The Yeast Inositol Polyphosphate 5-Phosphatase Inp54p Localizes to the Endoplasmic Reticulum via a C-terminal Hydrophobic Anchoring Tail

REGULATION OF SECRETION FROM THE ENDOPLASMIC RETICULUM*

The budding yeast Saccharomyces cerevisiae has four inositol polyphosphate 5-phosphatase genes, INP51, INP52, INP53, and INP54, all of which hydrolyze phosphatidylinositol (4,5)-bisphosphate. INP54 encodes a protein of 44 kDa which consists of a 5-phosphatase domain and a C-terminal leucine-rich tail, but lacks the N-terminal Sac1 domain and proline-rich region found in the other three yeast 5-phosphatases. We report that Inp54p belongs to the family of tail-anchored proteins and is localized to the endoplasmic reticulum via a C-terminal hydrophobic tail. The hydrophobic tail comprises the last 13 amino acids of the protein and is sufficient to target green fluorescent protein to the endoplasmic reticulum. Protease protection assays demonstrated that the N terminus of Inp54p is oriented toward the cytoplasm of the cell, with the C terminus of the protein also exposed to the cytosol. Null mutation of INP54 resulted in a 2-3 fold increase in secretion of a reporter protein, compared with wild-type yeast or cells deleted for any of the Sac1 domain-containing 5-phosphatases. We propose that Inp54p plays a role in regulating secretion, possibly by modulating the levels of phosphatidylinositol (4,5)-bisphosphate on the cytoplasmic surface of the endoplasmic reticulum membrane.

Phosphoinositides are ubiquitous membrane components of various intracellular compartments, which regulate many diverse cellular functions including membrane trafficking events, secretion, actin cytoskeletal organization, cellular proliferation, and inhibition of apoptosis (reviewed in Refs. 1-4). Many of these functions are mediated by binding and recruiting signaling proteins which contain specific phosphoinositide-binding domains such as SH2 domains, pleckstrin homology domains, FYVE domains, C2 domains or polybasic domains, thereby localizing these effector proteins to specific membranes (reviewed in Refs. 3 and 4).

Phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P₂) serves as a precursor to second messenger molecules such as inositol (1,4,5)-trisphosphate and phosphatidylinositol (3,4,5)-trisphosphate, but also independent of further modification regulates the actin cytoskeleton and membrane trafficking (1, 5). PtdIns(4,5)P₂ binds to actin-binding proteins such as profilin and gelsolin (6) and displaces capping proteins from actin filaments, allowing polymerization and formation of actin stress fibers (7-9). PtdIns(4,5)P₂ also plays a role in regulating vesicle budding and in the recruitment and activation of proteins involved in the coating of vesicles (2).

Cellular levels of PtdIns(4,5)P₂ are regulated by a series of lipid phosphorylation and dephosphorylation reactions mediated by specific lipid kinases and phosphatases. Inositol polyphosphate 5-phosphatases (5-phosphatases) regulate cellular PtdIns(4,5)P₂ levels by hydrolyzing the 5-position phosphate from the inositol ring forming phosphatidylinositol 4-phosphate (PtdIns(4)P) (10, 11). The budding yeast Saccharomyces cerevisiae has four 5-phosphatase genes, INP51, INP52, INP53, and INP54. Inp51p, Inp52p, and Inp53p each comprise an N-terminal Sac1 domain, a central 5-phosphatase domain, and a C-terminal proline-rich region (12, 13). These enzymes share significant sequence homology with the mammalian homologue synaptojanin, which regulates the recycling of synaptic vesicles in nerve terminals (14). Synaptojanin, Inp52p, and Inp53p contain two catalytic domains, a central 5-phosphatase domain and an N-terminal Sac1 domain which hydrolyzes PtdIns(3,5)P₂, PtdIns(4)P, and PtdIns(3)P forming PtdIns (15). Null mutation of any of two Sac1 domain-containing 5-phosphatases results in plasma membrane invaginations and thickened cell walls, defects in polarization of the actin cytoskeleton, and impaired endocytosis (12, 13). However, double Sac1 domain-containing 5-phosphatase null mutants display normal secretion of invertase suggesting that Inp51p, Inp52p, and Inp53p do not play a role in regulating secretion (16). A triple Sac1 domain-containing 5-phosphatase null mutant is nonviable suggesting Inp54p cannot function to rescue the loss of these three 5-phosphatases.

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The abbreviations used are: PtdIns(4,5)P₂, phosphatidylinositol (4,5)-bisphosphate; 5-phosphatase, inositol polyphosphate 5-phosphatase; PtdIns(3)P, phosphatidylinositol (3)-phosphate; PtdIns(4)P, phosphatidylinositol (4)-phosphate; PtdIns(3,5)P₂, phosphatidylinositol (3,5)-bisphosphate; BPTI, bovine pancreatic trypsin inhibitor; B1P, binding protein; LRD, leucine-rich domain; bp, base pair(s); ORF, open reading frame; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein; ER, endoplasmic reticulum; HA, hemagglutinin.
Inp54p Is an ER-localized Tail-anchored Protein

