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Skp1p Regulates Soi3p/Rav1p Association with Endosomal Membranes but Is Not Required for Vacuolar ATPase Assembly▽

E. J. Brace, Leah P. Parkinson, and Robert S. Fuller*

Department of Biological Chemistry, University of Michigan Medical Center, Ann Arbor, Michigan 48109-0606

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Skp1p is an essential component of SCF-type E3 ubiquitin ligase complexes and associates with these through binding to F-box proteins. Skp1p also binds F-box proteins in a number of non-SCF complexes. The Skp1p-associated yeast protein Soi3p/Rav1p (hereafter referred to as Rav1p) is a component of the RAVE complex required for regulated assembly of vacuolar ATPase (V-ATPase). Rav1p is also involved in transport of TGN proteins and endocytic cargo between early and late endosomes. To evaluate the role of Skp1p in the RAVE complex, we made use of the fact that overexpression of Rav1p is toxic because it sequesters Skp1p from essential interactions. We isolated a separation of function allele of *SKP1*, *skp1*(*Asn108Tyr*), that completely abrogated the Rav1p interaction but allowed Skp1p to perform other essential cellular functions. Cells containing the *skp1*(*Asn108Tyr*) allele as the sole source of Skp1p exhibited normal V-ATPase assembly and activity. However, in the *skp1*(*Asn108Tyr*) mutant strain, the membrane-associated pool of Rav1-green fluorescent protein was increased, suggesting that Skp1p is important for the release of Rav1p from endosomal membranes where it functions in V-ATPase assembly. Thus, although part of the RAVE complex, Skp1p does not appear to be involved in V-ATPase assembly but instead in the cycling of the complex off membranes. This work also provides a generalizable approach to defining the roles of interactions of Skp1p with individual F-box proteins through the isolation of special alleles of *SKP1*.

The processing protease Kex2p is a type I transmembrane protein necessary for cleaving pro-α-factor and other proproteins in the secretory pathway of the budding yeast *Saccharomyces cerevisiae* (11). Steady-state localization of Kex2p to the *trans*-Golgi network (TGN) depends on cycling of the protein between the TGN and the late endosome/prevacuolar compartment (PVC) (6). Kex2p localization is mediated by two TGN localization signals (TLSs) in the Kex2p cytosolic tail (6, 41). TLS1 promotes retrieval of Kex2p from the PVC to the TGN; whereas, TLS2 slows Kex2p delivery to the PVC, either through retention in the TGN or by promoting an alternative pathway through the early endosome (6, 15). Disruption of either TLS1 or TLS2 results in increased trafficking of Kex2p to the vacuole, where it is degraded (6, 41).

The *SOI3* gene was identified by a mutation (*soi3-1*) that suppressed mislocalization of Kex2p bearing a $Tyr_{713}Ala$ mutation in TLS1 (27). Deletion of *SOI3* disrupted trafficking of TGN membrane proteins Kex2p and A-ALP, a fusion protein consisting of the cytosolic domain of dipeptidyl aminopeptidase A fused to the transmembrane and lumenal domains of the vacuolar protein alkaline phosphatase (24) but not the vacuolar sorting receptor, Vps10p, between the TGN and PVC (34). The *soi3*Δ mutation also delayed delivery of the plasma membrane-localized **a**-factor receptor, Ste3p, to the vacuole at a step after endocytic internalization (34). Although most of the protein is cytosolic, a fraction of tagged Soi3p was associated with dense membranes and localized to peripheral punctate structures (34). These data argued that Soi3p is required for transport between the early endosome and PVC.

