast actin cytoskeleton may be indirectly affected by interactions between Sjl1p and Pan1p, as suggested by the genetic interactions presented here. Consistent with this, synaptojanin activity modulates the levels of phosphoi-
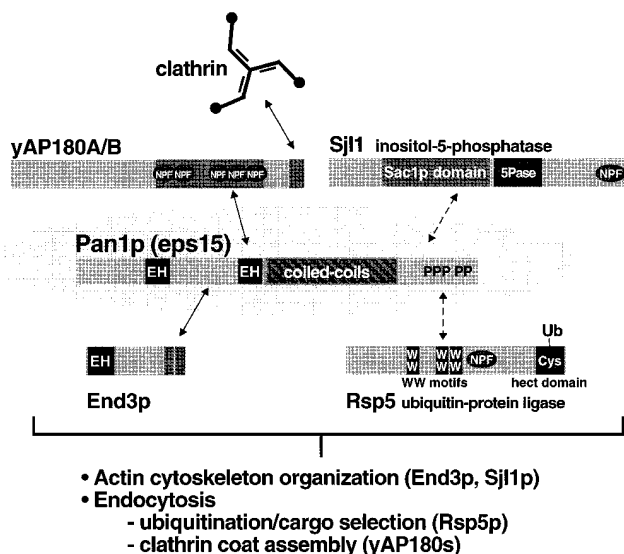

Figure 6. A model describing the interactions between Pan1p and proteins that regulate clathrin (*yAP180A/B*), the actin cytoskeleton (*End3p*), the ubiquitination pathway (*Rsp5p*), and inositol phospholipids (the synaptojanin-like *inositol-5-phosphatases*). Solid arrows indicate physical interactions between the proteins, and the dashed arrows denote genetic interactions.

nositides to influence the structure of the actin cytoskeleton (Sakisaka et al., 1997). Recently, an important role for actin cytoskeleton dynamics in endocytosis has been recognized in mammalian cells (Lamaze et al., 1997). Local depolymerization of actin may be necessary to allow for assembly of clathrin and/or other coat proteins that mediate the formation of endocytic vesicles. Using the actin depolymerizing agent latrunculin A, it was found that endocytosis in both yeast and mammalian cells requires the turnover of actin filaments (Lamaze et al., 1997; Lappalainen and Drubin, 1997). Interestingly, *end3* and *sla1* mutants, each of which are synthetically lethal in combination with *pan1* alleles, are resistant to latrunculin A (Ayscough et al., 1997). This suggests that End3p and Sla1p normally destabilize actin filaments; in their absence, the stabilized actin filaments require more latrunculin A for depolymerization (Ayscough et al., 1997). It is likely that Pan1p may also regulate actin filament turnover, as elongated and thickened actin structures extending from the plasma membrane appear in *pan1* mutants at nonpermissive temperature (Wendland et al., 1996). Whether Pan1p regulation of the actin cytoskeleton is through its interactions with End3p, synaptojanin, or other as yet unrecognized interactions remains to be determined.

Based on our data combined with findings from others, we propose that Pan1p participates in a dynamic series of protein interactions (Fig. 6) which serve both to regulate and coordinate the activity of the endocytic and actin cytoskeletal machinery. Activities included in this machinery are: (*a*) clathrin coat assembly (yAP180 proteins), (*b*) ubiquitination enzymes (Rsp5p), and (*c*) actin-regulatory proteins (Sjlp, End3p). These observations highlight the complexity of the endocytic system in yeast and mammalian cells. Further genetic and biochemical characterization should define the precise order of the reactions and the regulatory role Pan1p plays in these reactions.

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