Expression and Purification of Recombinant (His)_6-Inp54p—Two × 100-ml cultures of pTrcHisB-Inp54p were grown at 37 °C to an A_600 of 0.5–0.6 prior to induction with 0.1 mM isopropyl-β-D-galactoside for 2 h at 37 °C. Following induction, cells were pelleted and soluble proteins were extracted in 1/10 volume of buffer B (20 mM Tris, pH 8, 250 mM NaCl) supplemented with 12 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotonin, 2 μg/ml leupeptin, 1 mM aminoguanidine, and 1% Triton X-100 at 4 °C overnight with gentle agitation. Triton extracts were centrifuged at 15,000 × g for 15 min then the 20-ml supernatant was incubated with 2.5 ml of Talon resin (CLONTECH) with gentle agitation in a 50-ml tube for 4 h at 4 °C. Following incubation, the resin was poured into a column and washed with 20 column volumes of buffer B. Bound proteins were eluted with 4 column volumes of buffer B at pH 6.5 supplemented with 100 mM imidazole and 700-μl fractions were collected. 50 μl of the starting material, flow through, and the eluted fractions were analyzed by 12% SDS-PAGE (22), and either visualized by Coomassie Brilliant Blue staining or transferred to polyvinylidene fluoride membranes according to standard protocols (23) and immunoblotted using monoclonal antibodies to the (His)_6-tag (Silenus). Immunoblots were developed using enhanced chemiluminescence (ECL) reagent (PerkinElmer Life Sciences) according to the manufacturer’s instructions. The protein concentration in Coomassie-stained gels was quantitated using densitometry by comparison with a standard amount of protein loaded on the gel.

PtdIns(4,5)P_2 5-Phosphatase Enzyme Assays—PtdIns(4,5)P_2 substrate was a mixture of 33.3 μM PtdIns(4,5)P_2 and 3 μg of phosphatidylinerse, dried under nitrogen, resuspended in 50 μl of lipid resuspension buffer (20 mM Hepes, pH 7.5, 1 mM MgCl_2, 1 mM EGTA), and sonicated for 5 min. The recombinant Inp54p was incubated with the substrate in the presence of kinase buffer (20 mM Hepes, pH 7.4, 1 mM EGTA, 5 mM MgCl_2) in a 100-μl total reaction volume. Assays were performed at 37 °C for 30 min using two linear protein concentrations in duplicate. The reaction was terminated by incubating with 1 ml of Biotinol Green™ reagent at room temperature for 30 min. The absorbance at 620 nm was measured and the amount of phosphate released calculated using known standards supplied in the supplied in the Biomol kit. The release of phosphate was measured as the change in absorbance at 620 nm due to release of 1 μM PtdIns(4,5)P_2 per minute. The identity of all constructs was confirmed using deoxy sequencing analysis.

Expression of GFP-tagged Inp54p in S. cerevisiae—All INP54 constructs were amplified by PCR using appropriate primers listed in Table II, from SEY6210 genomic DNA. The primers incorporated a BamHI site for cloning INP54 into the pPS1303 vector, in-frame with the C-terminal GFP. The PCR product was ligated into pCRBlunt, released by BamHI digestion, and ligated to the compatible BglII site in pPS1303H, GFP-tagged LbFG3, 12, 11, and 10 were amplified from pPS1303-IPN54 using oligonucleotides listed in Table II and a 3′ primer specific for the 3′ end of the GFP ORF. The resulting LRD-GFP products were cloned into pCRBlunt, excised using BamHI and cloned into the BglII site of pPS1303H. pPS1303H lacked the GFP ORF and was constructed by excising GFP from pPS1303 via HindIII digestion followed by religation of the vector. The INP54-psPS1303 constructs were transformed into an inp54 null mutant strain using the S. cerevisiae EasyComp Transformation kit (Invitrogen) and the transformants were selected on complete minimal media agar plates lacking uracil. The identity of all constructs was confirmed using deoxy sequencing analysis.

Yeast strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| W303   | MATα/α ade2-1/ade2-1 trp1-1/1 trp1-1, leu2-3,112/leu2-3,112 his3-11,15/his3-11,15, ura3-1/1 ura3-1 | a |
| W303a  | MATα ade2-1/1 trp1-1 leu2-3,112 his3-11,15,15 ura3-1/1 ura3-1 | “ |
| SEY6210| ura3-52 leu2-3,112 his3-32,000 trp901 lys2-801 suc2-Δ9 | Ref. 70 |
| inp54  | ade2-1/1 trp1-1 leu2-3,112 his3-11,15 ura3-1, inp54::LEU3 | This study |
| inp52  | ade2-1/1 trp1-1 leu2-3,112 his3-11,15 ura3-1, inp52::URA3 | Ref. 21 |
| inp53  | ade2-1/1 trp1-1 leu2-3,112 his3-11,15 ura3-1, inp53::TRP1 | Ref. 21 |

*These yeast strains were the kind gift of Dr. D. Germain.
stained with an anti-GFP antibody (CLONTECH) to amplify the signal, and analyzed using confocal microscopy as described above.

Expression of HA-tagged Inp54p in *S. cerevisiae*—The HA-tagged Inp54p was cloned into the pPS1303H expression vector lacking the GFP coding region. The primers used to amplify *INP54* incorporated a HA tag at either the 5′ or 3′ end, with a BamHI site (see Table II), this facilitated cloning into the *BglII* site of pPS1303H vector. The resulting constructs were transformed into *inp54* null mutants as described above.

Analysis of GFP and HA-tagged Inp54p in *S. cerevisiae* by Confocal Microscopy—Yeast cells were grown overnight in complete minimal media + 2% glucose, diluted 1/200 in 2% raffinose, and induced the next day in 2% galactose for 4 h. Cells were fixed according to Franzusoff et al. (24) and stained with either an anti-HA antibody (diluted 1/1000) to amplify the signal, or an anti-GFP antibody (undiluted 1/25) to co-localize the GFP-tagged proteins or an anti-BiP antibody (diluted 1/25) to visualize BiP (binding protein) in the same fraction. Glass slides were mounted with SlowFade (Molecular Probes).

