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

Beverly Wendland and Scott D. Emr
Howard Hughes Medical Institute, Division of Cellular and Molecular Medicine, University of California at San Diego, School of Medicine, La Jolla, California 92093-0668

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 *SIL1* 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.

The 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 (Rothenberg 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 (*chc1*) 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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*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.

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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 heterotrimeric 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., 1997b). 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 heterotrimeric adaptor complex AP-2 is required for clathrin-dependent endocytosis in mammalian cells (Lin et al., 1991; Smythe et al., 1992). Interestingly, a distinct, nonheterotrimeric 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 (Kohzt 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 clathrin assembly lymphoid myeloid 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 (Maniatis 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 YAPI801 and YAPI802 genes were obtained by PCR amplification of chromosomal DNA to produce pYAPI80A and pYAPI80B, respectively. The yap1801::HIS3 deletion construct removes 94% of the
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 SmalI. The deletion strains were generated by sequential deletion of YAP1801 and YAP1802 in an SEY6210/a 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- plates were replica plated and one set transferred to filter disks, (Whatman Inc, Clifton, NJ) lysed by freezing in liquid N2, and incubated at 30°C with 0.1 M NaPO4, 10 mM KCl, 1 mM MgSO4, 0.27% β-mercaptoethanol, 0.33 mg/ml Xgal, pH 7.0. Blue colonies were selected, restested, 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**

Lyastes 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 OD600 2–8. P16 membranes (for Pan1p binding) or or a mouse monoclonal recognizing yeast clathrin heavy chain (SKL1; Sachs, A., University of California, Berkeley, CA; Boeck et al., 1996), or rabbit polyclonal antibodies, recognizing Pan1p (Nos. 91 or 92; provided by for Pan1p–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 sjl3Δ 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 sjl3Δ genotype, and did not contain the pPA114 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. 1A), 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.

**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).

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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. 1B).

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]P3) that is also shared with mammalian AP180, centaurin α, and synaptotagmin (Ham-...
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_{1})R/K(X_{1})K(X_{3})F(X_{5})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. 1B). 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. 2A). 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 were incubated by SDS-PAGE, and analyzed by immunoblotting with a Pan1p-specific antiserum (Fig. 2B). 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 \( \text{d} \) in Fig. 2A; 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. 2C). 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 (Chclp) or a polyclonal antiserum directed against the clathrin light chain (Clc1p) are shown in Fig. 2D. 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...
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 (Q17) 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 yAPI80 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 immunoprecipitation 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 yAPI80 protein in the starting material. A distinct band which comigrated with yAPI80A was clearly apparent in the lane where wild-type extracts were used. Comparison with the total starting amount of yAPI80A indicated that ~3-5% of the yAPI80A 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 yAPI80A and yAPI80B proteins and from extracts lacking added antibodies. Furthermore, an increased signal was observed using extracts from cells overproducing yAPI80A (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 yAPI80A was complexed with Pan1p by native coimmunoprecipitation, these data nevertheless support a specific physical interaction between Pan1p and yAPI80A. Together, these three independent experimental techniques (two-hybrid, GST binding, and native coimmunoprecipitation) all reveal interactions between Pan1p and yAPI80A.

Phenotypes of YAP1801 and YAP1802 Deletion Mutants

To investigate the function of the genes encoding the yAPI80 proteins, YAP1801 and YAPI802 were deleted either individually or together. Strains that lack both yAPI80 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 chc1ts 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 API80 proteins; Fig. 4 D). Although the precise function(s) of the yAPI80 proteins may not mirror the roles of the AP-1 and AP-2 adaptor complexes, deletions in yeast of genes encoding either the yAPI80A or yAPI80B proteins or subunits of the AP-1 and AP-2 adaptor complexes are all notable for their absence of phenotypes.

yAPI80 Protein Characterization by Subcellular Fractionation

A polyclonal rabbit antiserum was generated against a GST fusion protein corresponding to amino acids 24–637
of yAP180A. The specificity of this antiserum, its cross-
reactivity with the homologous yAP180B protein, and sub-
cellular 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 sub-
jected 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 pel-
let (P100) and supernatant (S100) fractions. Differential
centrifugation was performed on extracts from wild-type,
_yap1801Δ_, _yap1802Δ_, and _yap1801Δ yap1802Δ_ strains, fol-
lowed by analysis of the fractions by immunoblotting (Fig.
4). Similar results were obtained using metabolically la-
beled cells followed by fractionation, immunoprecipita-
tion, and fluorography (data not shown).

A strain in which both _YAP180_ genes were deleted re-
vealed 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 ex-
plain the absence of major phenotypes in the double dele-
tion strain (see above). The _yAP180A_ and _yAP180B_ pro-
tein 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 overex-
pressing the _yAP180A_ protein (data not shown). The pre-
dicted 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 ubiquiti-
nation. Alternatively, the aberrant migration of _yAP180A_
may be due to primary sequence and/or secondary struc-
ture 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 mito-
chondrial membranes (Marcusson et al., 1994); however,
given the putative role(s) of _yAP180_ proteins and localiza-
tion of GFP–yAP180 fusion proteins (see below), their lo-
calization to these compartments seems unlikely. The ma-
jority 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 ob-
served to float when loaded at the bottom of a sucrose gra-
dient (data not shown), consistent with P13 membrane as-
sociation. 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 dis-
tributed between the P13 and P100 fractions, whereas the
90-kD form was observed only in the P13 fraction. Typi-
ically, 100,000 g pellets are enriched in Golgi membranes,
transport vesicles, secretory vesicles, and endosomal mem-
branes (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 frac-
tion 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 micro-
copy, 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 Silicon 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)P3 and PI(4,5)P2 and may promote down regulation of signal transduction pathways that are stimulated by these important second messengers (De Camilli et al., 1996; McPherson et al., 1996). PI(4,5)P2 levels affect many important cellular systems. Among these is the actin cytoskeleton, as several actin cytoskeleton (Tang and Cai, 1996) and PI(4,5)P2 and may promote down regulation of signal transduction pathways that are stimulated by these important second messengers (De Camilli et al., 1996; McPherson et al., 1996). PI(4,5)P2 levels affect many important cellular systems. Among these is the actin cytoskeleton, as several actin regulatory proteins are modulated by PI(4,5)P2 binding (Janmey et al., 1987; Yonezawa et al., 1990). Pan1p contains a consensus sequence for binding to PI(4,5)P2 (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 sjl1Δ sjl2Δ strain. This was followed by sporulation and tetrad dissection. Haploid spores that were deduced to have the double mutant phenotypes pan1–20 sjl1Δ 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 implicate 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 endocytosis. 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 NH2-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–yAPI180 interaction because (a) End3p does not contain an NPF motif, and (b) the yAPI180 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 yAPI180 proteins bind to EH domains in other yeast proteins; however, because yAPI180A interacted with Pan1p from both end3Δ and pan1-likeΔ extracts (our unpublished results),
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 fractionation 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 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).

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 Slalp and Abp1p (Holtzman et al., 1993). Like Rsp5p, Slalp 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-

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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 regulates 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 (γAP180 proteins), (b) ubiquitination enzymes (Rsp5p), and (c) actin-regulatory proteins (Sip1, 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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