A clue to the molecular function of Soi3p in early endosome-to-PVC trafficking comes from the fact that the protein was independently isolated as Rav1p, a Skp1p-interacting protein that forms part of a complex termed RAVE (*r*egulator of the H^+-*A*TPase of the *v*acuolar and *e*ndosomal membranes), which includes a third polypeptide, Rav2p (33) (Soi3p will hereafter be referred to as Rav1p). RAVE is associated with the V_1 subcomplex ("sector") of the vacuolar ATPase (V-ATPase), a multisubunit proton-translocating ATPase responsible for the acidification of intracellular compartments (13, 38). In yeast grown on a poor carbon source, V_1 is largely cytosolic but can be induced, upon addition of glucose, to assemble with the integral membrane V_o sector to form an active holoenzyme (17). Rav1p is important for efficient assembly and for activation of V-ATPase (33, 36). We hypothesized that the role of Rav1p in early endosome-to-PVC transport reflects a requirement for assembly and activation of V-ATPase at the early endosome (34). This may be a conserved function in that V-ATPase activity is required for early endosome maturation in mammalian cells (8) and Rav1p has homologues in both *Drosophila* and humans (18, 19).

The Rav1p-Skp1 interaction is intriguing because Skp1p is a component of SCF (*S*kp1, *C*dc53/Cullin, *F*-box protein)-type E3 ubiquitin-protein ligases. SCF complexes are responsible for ubiquitination of numerous substrates (10). The specificity of SCF complexes is achieved, in part, through the interchangeable F-box proteins that contain a Skp1p-binding motif (the F-box sequence) and a substrate recognition domain often consisting of WD-40 or leucine-rich repeats. The F-box protein thus acts as an adaptor between the ubiquitin transferase and its substrate (25). Although not an identified F-box protein,

* Corresponding author. Mailing address: Department of Biological Chemistry, University of Michigan Medical Center, Ann Arbor, MI 48109-0606. Phone: (734) 936-9764. Fax: (734) 763-7799. E-mail: bfuller@umich.edu.

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TABLE 1. Yeast strains

Strain	Relevant genotype	Parental strain
CRY1	MAT**a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	W303-1A
CRY2	MATα *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	W303-1B
DC14	MAT**a** *his1*	
KRY18-1A	MATα *kex2*Δ::*TRP1*	CRY2
GSY11-2A	MATα *soi3*Δ::Kan^r	CRY2
GSY11-r1	MATα *soi3*Δ::Kan^r *kex2*Δ::*TRP1*	CRY2
EBY23	MAT**a** Kan^r::P_{GAL1}-*SOI3*	CRY1
EBY72-2	MATα *vma2*Δ::Kan^r	CRY2
EBY79-12	MATα *doa4*Δ::*HIS3 soi3*Δ::Kan^r	CRY2
EBY94	MATα *vma2*Δ::*LEU2*	CRY2
EBY116-4	MATα *skp1*Δ::*HIS3 soi3*Δ::Kan^r [p*skp1-Asn108Tyr*]	CRY2
EBY116-6	MATα *skp1*Δ::*HIS3* [p*skp1*(*Asn108Tyr*)]	CRY2
EBY127-2	MATα *kex2*Δ::*TRP1 skp1*Δ::*HIS3*	CRY2
LPY01-4	MATα *soi3*Δ::Kan^r *skp1*Δ::*URA3* [p*skp1*(*Asn108Tyr*)]	CRY2
LPY02-4	MATα *soi3*Δ::Kan^r *skp1*Δ::*URA3* [pCB6]	CRY2

Rav1p does contain potential F-box motifs. Rav1p and the RAVE complex are hypothesized to be a non-SCF complex since additional components of the SCF complex cannot be coimmunoprecipitated (33).

Here we demonstrate that overexpression of Rav1p is toxic and that Skp1p acts as a multicopy suppressor of this toxicity. Taking advantage of the Rav1p overexpression phenotype, we designed a screen to identify *skp1* mutants that would not interact with Rav1p but would maintain other essential interactions. One such mutant completely abrogated the Rav1p interaction and allowed us to dissect the role of the Rav1p-Skp1p interaction in Kex2p trafficking and V-ATPase assembly. We determined that the Rav1p-Skp1p interaction is not required for V-ATPase assembly/activation. Moreover, loss of the interaction alters Kex2p trafficking, but it does so differently than a *rav1*-null mutation. Finally, we found that loss of Skp1p-Rav1p binding alters localization of a Rav1-green fluorescent protein (GFP) fusion, suggesting that Skp1p functions in release of Rav1p from early endosomes.