Extraction of Recombinant GFP- and HA-Inp54p from Yeast—This extraction method is a slight modification of that described in Seedorf and Silver (25). Yeast cells expressing Inp54p tagged with GFP or HA were harvested and resuspended in PBSM buffer (phosphate-buffered saline, 5 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride, and 3 μg/ml each of leupeptin, aprotinin, pepstatin, and chymostatin. Glass beads (250 μm) were added to the suspension, vortexed, and incubated on ice at 30-s intervals for 12 cycles, respectively. The lysate was centrifuged at 10 min at 4 °C, the supernatant represented cytosolic fraction, the pellet was resuspended in PBSM buffer with 1% Triton. After overnight Triton extraction at 4 °C, the lysate was spun for 10 min to separate the Triton-soluble and Triton-insoluble fractions. These were separated on 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes according to standard protocols (23), and immunoblotted using an antibody specific for GFP (P. Silver) or HA (Covance). To determine the 5-phosphatase activity of Inp54p tagged with GFP or HA, the fusion protein was immunoprecipitated from the Triton-soluble fraction using 50 μl (50% v/v) of protein A-Sepharose, 0.8 μg of anti-GFP antibody (Roche Molecular Biochemicals) or 6 μg of anti-HA antibody (Covance), and 5 μg of rabbit anti-mouse immunoglobulin (Dako) which served as a linker. Immunoprecipitation was performed at 4 °C overnight with gentle agitation. The protein A-Sepharose pellet was washed 6 times with ice-cold Tris saline (20 mM Tris, pH 7.2, 150 mM NaCl), and the pellet used in PtdIns(4,5)P₂ 5-phosphatase enzyme assays as described previously.

Protease Protection Assays on Yeast Expressing Recombinant HA- or GFP-Inp54p—Micromones were extracted from yeast by differential centrifugation fractionation according to Parlati et al. (26) and Paddon et al. (27). The medium-speed microsomal pellet, which was found to be enriched in ER markers, was treated with protease K according to the methods described by Bascou et al. (28). The control fractions (untreated) and protease K-treated fractions were separated by 10% SDS-PAGE, and immunoblotted with either an anti-HA antibody or anti-GFP antibody. The same fractions were immunoblotted with an anti-BiP antibody as a control to ensure that the microsomal membranes were intact.

**TABLE II**

| Construct | Features | Primers |
|-----------|----------|---------|
| pPS1303   | GFP      |         |
| pTrcHisB  | 6 × His tag | Invitrogen |
| *INP54*-pTrcHisB | Full-length His-Inp54p | This study |
| *INP54*-pPS1303 | His-Inp54p | This study |
| *INP54*-pPS1303 | Full-length Inp54p-GFP | This study |
| *INP54*-pPS1303 | Inp54p-GFP | This study |
| *INP54*-pPS1303 | Inp54p-GFP | This study |
| HA-*INP54*-pPS1303H | Full-length HA-Inp54p | This study |
| LRD13-pPS1303 | LRD13-GFP | This study |
| LRD12-pPS1303 | LRD12-GFP | This study |
| LRD11-pPS1303 | LRD11-GFP | This study |
| LRD10-pPS1303 | LRD10-GFP | This study |
| *INP54*-pRS416-GFP | Inp54p-GFP under its native promoter | This study |
| pEB316U | BPTI expression plasmid with uracil marker | This study |
| YEplac181-GalBPTI | BPTI expression plasmid with uracil marker | This study |

a This plasmid was a kind donation from Prof. Pamela Silver.
b pPS1303 vector with the GFP ORF deleted, bold letters represent the HA sequence.
c This plasmid was a gift from Prof. Dane Wittrup.
cleotides listed in Table II and cloning the PCR product into the EcoRI/HindIII site of YEplac181 vector (29). W303a and the inp54 mutant strain were transformed with a BPTI expression plasmid, pEB316U, containing a uracil nutritional marker. Yeast cells expressing BPTI were grown at 30 °C overnight in complete minimal media lacking either leucine or uracil, supplemented with 2% glucose. The next day, cultures were diluted 1/200 in raffinose containing media, grown overnight until they reached 10⁷ cells per ml, and induced with galactose for the specified time frames. The amount of BPTI secreted in the culture media was quantified according to the method described by Parekh et al. (30). A rescue experiment was done to investigate whether ∆inp54 mutant could remove a sufficient amount of BPTI from the intracellular space and maintain viability when grown continuously on galactose-supplemented media. Since accumulation of high levels of BPTI is toxic, continuous induction would be lethal to the cells. Raffinose cultures of yeast cells were spotted in 10-fold serial dilutions in 5-μl aliquots onto complete minimal agar plates supplemented with either 2% glucose or galactose.

RESULTS

Inp54p Sequence Predicts for a C-terminal Tail-anchor—Inp54p is one of four inositol polyphosphate 5-phosphatases (5-phosphatases) found in the yeast S. cerevisiae. Unlike the other previously characterized yeast 5-phosphatases, Inp54p predicts for a smaller molecular mass, 44 kDa, comprising a central 300-amino acid 5-phosphatase domain and no other defined signaling motif. However, we noted that the C terminus of Inp54p strongly resembled that found in many C-terminal tail-anchored proteins (Fig. 1B). Tail-anchored proteins are a class of integral membrane proteins which lack any N-terminal targeting sequence and insert into membranes via a single C-terminal hydrophobic sequence (32–36). These proteins, which include cytochrome b₅, UBC6, Bcl2, and numerous SNARE proteins have been identified in both mammalian cells and S. cerevisiae (32, 37). A comparison of the C-terminal hydrophobic domain of Inp54p versus previously characterized tail-anchored proteins is shown in Fig. 1B. Tail-anchored proteins are predominantly localized on the membranes of the endoplasmic reticulum and/or mitochondria, with the bulk of the protein oriented toward the cytoplasm (33, 34, 36–38).

