FYVE Domain Targets Pib1p Ubiquitin Ligase to Endosome and Vacuolar Membranes*

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Signaling by phosphatidylinositol 3-kinases (PI3Ks) is often mediated by proteins which bind PI3K products directly and are localized to intracellular membranes rich in PI3K products. The FYVE finger domain binds with high specificity to PtdIns(3)P and proteins containing this domain have been shown to be important components of diverse PI3K signaling pathways. The genome of the yeast Saccharomyces cerevisiae encodes five proteins containing FYVE domains, including Pib1p, whose function is unknown. In addition to a FYVE finger motif, the primary structure of Pib1p contains a region rich in cysteine and histidine residues that we demonstrate binds 2 mol eq of zinc, consistent with this region containing a RING structural domain. The Pib1p RING domain exhibited E2-dependent ubiquitin ligase activity in vitro, indicating that Pib1p is an E3 RING-type ubiquitin ligase. Fluorescence microscopy was used to demonstrate that a GFP-Pib1p fusion protein localized to endosomal and vacuolar membranes and deletional analysis of Pib1p domains indicated that localization of GFP-Pib1p is mediated solely by the FYVE domain. These results suggest that Pib1p mediates ubiquitination of a subset of cellular proteins localized to endosome and vacuolar membranes, and they expand the repertoire of PI3K-regulated pathways identified in eukaryotic cells.

The genome of the budding yeast, Saccharomyces cerevisiae, encodes a single phosphatidylinositol 3-kinase (PI3K),1 encoded by the VPS34 gene, that is responsible for all PtdIns(3)P synthesis within the cell (1). The only known function of the yeast Vps34 PI3K is in sorting of proteins to the lysosome-like vacuole via multiple pathways (1−3). Vps34p directly regulates Golgi-to-endosome vesicle-mediated transport via the PtdIns(3)P-binding protein, Vac1p/Pepl7p (4−8), required for docking and fusion of Golgi-derived vesicles with the prevacuolar endosome. Vac1p, and the related human protein EEA1, link PI3K signaling directly to SNARE-mediated vesicle trafficking within endosomal pathways by directly binding PtdIns(3)P, SNAREs, and other required factors such as the SEC1 family protein, Vps45p (8, 9). In addition to this role in regulating fusion of post-Golgi membranes, Vps34p produces substrate for the Fab1 phosphatidylinositol 3-phosphate 5-kinase, which has been implicated in protein sorting within late endosomes and multivesicular body endosomes (10). Besides these roles for PtdIns(3)P, other roles are likely to be discovered as more PtdIns(3)P-binding proteins are characterized.

The downstream effects of PI3K signaling are, in many cases, mediated by proteins containing a PtdIns(3)P-binding protein module called a FYVE finger domain. FYVE domains comprise a subfamily of RING domains, identified by the spacing of cysteine and/or histidine residues which bind two atoms of zinc and are required for the overall structure of the domain (11−13). The hallmark of the FYVE finger subfamily is a patch of basic residues surrounding the third zinc-coordinating cysteine residue which forms, together with other conserved basic residues, the PtdIns(3)P binding pocket (14). The yeast genome encodes five FYVE domain-containing proteins, and three of these proteins, Vac1p, Vps27p, and Fab1p, regulate aspects of endosomal protein sorting. No functions have been described for the remaining two FYVE domain proteins, which we have named Pib1p and Pib2p (PtdIns(3)P-binding). We have discovered that Pib1p, in addition to a FYVE domain, contains a second RING-related domain containing binding sites for two zinc atoms. Localization of Pib1p to endosomal and vacuolar membranes is mediated solely by the FYVE domain. We demonstrate that the RING domain exhibits E3 ubiquitin ligase activity, suggesting that Pib1p may function in ubiquitination of endosomal and vacuolar proteins.