MATERIALS AND METHODS

Reagents, strains, plasmids, and media. Unless otherwise noted, chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and used as received. Antibodies are described where used. Yeast media were as described previously (31) except as noted. SGC-Leu plates were equivalent to SC-Leu dropout plates but contained 2% (wt/vol) galactose in place of glucose. YPAD, pH 5.6, plates were buffered with 50 mM Na-morpholineethanesulfonic acid (MES), pH 5.6, and 50 mM Na-morpholinepropanesulfonic acid (MOPS), pH 5.6. YPAD, pH 7.5, plates were buffered with 100 mM Tris-HCl, pH 7.5. YPAD zinc plates were supplemented with 5 mM $ZnCl_2$ and buffered with 50 mM Na-MOPS, pH 5.6, and 50 mM Na-MES, pH 5.6. YPAD cobalt plates were supplemented with 750 μM $CoCl_2$.

Plasmids and strains are outlined in Tables 1 and 2. Plasmid p*SOI3*.HA.HIS6-SL was created by replacing the SacI site in p*SOI3*.HA.HIS6, in which sequences encoding a triple hemagglutinin (HA) epitope tag followed by six His codons are fused to the 3′ end of the wild-type *RAV1* gene (34), with a SalI site by linker ligation. Plasmid p413.ADH.*SOI3*.HA.HIS6, in which the *RAV1* fusion is under the control of the *ADH1* promoter on *CEN6 ARS4 HIS3* plasmid p413 (22), was created by ligating the NarI/SalI fragment of p*SOI3*.HA.HIS6 containing the C terminus of *SOI3.HA.HIS6* into p413ADH.*SOI3* cleaved by NarI and SalI. Plasmid p416.ADH.*SOI3*.GFP was created by inserting an EcoRI/SalI fragment containing the *SOI3.GFP* fragment from p413.ADH.*SOI3.GFP* (34) into plasmid p416.ADH cleaved by EcoRI and SalI. Plasmid p413GAL.*SOI3* was constructed by PCR amplifying the *RAV1* structural gene with primers containing EcoRI sites and inserting the resulting fragment into the EcoRI site of p413.*GAL*.

All yeast strains were derived from the W303 background (28) (Table 1). Gene disruptions were created by homologous recombination of a marker into the desired gene locus and confirmed by PCR (14), and subsequent strains were created by standard genetic crosses. The *GAL1* promoter was integrated upstream of *RAV1* using the Kan^r marker to create EBY23 (21).

Identification of Skp1p and isolation of *skp1*(*Asn108Tyr*). EBY23 was transformed with a multicopy YEp13 library (42) and plated on SGC-Leu plates after a 4-h outgrowth in YPAD media. Transformants that grew on galactose-containing plates were isolated, and the resulting plasmids were retested for suppression of the toxicity of Rav1p overexpression. Inserts of confirmed suppressor plasmids were partially sequenced to identify the genomic insert.

A library of *SKP1* mutants was created by PCR mutagenesis of wild-type *SKP1* in plasmid pCB6 (4). Plasmid pCB6-XhoIΔ was created by digesting pCB6 with XhoI and blunting the ends with Klenow polymerase before ligation. *Taq* polymerase was used under standard conditions to maximize the yield of single-point mutations. Mutagenized PCR products were cotransformed into EBY23 along with pCB6-XhoIΔ, digested with AvaI/NsiI, to generate pCB6 containing mutagenized *SKP1* inserts by homologous recombination. Transformants were plated on SGC-Leu plates after a 4-h outgrowth in liquid YPAD medium. Suppressors of Rav1p toxicity were isolated, and the phenotypes were confirmed. Confirmed plasmids were sequenced to identify the mutations.