Inp54p Localizes to the ER of the Cell—To characterize the intracellular localization of Inp54p, the protein was expressed as a fusion protein with GFP under the control of a galactose-inducible promoter in inp54 null mutant cells. Cells cultured in the presence of raffinose, which does not induce production of the fusion protein, demonstrated no fluorescence (Fig. 2A). Incubation of cells in media supplemented with galactose resulted in expression of Inp54p-GFP fluorescence in the perinuclear region, as shown by co-localization with propidium iodide, which stains the nucleus (Fig. 2B). Co-localization using an antibody directed against the yeast protein Kar2p or BiP which localizes to the ER, demonstrated co-localization with Inp54p-GFP expression. Peripheral and reticular staining was also detected by both Inp54p-GFP fluorescence and the anti-BiP

Fig. 1. A, hydropathic profile of Inp54p calculated using the Kyte-Doolittle x–1 scale. In this plot, a value of –2 indicates strong hydrophobicity corresponding to a transmembrane topology. The leucine-rich sequence of the C-terminal 13 amino acids is shown. B, table showing the C-terminal portions of several known tail-anchored proteins. The hydrophobic tail which serves as a membrane anchor is highlighted in gray. The following abbreviations are used: Inp54p (Inp54p; Saccharomyces cerevisiae, GenBank accession number NP_014576), PTN1_HUMAN (protein-tyrosine phosphatase 1B; Homo sapiens, P10415), SYB1_HUMAN (synaptobrevin 1; H. sapiens, P23763), VAMP_1B (synaptobrevin 1 isoform Vamp-1B; H. sapiens, AAC28336), SYB2_HUMAN (synaptobrevin 2; H. sapiens, P19066), SNC1_Yeast (synaptobrevin homolog 1; S. cerevisiae, P31109), U61p (t-SNARE; S. cerevisiae, AAC13730), CYB5_HUMAN (cytochrome b₅; H. sapiens, P00167), and CAATJ7117 (cytochrome b₅, Rattus norvegicus, mitochondrial isoform, CAATJ7117).
antibody. To confirm that GFP did not play any role in targeting the fusion protein to the ER, cells expressing the GFP alone were induced and analyzed. GFP was detected throughout the entire cell (Fig. 2B).

To ensure that the ER localization of Inp54p-GFP did not result from overexpression, which may cause proteins to distribute aberrantly, Inp54p-GFP was expressed under the control of its native promoter. The entire INP54 open reading frame and 968 bp upstream of the initiating ATG was amplified by PCR and cloned into a single copy yeast expression vector pRS416-GFP, which lacks a promoter. The construct was transformed into inp54 null mutant cells, which were grown to mid-log phase and analyzed by confocal microscopy. As the expression level was very low (results not shown) the signal was amplified using an antibody specific for GFP. Inp54p-GFP expressed under the Inp54p native promoter localized to a perinuclear ring-like structure, comparable to that observed in Inp54p overexpressing cells (Fig. 2C), indicating that the ER localization of Inp54p is not affected by the level of expression of the recombinant protein.

To further validate the intracellular distribution of Inp54p in yeast cells and demonstrate that the position of the tag did not influence the localization of the protein, a hemagglutinin (HA) tag was fused at either the N or C terminus of Inp54p. Inp54p was amplified by PCR as described under “Experimental Procedures,” with the HA tag incorporated in either the 5' or 3' oligonucleotide primer, and cloned into the pPS1303H vector from which the GFP sequence had been removed. Following induction, cells were fixed and stained with an antibody specific for HA to visualize the HA-tagged Inp54p. Fig. 2D shows that

**Fig. 2. Intracellular localization of Inp54p-GFP.** INP54 was cloned in-frame with GFP under the control of a galactose-inducible promoter and transformed into an inp54 null mutant strain. A, yeast were grown in the presence of raffinose to inhibit the production of the Inp54p-GFP fusion protein, and analyzed by confocal microscopy. The expression of GFP under the same conditions is included as a control. Bar indicates 5 \( \mu m \). B, expression of recombinant GFP, or Inp54p-GFP was induced following incubation for 4 h in galactose-containing media. The cells were fixed and stained with propidium iodide to visualize the nucleus, or an antibody directed against the Kar2p protein (BiP) in the endoplasmic reticulum. Cells induced for 2 h, which represent a lower level of expression, are also shown. Bar indicates 5 \( \mu m \). C, cells expressing Inp54p-GFP under the control of the native INP54 promoter were grown to mid-log phase, fixed, and stained with an anti-GFP antibody. Bar indicates 5 \( \mu m \). D, expression of Inp54p tagged with HA at either the N or C terminus was induced by growing the cells in galactose-containing media for 4 h. The cells were fixed and stained with anti-HA antibody to visualize the HA-tagged Inp54p. Bar indicates 5 \( \mu m \).
Inp54p tagged with HA was expressed in a perinuclear distribution consistent with ER localization, as observed with the GFP-tagged protein (Fig. 2B). In addition, these results demonstrate that the position of the tag at the N or C terminus did not affect the localization of the Inp54 protein, as has been shown previously for other ER tail-anchored proteins such as UBC6 (34) and cytochrome b$_5$ (33).