EXPERIMENTAL PROCEDURES

Strains and Microbiological Methods—Standard media (YPD, SM dropout) were used for culturing S. cerevisiae strains (15). All yeast strains were derivatives of SEY6210 [MATa, ura3−52, his3−200, trp1−
Δ901, lys2−801, suc2−Δ9, leu2−3, 11]. The pib1Δ:HIS3 deletion strain, CYB56, was constructed by transformation of SEY6210 with a DNA cassette generated by the polymerase chain reaction (PCR). Oligonucleotide primers were designed to amplify the HIS3 locus and were tailed with 40 bases of sequence identical to a region of the PIB1 locus immediately upstream of the start codon and immediately downstream of the stop codon (16). The resultant transformants were screened by PCR to confirm that the PIB1 locus was replaced with the HIS3 cassette and by immunoblotting with anti-Pib1p antibodies to confirm loss of Pib1 protein.

Molecular Biology Methods—Standard DNA cloning methods were used (17). For culturing Escherichia coli DH5α and BL21 strains, LB media containing selective antibiotics was used (17). The cloned PIB1 gene was obtained by PCR using Taq polymerase and oligonucleotide primers which were designed to amplify PIB1 and 400 base pairs upstream and downstream of the start and stop codons. Nucleotides, Taq polymerase, EXPAND polymerase, and DNA modifying enzymes were purchased from Roche Molecular Biochemicals, New England
Biolabs, and Promega. PCR products were first cloned into pTOPO using a TOPO cloning kit (Invitrogen), then moved into appropriate bacterial or yeast expression vectors (see below). Gene overlap extension PCR was used to construct the C235S and C225S pib1 point mutants (18). Primers were designed to change one base of the appropriate cysteine codon. PCR-amplified DNA sequences were confirmed by sequencing. DNA fragments were purified using a Gel Extraction Kit (Qiagen).

Pib1p derivatives were expressed in E. coli as fusions to GST using pGEX-KG as vector, or with an amino-terminal fusion to a polyhistidine tag using pET28. Recombinant GST fusion protein was purified on glutathione-agarose (Amersham Pharmacia Biotech), and His-tagged protein on His-Bind resin (Novagen) according to the manufacturer's instructions. Eluted His-tagged protein was dialyzed against phosphate-buffered saline overnight. GST fusion proteins were eluted using 50 mM reduced glutathione, and elution buffer was immediately exchanged into storage buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 5% glycerol) using PD10 desalting columns (Amersham Pharmacia Biotech). The concentration of recombinant proteins was diluted to 50% glycerol. The concentration of recombinant proteins was determined using a Bio-Rad Protein Assay kit according to the manufacturer's instructions using bovine serum albumin as a standard. To calculate the concentration of GST-Pib1(RING) fusion proteins, a molecular weight of 49,600 was used.

ZnCl2- saturated GST-Pib1 fusion protein was determined using a colorimetric assay (19) and compared to a standard curve generated using known amounts of ZnCl2. The standard curve was generated by determining the absorbance of 4-(2-pyridylazo)resorcinol at 500 nm of 1, 5, 10, 15, and 20 μM ZnCl2. Triplicate measurements were made and the average of the three measurements was used to generate the standard curve. The concentration of GST-Pib1(RING) in the analysis of bound zinc was 3.57 μM for wild-type GST-Pib1(RING) and 22.2 μM for the C225S mutant GST-Pib1(RING). More mutant protein was used compared with wild-type protein so that an accurate value of bound zinc could be obtained. Zinc content of three aliquots of each protein was measured. The concentration of bound zinc was determined from the standard curve and used to calculate the mole ratio of released zinc to protein. The average and standard deviations of these values were plotted in Fig. 1.

Gene fusions to green fluorescent protein were constructed by cloning PCR-derived PIB1 DNAs into pGO-GFP (20). The full-length GFP-Pib1 fusion vector has been described (6). To construct the GFP-Pib1(FYVE) fusion, the full-length GFP-Pib1 vector was cut with KpnI, and then released. This vector directed the expression of Pib1p amino acids 1–107. A GFP-Pib1(RING) fusion vector was constructed as follows. A DraI, SphI fragment of PIB1 (native Pib1p sites) was cloned into the Smal site of pGO-GFP (20). This vector directed the expression of Pib1p amino acids 91–286 fused to GFP.