Rav1p-Skp1p coimmunoprecipitation. Cell extracts were prepared by gentle freeze-thaw lysis of yeast spheroplasts as described by Baker et al. and Sipos and Fuller (5, 35). S13 fractions were obtained from the supernatant by centrifuga-

TABLE 2. Plasmids

Plasmid name	Relevant characteristic(s)	Source or reference
P413.GAL	Galactose-regulated expression plasmid	D. Thiele
P413.GAL.*SOI3*	*SOI3* under *GAL1* promoter	This study
YEp13	2μm plasmid (library backbone)	42
YEp96	Wild-type ubiquitin under *CUP1* promoter	Ellison and Hochstrasser
pLib.*SKP1*	Library vector containing *SKP1*	This study
p*skp1*(*Asn108Tyr*)	Soi3p-resistant *skp1* allele	This study
p413ADH.*SOI3*	*SOI3* under *ADH* promoter	34
p413.ADH.*SOI3.HA.HIS6*	*SOI3*.HA.HIS6 under *ADH* promoter	This study
p413.ADH.*SOI3*.GFP	*SOI3.GFP* under *ADH* promoter (*HIS3*)	34
p416.ADH.*SOI3*.GFP	*SOI3.GFP* under *ADH* promoter (*URA3*)	This study
pCWKX20	Wild-type *KEX2* under *GAL1* promoter	41
pCWKX21	Y_{713}A-*KEX2* under *GAL1* promoter	41
pCWKX20-I718	I718tail-*KEX2* under *GAL1* promoter	41
pCWKX21-I718	Y_{713}A, I718tail-*KEX2* under *GAL1* promoter	41
pCB6	Wild-type ubiquitin under *CUP1* promoter	4
pCB6-Xho1Δ	XhoI digest and blunt ligation of pCB6	This study