To confirm that Inp54p-GFP and HA-Inp54p were functional proteins, following induction in galactose-containing media, recombinant fusion proteins were extracted from yeast cells with Triton X-100, immunoprecipitated with antibodies specific for GFP or HA, and assayed for PtdIns(4,5)P$_2$ 5-phosphatase activity as described under “Experimental Procedures.” Immunoprecipitated GFP displayed no activity against PtdIns(4,5)P$_2$. Both Inp54p-GFP and HA-Inp54p were able to hydrolyze PtdIns(4,5)P$_2$, confirming that the constructs encoded functional proteins (results not shown).

**Inp54p Attaches to the Endoplasmic Reticulum Membrane with the Bulk of the Protein Oriented toward the Cytosol**

The C terminus of tail-anchored proteins inserts into the cytoplasmic surface of the endoplasmic reticulum or mitochondrial membrane with the N-terminal domain located in the cytosol (33, 37, 39–41). Inp54p lacks a signal sequence suggesting the enzyme does not represent an integral endoplasmic reticulum protein, but post-translationally associates with this compartment. The membrane orientation of Inp54p in the endoplasmic reticulum was determined by a protease protection assay. Cells expressing HA-Inp54p, or Inp54p-GFP, were subjected to differential centrifugation to isolate the ER-enriched microsomal membrane fraction (36). Recombinant Inp54p association with the membrane fraction was sensitive to alkaline treatment using 0.1 M NaCO$_3$, pH 11.5 (results not shown), therefore it is not an integral membrane protein. Inp54p associates tightly with the membrane, however, since treatment with 0.5 and 1 M NaCl failed to release recombinant Inp54p from membranes (results not shown). ER-enriched microsomes were incubated in the presence or absence of Proteinase K for 1 or 2 h at 4 °C, then centrifuged at 25,000 × g to obtain supernatant (S) and microsomal (M) fractions. Proteins were separated by 10% SDS-PAGE and immunoblotted with anti-HA (A) or anti-GFP antibodies (B). Molecular weight markers are shown on the left. The same fractions were probed with an anti-BiP antibody to confirm the integrity of the microsomal membranes. Untreated microsomes are included as a control.

**FIG. 3.** Inp54p attaches to the endoplasmic reticulum membrane with the bulk of the protein oriented toward the cytosol. Cells expressing HA-Inp54p (A) or Inp54p-GFP (B) were subjected to differential centrifugation to isolate ER-rich microsomes. Microsomes were treated with proteinase K for 1 or 2 h at 4 °C, then centrifuged at 25,000 × g to obtain supernatant (S) and microsomal (M) fractions. Proteins were separated by 10% SDS-PAGE and immunoblotted with anti-HA (A) or anti-GFP antibodies (B). Molecular weight markers are shown on the left. The same fractions were probed with an anti-BiP antibody to confirm the integrity of the microsomal membranes. Untreated microsomes are included as a control.

**The C-terminal Hydrophobic Domain of Inp54p Mediates Localization to the ER**

To determine whether the C-terminal hydrophobic domain mediates ER localization a series of C-terminal Inp54p truncation mutants were constructed and expressed in *inp54* null mutant yeast. Previous studies have demonstrated removal of the hydrophobic tail of tail-anchored proteins results in the relocalization of the truncated protein to the cytoplasm (42–47).

Mutant Inp54p$_{p331}$ contained the 5-phosphatase domain from amino acids 1–331 and lacked the entire hydrophobic C-terminal 53 amino acids. The second mutant, Inp54p$_{p373}$, lacked amino acids 354–384. These residues are predicted to have a transmembrane topology by a Kyte-Doolittle hydrophobicity plot analysis. Inp54p$_{p43}$ lacks the leucine-rich area spanning the last 13 amino acids (residues 372–384), which is the minimal hydrophobic domain in the C terminus. All mutant sequences were amplified by PCR using appropriate primers as described under “Experimental Procedures,” and cloned into the pPS1303 vector. Expression of the mutant fusion proteins was induced by incubating the cells in galactose-containing media for 4 h and cells were examined live by confocal microscopy. All three mutant proteins were expressed in the cytosol although Inp54p$_{p43}$-GFP still showed some ER staining, but to a much lesser degree than that observed in the full-length protein (Fig. 4A). This indicates that the C-terminal tail of Inp54p mediates ER localization and that the last 13 amino acids are required to maintain this localization.

To confirm the cytosolic localization of the mutant fusion proteins, yeast cells were fractionated into cytosol, Triton-soluble, and Triton-insoluble fractions as outlined under “Experimental Procedures.” Proteins were separated by SDS-PAGE and immunoblotted with an antibody specific for GFP (Fig. 4B). Full-length Inp54p-GFP which localized to the ER, was present intact, as a 71-kDa protein consistent with the predicted molecular mass of the recombinant protein, predominantly in the Triton-soluble fraction, with only a very small amount in the Triton-insoluble fraction. In contrast, the three Inp54p truncation mutants were expressed predominantly in the cytosol. Occasionally, a small, variable proportion of the mutant protein was detected in the Triton-soluble and Triton-insoluble fractions. Minimal proteolysis of recombinant mutant proteins was detected. These studies collectively indicate that the Inp54p C-terminal domain is critical for mediating membrane association, specifically to the endoplasmic reticulum.