A rabbit antisera was raised against a His-tagged Pib1 fusion protein (amino acids 91–286) by Cocalico Biologicals (Reamstown, PA). The GST-Pib1 fusion protein (amino acids 91–286) was used to affinity purify the GST-Pib1 fusion protein from 10 ml of cells. To do so, 1 ml of fusion protein was cross-linked to cyanogen bromide-activated Sepharose (Amersham Pharmacia Biotech) and observed at 10-min intervals. Images were captured on Leica DM IRBE inverted microscope fitted with a ×63 plan-apo lens. GFP-Pib1p and FM4-64 signals were observed using a Chroma 41001 and Chroma 4104 filter sets. There was no interference of GFP and FM4-64 signals. Images were captured and processed using Openlab version 2.2.5 (Leica) Adobe Photoshop version 5.0. Strains were grown in selective media overnight and the following morning a fresh culture was inoculated and grown at 26 °C until an A600 of ~0.5. Approximately 3 μl of culture was mounted on a glass slide and visualized.

**Ubiquitination Assays—**E1 (rabbit) and E2 (human Ubc5a, yeast Ubc10) enzymes were purchased from Affiniti Research Products Limited (Oxon, United Kingdom). Ubiquitin was purchased from Sigma-Aldrich. Ubiquitination assays contained the following: 50 mM Tris, pH 7.5, 2.5 mM MgCl2, 0.5 mM dithiothreitol, 100 mM E1, 2.5 mM GST-Ubc5a, 5 μM ubiquitin, and 5 μg of recombinant protein to be tested. Reactions were mixed on ice, incubated at room temperature for 2 h, then stopped by the addition of 5 reaction volumes of SDS-PAGE sample buffer. Ubiquitin conjugates were detected after SDS-PAGE by immunoblotting with a rabbit anti-ubiquitin antiserum (Santa Cruz Biotechnology) used at a 1:1000 dilution.

**RESULTS AND DISCUSSION**

**Pib1p Contains a Second RING Domain—**The amino-terminal domain of Pib1p contains a FYVE domain and a RING domain. As a control, a mutation in the codon of a predicted RING domain, in addition to the previously reported FYVE domain, is predicted to bind 2 mol eq of zinc. To test this, a region of Pib1 containing the putative RING domain (amino acids 91–286) was expressed in bacteria as a fusion to GST, purified, and zinc content was determined using a colorimetric assay (Fig. 1). This region of Pib1p bound ~2 mol eq of zinc (average of three determinations, 2.3 ± 0.14), consistent with the prediction that this region forms a RING structural domain. As a control, a mutation in the codon of a predicted zinc-coordinating residue in one of the zinc-binding pockets, C225S, was constructed and the zinc content of the mutant protein was determined. Based on analogous mutations in other RING domains (22), this mutation is predicted to reduce or completely abolish zinc binding. The mutant protein bound ~0.24 ± 0.01 mol eq of zinc, suggesting that the global structure of this domain is dependent on zinc binding. Moreover, in multiple preparations this mutant fusion protein was partially degraded (data not shown), whereas the wild-type fusion protein was highly stable, consistent with this region forming a stable structure that is dependent on zinc binding. These results strongly suggest that Pib1p contains a carboxyl-terminal RING domain, in addition to the previously reported FYVE domain.