To confirm that the 5-phosphatase catalytic function is not
regulated by the C-terminal hydrophobic domain, the activity of purified recombinant full-length Inp54p<sub>384</sub> versus mutant Inp54p<sub>331</sub>, which lacks the entire C-terminal hydrophobic domain, was determined. Recombinant wild-type versus C-terminal mutant (His)₆-tagged Inp54p were expressed in *Escherichia coli*, and purified using Talon metal affinity chromatography and assayed for PtdIns(4,5)P₂ 5-phosphatase activity. No significant difference in PtdIns(4,5)P₂ 5-phosphatase activity was detected between full-length Inp54p<sub>384</sub> or mutant Inp54p<sub>331</sub> (Fig. 4C) confirming that the C-terminal leucine-rich domain does not play a role in regulating enzyme activity.

The Last 13 Amino Acids of Inp54p Constitute the ER Localizing Sequence—Previous studies have demonstrated that C-terminal hydrophobic domains are sufficient to direct heterologous proteins to specific subcellular compartments (34, 38, 48). To determine whether the last 13 amino acids of Inp54p (372–384) comprise the ER targeting region, a series of leucine-rich domain (LRD) mutant constructs were generated, encoding the C-terminal 13, 12, 11, and 10 amino acids of Inp54p fused to GFP (designated LRD13, LRD12, LRD11, and LRD10, respectively). Following induction of the recombinant fusion proteins, expressing cells were analyzed by confocal microscopy. LRD13 was sufficient to localize GFP to the ER (Fig. 5). In contrast, LRD12, LRD11, and LRD10 fused to GFP predominantly demonstrated diffuse cytoplasmic staining, although minimal ER staining was still detected. This indicates that the last 13 amino acids of Inp54p constitute the minimum ER targeting motif, and while the shorter sequences contain some targeting information, it is not as efficient as LRD13.

Substitution of a Single Charged Amino Acid in the Tail-anchor Domain Has No Effect on Localization—Several models for the insertion of the C-terminal targeting domain of tail-anchored proteins to the membrane have been proposed. It has been suggested that the absolute sequence of the tail is not important, rather, the amino acid composition in terms of hydrophobicity and length of the domain dictate the intracellular localization (34, 49, 50). Furthermore, several studies indicate that charged amino acids within the tail-anchor are the determinants of specific subcellular location to the endoplasmic reticulum versus mitochondria. Kuroda *et al.* (48) demonstrated that charged amino acids within the last 10 amino acids of two isoforms of cytochrome *b*₅ (outer mitochondrial membrane *b*₅ and ER *b*₅) determined their localization in the cell. Mutation of the positively charged amino acids (Arg¹³⁷ and Lys¹⁴⁴) to noncharged residues altered the localization of outer mitochondrial membrane *b*₅ from the mitochondria to the ER. Mutation of the negatively charged residue (Asp¹³⁴) to a neutral Ala or a positive Lys in ER *b*₅ redirected this protein from the ER to the mitochondria.

Examination of the hydrophobic tail of Inp54p, in particular the last 13 amino acids at the extreme C terminus, revealed a single charged residue (Lys) at position 382, and no other

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**Fig. 4. The C terminus of Inp54p is required for ER localization.** A, mutant constructs of INP54 with C-terminal deletions were generated as described under “Experimental Procedures.” A schematic representation of each construct is shown on the left with the full-length Inp54p<sub>384</sub> included for comparison. The subscript on each construct refers to the last amino acid in the Inp54p mutant proteins. Expression of Inp54p-GFP C-terminal deletion mutants was induced by incubation with galactose for 4 h and following fixation the cells were analyzed by confocal microscopy. *Bar* indicates 5 μm. B, yeast cells expressing full-length or mutant Inp54p-GFP were fractionated into cytosol, Triton-soluble, and Triton-insoluble fractions as described under “Experimental Procedures.” The fractions were separated by 10% SDS-PAGE and immunoblotted with an anti-GFP antibody. C, *E. coli* cultures harboring recombinant full-length (His)₆-Inp54p<sub>384</sub> or mutant (His)₆-Inp54p<sub>331</sub> were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 23 °C. The induced cultures were extracted with 1% Triton X-100 overnight, and the soluble extract was purified by Talon resin immobilized metal affinity chromatography as described under “Experimental Procedures.” 50 μl of eluted fractions were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. Peak eluted fractions 6 and 7 for each recombinant wild-type or mutant Inp54p were assayed for PtdIns(4,5)P₂ 5-phosphatase activity in triplicate.
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charged amino acids. To investigate whether this residue plays a role in ER targeting, Lys382 was mutated to a neutral alanine. An HA tag was fused to the N terminus of Inp54p (K382A) and the mutant recombinant 5-phosphatase expressed in the 

null mutant strain. As can be seen from Fig. 6, mutation of Lys382 to Ala did not affect the ER localization of the protein. The ER isoform of cytochrome b6 has 2 charged residues at the C-terminal tail, an arginine and an aspartic acid. The negatively charged aspartic acid (Asp134) mediates ER localization, whereas the positively charged arginine (Arg128) demonstrated no role in localization (48). Since Lys 382 is the only charged residue in the tail of Inp54p, the “charged amino acids as determinant of intracellular localization” model cannot be applied in this case. A more detailed analysis of the 13-residue tail-anchor of Inp54p has to be performed to delineate the targeting information, and is the subject of ongoing studies.

**Null Mutation of inp54 Results in Increased Secretory Capacity Compared with Wild-type Cells**—In mammalian cells PtdIns(4,5)P2 recruits ADP-ribosylation factor to the Golgi membrane where GTP-bound ADP-ribosylation factor engages coatomer proteins and stimulates phospholipase D, followed by vesicle budding (2). PtdIns(4)P and PtdIns(4,5)P2 have been proposed to act as receptors for the coatomer complex COPII, which coats vesicles budding from the ER (51). PtdIns(4,5)P2 is also required for fusion of secretory granules with the plasma membrane in exocytosis and clathrin-mediated endocytosis (4). To date, PtdIns(4,5)P2 has not been shown to be directly involved in regulating vesicular transport from the ER in yeast. However, its precursor PtdIns(4)P is required for normal secretion in S. cerevisiae. Mutation of the PtdIns 4-kinase Pik1p results in impaired secretion from the Golgi and abnormal Golgi morphology (52, 53).