**Identification of PIB1 Gene Product—**To determine whether Pib1p is expressed in vegetative cells, we raised a polyclonal antisera to a region of Pib1p encompassing amino acids 91–266 and affinity purified it on a GST-Pib1(RING) column. This antisera specifically detected a protein of ~43 kDa in wild-type cells that was absent in an extract prepared from cells deleted for the PIB1 gene and two prominent species at ~48 and 60 kDa that are, however, present in the extract of pib1Δ
Fig. 1. Pib1p contains a FYVE domain and a RING domain. A, Pib1p constructs used in these studies. The FYVE domain and the RING domain of Pib1p are illustrated, and the amino acid positions indicating the boundaries of the various constructs are indicated. The positions of two cysteine residues that were mutagenized are indicated. B, Pib1p RING domain is homologous to RING domains demonstrated to have ubiquitin ligase activity. Proteins are identified to the left. Light shaded residues with a dot above are putative zinc-coordinating residues. Dark shaded boxes indicate positions which are highly conserved between RING domains of this family. The boxed cysteine residue in the Pib1p sequence indicates the amino acid which was mutagenized in this study (see text). Accession numbers and amino acid positions shown in the alignment are as follows: S. cerevisiae Pib1p, NP_010599, amino acids 154–283; Mus musculus PRAJA, accession AAK15764, amino acids 531–572; Homo sapiens c-Rel, accession P22681, amino acids 381–420; S. cerevisiae Hrd1p, accession NP_014630, amino acids 349–400; H. sapiens TRC8, accession AAC39930, amino acids 547–586; H. sapiens ROC1, accession NP_055060, amino acids 61–103; H. sapiens c-cbl, accession NP_06219, amino acids 355–409; H. sapiens kf-1, accession BAB20900, amino acids 621–663. C, the RING domain of Pib1p binds 2 mol eq of zinc. A GST fusion protein containing residues 91–286 of Pib1p was expressed in E. coli, purified, and the zinc content was used to determine the proportion of Pib1p that is membrane associated using subcellular fractionation. Spheroplasts prepared from a wild-type strain were lysed and several particulate fractions and a soluble fraction were generated by successive centrifugations, and the proportion of Pib1p in various fractions was determined using anti-Pib1p immunoblotting (Fig. 2). Approximately equal proportions of Pib1p were present in the particulate (sum of all pellet fractions) and soluble fractions. Pib1p in the particulate fractions was distributed between the 300 × g, 13,000 × g, and 100,000 × g fractions (Fig. 2).

FYVE Domain of Pib1p Is Required for Endosome and Vacuole Membrane Localization—We have shown previously that in living cells a GFP-Pib1p fusion protein localizes to punctate structures and to the vacuole membrane, which is similar to the localization of a GFP human EEA1 FYVE domain fusion protein (6). The punctate structures decorated by GFP-Pib1p fusion protein localizes to punctate structures and to the vacuole membrane, which is similar to the localization of a GFP human EEA1 FYVE domain fusion protein (6). The punctate structures decorated by GFP-Pib1p are probably late endosomes. After 60 min, most GFP-Pib1p-labeled structures initiate a wave of endocytic uptake of FM4-64 and at 10-min intervals FM4-64 and GFP-Pib1p fluorescence were examined by fluorescence microscopy. No co-localization was observed between time 0 and 20 min (not shown), times when early endocytic intermediates are labeled by FM4-64 (23). At the 30-min time point, however, most GFP-Pib1p-labeled structures were also stained by FM4-64 (Fig. 3), indicating that the puncta labeled by GFP-Pib1p are endosomes. Co-localization at this relatively late time point suggests that the puncta decorated with GFP-Pib1p are probably late endosomes. After 60 min, all FM4-64 had been delivered to the vacuole (data not shown). To investigate the importance of each of the Pib1p domains for intracellular localization, we constructed GFP fusion proteins with Pib1p point mutants in which zinc-coordinating cysteine residues of the FYVE domain (C225S) and the RING domain (C225S) had been changed. Each of these proteins was
expressed in wild-type cells and localization determined by fluorescence microscopy (Fig. 4). Full-length GFP-Pib1p localized to 1–5 small puncta per cell that correspond to endosomal compartments, and to the vacuole membrane as observed previously (6). Note that in these micrographs the vacuole membrane signal is difficult to fully appreciate because of the intense fluorescence of the puncta. In contrast, the C23S FYVE domain mutant did not localize to endosomal/vacuole compartments, but rather, it was diffusely distributed throughout the cell. Surprisingly, in many cells GFP signal from this mutant protein was observed in the nucleus, but a GFP-Pib1p(RING) domain construct lacking the FYVE domain does not localize to the nucleus (see below), so the physiological significance of the localization of the C23S mutant protein is not apparent. Localization of the C225S RING mutant was indistinguishable from wild-type GFP-Pib1p.