Defects in endocytosis have been noted in the yeast SacI domain-containing 5-phosphatases, Inp51p, Inp52p, and Inp53p null mutants, however, secretion was reported to be normal (16). The functional role of Inp54p in yeast is as yet unclear and although null mutation of inp54 is not lethal, no other phenotype has been described. inp54 null mutant was generated as described under “Experimental Procedures” and the morphology of the null mutant strain compared with wild-type. Electron microscopy analysis of inp54 null mutant did not reveal any significant phenotype that differed from the wild-type (results not shown). The growth of inp54 null mutant was tested on media supplemented with either 0.9 m NaCl or 1.4 m sorbitol. The degree of viability on hyperosmotic media was the same for wild-type and null mutant strains (results not shown).

Unlike double null mutants of the SacI domain-containing 5-phosphatases which have abnormal actin and chitin organization (16), inp54 null mutant demonstrated normal actin patches and chitin deposition as compared with the wild-type, assessed by staining cells with phalloidin or calcofluor, respectively, and analysis by confocal microscopy (results not shown).

Inp54p is a PtdIns(4,5)P2 5-phosphatase which localizes on the cytosolic face of the ER. PtdIns(4,5)P2 and PtdIns(4)P have been demonstrated to enhance binding of coatomer complexes to liposomes suggesting that these phosphoinositides are important in protein transport from the ER, possibly by promoting vesicle budding (51). Since proteins that are to be secreted have to be synthesized in the ER and transported further along the secretory pathway, Inp54p may also contribute indirectly to the regulation of secretion. Therefore, we investigated the role of Inp54p in secretion from the ER. We utilized a reporter protein assay which measures the amount of BPTI secreted from the cell (30, 54). Wild-type yeast and cells with null mutation of inp51, inp52, inp53, or inp54 were transformed with an expression plasmid containing BPTI under the control of a galactose promoter. Cells were grown in the presence of galactose for 0, 16, 24, and 48 h at 30 °C and the media containing secreted BPTI was collected. The level of BPTI present in the cytosolic media was determined by measuring the inhibition of tryptophan cleavage of a synthetic substrate L-BAPNA (N α-benzoyl-arginine-p-nitroanilide), as previously described (30). Similar levels of BPTI were secreted into the media at all time points assayed for wild-type cells, the inp51, inp52, and inp53 single null mutant strains (Fig. 7). The results are consistent with studies by Singer-Krueger et al. (16) who showed no abnormalities in secretion in the inp51, inp52, and inp53 mutants.
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Inp54p localizes to the ER of yeast cells by the C-terminal hydrophobic tail comprising the last 13 amino acids of the protein. Deletion of Inp54p from 16 to 24 amino acids, by the insertion of eight hydrophobic residues, resulted in the protein being mislocalized to the plasma membrane (50). Increasing the length of the C-terminal membrane anchor of UBC6 from 17 to 21 amino acids caused mistargeting of the protein from the ER to the Golgi, whereas a further increase to 26 amino acids resulted in expression of the protein at the plasma membrane (54). Based on these previous studies, the short 13-amino acid targeting sequence of Inp54p would be consistent with the ER localization of the protein.

The exact mechanism by which C-terminal anchored proteins are targeted to specific membranes remains unclear. Several studies have shown that the targeting of various isoforms of synaptobrevin to the ER requires ATP, but is independent of the Sec61p/SRP pathway, relying instead on an as yet uncharacterized receptor system (37, 40, 62). The C-terminal tails of VAMP-2 and Ufe1p form an amphipathic helix critical for localization of the proteins to the ER (50, 63). Mutation of the residues forming the polar face of the helix of Ufe1p to leucine abolished ER targeting of the protein (50). Mapping of the C-terminal hydrophobic sequence of Inp54p to a helical wheel...
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reveals that the lysine, tyrosine, and serine are predicted to line one face of the helix, however, this would not be predicted to line an amphipathic face. Mutation of the positively charged lysine residue at position 382 had no effect on the localization of the protein, as has been shown for mutation of the arginine residue within the C terminus of cytochrome b5 (48).

We predict Inp54p is oriented on the ER membrane with the bulk of the protein located on the cytoplasmic side of the ER membrane. Location of the tag did not affect the cytoplasmic orientation of Inp54p, or its intracellular localization. Previous studies have shown that C-terminal tags attached to cytochrome b5 and synaptobrevin did not affect their insertion or orientation in the membrane (33, 37, 41). The topology of tail-anchored proteins in the membrane is predicted to mimic classical transmembrane segments, whereby the tail spans the width of the membrane bilayer, with only a few residues on the other side of the membrane (32). At only 13 amino acids in length, the hydrophobic region of Inp54p is too short to span the membrane as typically integral membrane proteins contain a hydrophobic span of 20 amino acids (64). We have also demonstrated that although Inp54p membrane association is resistant to salt extraction, it is sensitive to alkaline treatment, indicating that it is not an integral membrane protein. Since a GFP tag fused to the extreme C terminus of Inp54p was degraded by protease K, it is most likely that the C terminus of Inp54p is also exposed to the cytoplasm. Thus, we propose the hydrophilic tail of Inp54p dips into the membrane to form a hairpin loop configuration whereby both the N and C termini are located in the cytosol (Fig. 8). As Inp54p membrane association is sensitive to high salt treatment, it is also possible that Inp54p could form a tight noncovalent interaction with an ER membrane protein. Protein-tyrosine phosphatase 1B has been proposed to adopt such a membrane topology (47).

The localization of Inp54p on the cytoplasmic surface of the ER would place the enzyme in an optimal position to regulate PtdIns(4,5)P2 levels on vesicles budding from the ER. In mammalian cells PtdIns(4,5)P2 binds ADP-ribosylation factor which results in the recruitment of coatomer proteins and activation of phospholipase D, followed by vesicle budding from the Golgi membrane (65–67). In yeast, the PtdIns 4-kinase Pik1p is of phospholipase D, followed by vesicle budding from the Golgi membrane (65–67). In yeast, the PtdIns 4-kinase Pik1p is

REFERENCES

1. De Camilli, P., Emer, S. D., McPherson, P. S., and Novick, P. (1996) Science 271, 1533–1539
2. Toker, A. (1998) Curr. Opin. Cell Biol. 10, 254–261
3. Hinchliffe, K. A., Ciruela, A., and Irvine, R. F. (1998) Biochim. Biophys. Acta 1436, 87–104
4. Martin, T. F. (1996) Annu. Rev. Cell Dev. Biol. 14, 231–264
5. Odorizzi, G., Babst, M., and Emer, S. D. (2000) Trends Biochem. Sci. 25, 229–235
6. Janney, P. A. (1994) Annu. Rev. Physiol. 56, 169–191
7. More, A. P., and Burridge, K. (1996) Nature 381, 531–535
8. de Corte, V., Gettemans, J., and Vandekerckhove, J. (1997) FEBS Lett. 401, 191–196
9. Ben, X. D., and Schwartz, M. A. (1998) Curr. Opin. Genet. Dev. 8, 63–67
10. Marmor, P. W. (1996) Gene Dev. 10, 1051–1053
11. Mitchell, C. A., Brown, S., Campbell, J. K., Munday, A. D., and Speed, C. J. (1996) Biochem. Soc. Trans. 24, 994–1000
12. Sinivasan, S., Seaman, M., Dube, Y., Danieli, L., Suchy, F. S., Emr, S., De Camilli, P., and Nussbaum, R. (1997) Eur. J. Cell Biol. 74, 350–360
13. Stolt, L. E., Huynh, C. V., Thorner, J., and Yurd, J. D. (1998) Genes 148, 1715–1729
14. McPherson, P. S., Garcia, E. P., Slepeniv, V. I., David, C., Zhang, X., Grabs, D., Sossin, W. S., Bauerfeind, R., Nemoto, Y., and De Camilli, P. (1990) Nature 379, 353–357
15. Guo, S., Stolt, L. E., Lemmrow, S. M., and Yurd, J. D. (1999) J. Biol. Chem. 274, 12990–12995
16. Singer-Kruger, B., Nemoto, Y., Danieli, L., Ferro-Novick, S., and De Camilli, P. (1999) J. Cell Sci. 111, 3347–3356
17. Bauecher, D., Stauffer, T., Chen, W., Shen, K., Guo, S., Yurd, J. D., Sheets, M. P., and Meyer, T. (2000) Cell 100, 221–228
18. Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Bocke, J. D., Bussey, H., Chia, A. M., Connelly, C., Davis, K., Dietrich, F., Dow, S. W., Elm Kakkoury, M., Feury, F., Friend, S. H., Gentelan, E., Gieser, V., Hegeman, J. H., Jones, T., Laub, M., Liao, H., Davis, R. W., and et al. (1999) Science 285, 901–906
19. Jones, J. S., and Prakash, L. (1998) Yeast 6, 363–367
20. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1997) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., New York.

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57. Zhang, X., Hartz, P. A., Philip, E., Racusen, L. C., and Majerus, P. W. (1998) J. Biol. Chem. 273, 1574–1582
58. Kong, A. M., Speed, C. J., O’Malley, C. J., Layton, M. J., Meehan, T., Loveland, K. L., Cheema, S., Ooms, L. M., and Mitchell, C. A. (2000) J. Biol. Chem. 275, 24052–24064
59. Mitoma, J., and Ito, A. (1992) EMBO J. 11, 4197–4203
60. Linstedt, A. D., Foguet, M., Renz, M., Seelig, H. P., Glick, B. S., and Hauri, H. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5102–5105
61. Whitley, P., Grahn, E., Kutay, U., Rapoport, T. A., and von Heijne, G. (1996) J. Biol. Chem. 271, 7583–7586
62. Kim, P. K., Hollerbach, C., Trimble, W. S., Leber, B., and Andrews, D. W. (1999) J. Biol. Chem. 274, 36876–36882
63. Grote, E., Hao, J. C., Bennett, M. K., and Kelly, R. B. (1995) Cell 81, 581–589
64. Rapoport, T. A. (1991) FASEB J. 5, 2792–2798
65. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) Cell 75, 1137–1144
66. Palmer, D. J., Helms, J. B., Beckers, C. J., Orci, L., and Rothman, J. E. (1993) J. Biol. Chem. 268, 12083–12089
67. Randazzo, P. A. (1997) J. Biol. Chem. 272, 7688–7692
68. Walch-Solimena, C., and Novick, P. (1999) Nat. Cell Biol. 1, 523–525
69. Wiedemann, C., Schafer, T., and Burger, M. M. (1996) EMBO J. 15, 2094–2101
70. Wilsbach, K., and Payne, G. S. (1993) EMBO J. 12, 3049–3059