We next examined localization of individual domains of Pib1p as fusions to GFP in wild-type cells (Fig. 4). GFP-Pib1p(FYVE) (amino acids 1–88) localized similarly to full-length GFP-Pib1p, but there was slightly more vacuole membrane signal with GFP-Pib1p(FYVE). The complementary construct, containing the linker and RING domains, localized to the cytosol. Thus, localization of Pib1p in vivo requires an intact FYVE domain (Fig. 4) and active Vps34 PtdIns 3-kinase (6), leading us to conclude that the FYVE domain is necessary and sufficient for localization of Pib1p to endosome and vacuole membranes containing PtdIns(3)P. As an independent test of localization of these constructs, strains expressing each of these constructs were subjected to subcellular fractionation, indicating that localization of these constructs is due predominantly to Pib1p sequences. These results suggest that the FYVE domain of Pib1p is solely responsible for targeting Pib1p to endosomal and vacuolar membranes.

Localization of GFP-Pib1p(FYVE) to endosomes and the vacuole membrane indicates that the cytoplasmic leaflets of these membranes contain PtdIns(3)P. Steady state localization of PtdIns(3)P within the cell by immunoelectron microscopy, using a FYVE domain from the human EEA1 protein for PtdIns(3)P detection, indicated that the majority of PtdIns(3)P is localized to the internal membranes of late endosomes and the lumen of the vacuole/lysosome (24). In yeast, most PtdIns(3)P is turned over within the lumen of the vacuole (25) and it is likely that PtdIns(3)P is internalized to the vacuole lumen first by incorporation into internal vesicles of multivesicular endosomes and then delivery to the vacuole. In addition, PtdIns(3)P may be internalized from the vacuole membrane by a microautophagocytosis process in which lipid rich, protein-depleted regions of the vacuole membrane are internalized (26). Because GFP signal from GFP-Pib1p constructs (Fig. 3) and from GFP-EEA1(FYVE) (6) was not observed in the vacuole, a mechanism must exist to prevent PtdIns(3)P-binding proteins from internalization into internal vesicles of multivesicular endosomes and the vacuole lumen.

Pib1p RING Domain Has Ubiquitin Ligase Activity—Comparison of the sequence of the carboxyl-terminal RING domain of Pib1p to other RING domains revealed that it is highly similar to RING domains found in proteins recently shown to function as E3 ubiquitin ligases (Fig. 1). Importantly, the sequence of Pib1p contains several key residues, including a tryptophan residue conserved with human cbl oncoprotein, required for ubiquitin ligase activity (27). Ubiquitination of substrate proteins requires that ubiquitin be activated through a cascade of covalent transfers from ubiquitin activating enzyme, E1, to any of multiple ubiquitin carrier proteins called UBCs or E2s. Finally ubiquitin is transferred to a substrate protein. The presumed role of E3 ubiquitin ligases is to bind substrate proteins to be ubiquitinated and to present them to activated E2-ubiquitin.

Ubiquitin ligase activity can be assessed in vitro using purified components. In these assays, ubiquitin ligase activity is promiscuous in that ubiquitin can be transferred to non-native substrates such as GST or GST-RING fusion proteins, and ubiquitin conjugates can be detected by immunoblotting with antibodies to ubiquitin (28, 29). To determine whether the Pib1p RING domain has ubiquitin ligase activity, in vitro assays employing recombinant E1 and E2 proteins, native ubiquitin.
The Pib1p RING domain exhibits E2-dependent ubiquitin ligase activity in vitro. In vitro ubiquitin ligase assays were carried out using GST-Pib1(RING), or a mutant GST-Pib1(RING) with a mutation changing Cys-225 to Serine. "No +" indicates no GST fusion protein was added to the reaction. The experiment in the right panel employed wild-type GST-Pib1(RING) and the indicated reaction components were omitted.

Uitin, and GST-Pib1(RING) were carried out. Recombinant GST fusion proteins containing the Pib1p RING domain (amino acids 91–286), or a mutant RING domain (C225S) were tested for E3 ligase activity. As shown in Fig. 5, GST-Pib1p(RING) exhibited ubiquitin ligase activity, as evidenced by the ladder of anti-ubiquitin-reactive material present in lane 1. In contrast, the RING mutant exhibited little activity (Fig. 5, compare lanes 1 and 2). In reactions in which one component was omitted from the reaction, ubiquitin ligase activity by the GST-Pib1p(RING) fusion protein was found to require ATP, E1, and E2. These results indicate that the Pib1p RING domain has E2-dependent E3 activity.

What might the function of Pib1p be? E3 ubiquitin ligases are thought to function as adapter molecules which bind a target substrate protein and present it to the ubiquitination machinery. The crystal structure of a Cbl/UbcH7-ZAP-70 ternary complex, containing the Cbl RING domain, the UbcH7 E2 ubiquitin carrier protein, and a substrate peptide derived from ZAP-70, has been interpreted in this perspective (30). The surface of Cbl, including the RING domain, makes contacts with both the substrate and the E2 and is postulated to direct the substrate polypeptide via a putative substrate-binding "channel" leading directly to the E2 active site (30). We speculate that Pib1p may mediate ubiquitination of endosomal and vacuolar proteins by binding the target protein and recruiting an as yet unidentified E2 enzyme. It is not yet known with which E2(s) Pib1p functions in vivo, but the results of the in vitro ubiquitination assays clearly indicate that it can use yeast Ub4p (data not shown) and human Ubc5 (Fig. 4).

In yeast, it is well established that single ubiquitin chains (monoubiquitin) attached to membrane proteins can serve as a targeting signal to sort these proteins to the vacuole (31). In contrast, polyubiquitin chains usually target proteins for degradation by the proteasome, although recent evidence suggests that polyubiquitin may also target Gap1p to the vacuole (32). These observations raise the possibility that Pib1p may function in ubiquitin-dependent sorting of proteins to the vacuole, and is consistent with the fact that three of the five FYVE domain proteins encoded in the yeast genome, Vac1/Pep7p, Vps27p, and Fab1p, function in vacuole protein sorting pathways. To determine whether Pib1p functions in one or more known protein sorting pathways to the vacuole, we examined localization of multiple vacuolar proteins in a pib1Δ null mutant using several different methods. By pulse-chase analysis, no defects in the biosynthetic pathways which mediate vacuolar localization of newly synthesized carboxypeptidase Y, carboxypeptidase S, proteinase A, proteinase B, alkaline phosphatase, or aminopeptidase I were observed (data not shown). Moreover, steady state localization of carboxypeptidase S and vacuolar alkaline phosphatase were determined using well characterized GFP-carboxypeptidase S and GFP-alkaline phosphatase fusion genes which are localized to the vacuole of wild-type cells (10, 20). In pib1Δ cells, GFP-alkaline phosphatase was localized exclusively to the vacuole membrane, and GFP-carboxypeptidase S was localized exclusively to the vacuole lumen, exactly as in wild-type cells (data not shown).

We also tested for kinetic defects in endocytic targeting of proteins to the vacuole in pib1Δ cells by determining degradation kinetics of Ste2p, Ste3p, Ste6p, Tat2p, and Gap1p. Each of these proteins functions at the plasma membrane, but is degraded within the lumen of the vacuole, and a defect in vacuolar sorting would be predicted to increase the half-life of each protein. For all cases, degradation rates in pib1Δ cells were indistinguishable compared with wild-type strains (data not shown). Finally, we also examined uptake of FM4-64, a fluorescent lipophilic tracer for bulk membrane endocytosis and did not observe any differences (compared with wild-type cells) in either the kinetics of uptake, or in the overall morphology of endocytic intermediates stained with the dye (data not shown). Taken together, these results suggest that Pib1p does not perform an essential role in sorting or trafficking of proteins to the vacuole, at least for the indicated proteins and the conditions used for these experiments. Importantly, at least two other ubiquitin ligases, Rsp5p and Rcy1p, function in post-Golgi sorting of proteins to the vacuole, so it is possible that the function of Pib1p is partially redundant with these proteins. Another possibility is that Pib1p targets non-native (e.g. damaged) proteins in endosomal and vacuolar membranes for degradation via the proteasome or the vacuole, as a component of an endosomal quality control system. The identification of Pib1p as a ubiquitin ligase highlights the role of the FYVE domain in intracellular targeting and may provide a molecular link for understanding the diverse roles of effectors of phosphatidylinositol 3-kinase.

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