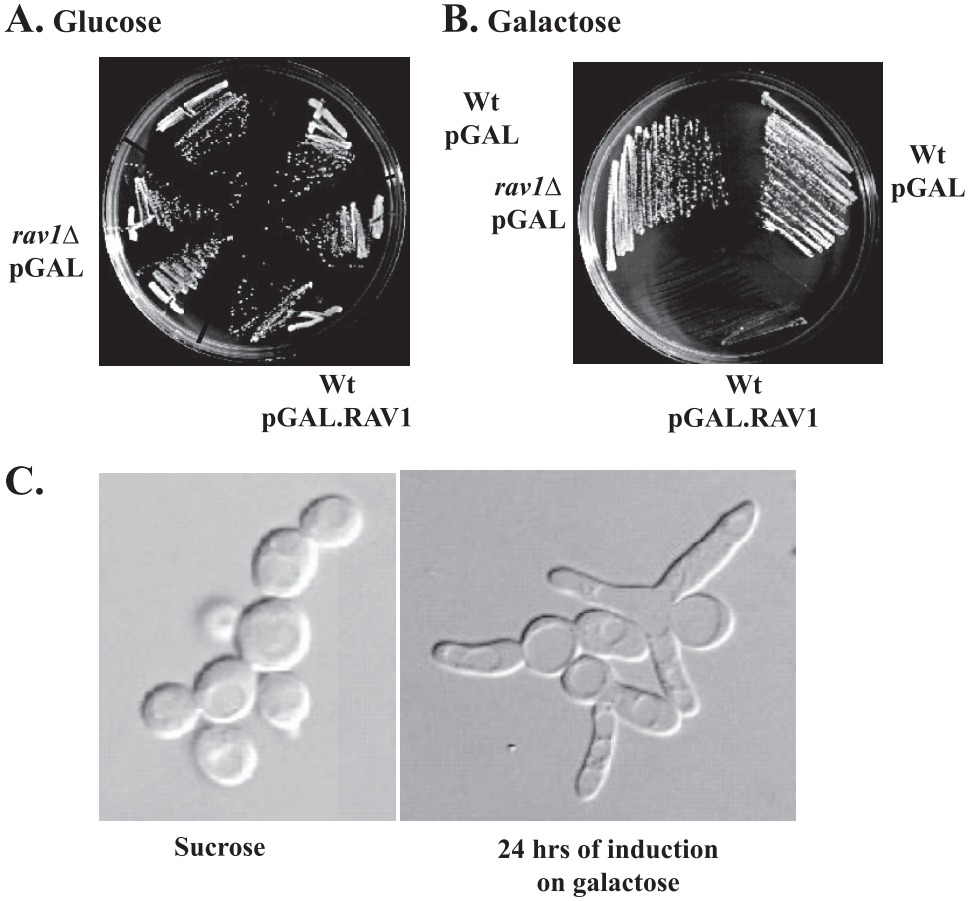

FIG. 1. Overexpression of Rav1p is toxic. (A and B) CRY1 (wild type) cells were transformed either with p413GAL or p413GAL.*RAV1*, and GSY11-1A (*rav1*Δ) was transformed with p413GAL. The p413GAL plasmid possesses the galactose-inducible *GAL1* promoter. Strains were tested on SC-His (glucose) and SGC-His (galactose) for the ability to grow when Rav1p was overexpressed. (C) CRY1 (wild type) cells transformed with p413GAL.*RAV1* were grown in log phase in either SSC-His (Sucrose) or SGC-His (galactose). Strains were visualized under differential interference contrast microscopy after 24 h.

tion of thawed spheroplasts, diluted in 800 μl of immunoprecipitation buffer containing protease inhibitors (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, and Complete Mini protease inhibitor cocktail tablets [Roche]), at 13,000 × *g* for 5 min at 4°C. Extracts from 10 optical densities (OD) of cells (equivalent to 10 ml of cells grown in log phase to an A_{600} of 1.0; ~1×10^8 cells) were immunoprecipitated with 0.25 μl of 12CA5 (5 mg/ml, anti-HA from Roche), 2 μl of rabbit anti-mouse (2 mg/ml; Jackson Laboratory), and 10 μl of Pansorbin (Calbiochem) for 2 h at 4°C. Immunoprecipitates were washed three times with immunoprecipitation buffer. Samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 11%), and gels were subsequently blotted to nitrocellulose (175 mA, 2 h). Nitrocellulose filters, probed with either anti-HA (1:5,000, 12CA5 from Roche) and sheep anti-mouse horseradish peroxidase (HRP, 1:2,000; Amersham) or rabbit anti-Skp1p antiserum (1:2,500; gift of Stephen Elledge, Harvard University) and protein A-HRP (1:5,000; Zymed), were developed using the ECL plus kit (Amersham).

Membrane fractionation. Cell extracts were prepared as described above except that thawed spheroplasts were homogenized with 15 strokes in a Dounce homogenizer prior to centrifugation. Sucrose gradients contained steps of 750 μl of 60% sucrose (in 20 mM Na-HEPES, pH 6.8) and 750 μl of 15% sucrose (in 20 mM Na-HEPES, pH 6.8) in 11- by 34-mm polyallomer centrifuge tubes (Beckman). S13 fractions from 125 OD of cells were loaded onto gradients and centrifuged at 200,000 × *g* for 4 h using a TLX centrifuge and TLS-55 swinging bucket rotor (Beckman). Membranes were collected from the interface of the two steps. S13 fractions were not examined because Rav1-HAp was unstable in this fraction (E. J. Brace and R. S. Fuller, unpublished data). The Golgi protein Mnn1p was used as a loading control (29). Membranes from 3.3 OD of cells were subjected to SDS-PAGE (8%), and gels were blotted to nitrocellulose (200 mA, 2 h). Blots were probed with either mouse monoclonal anti-GFP (1:100; Clontech) and sheep anti-mouse HRP (1:2,000; Amersham) or rabbit anti-Mnn1p (1:500; gift of Todd Graham, Vanderbilt University) and donkey anti-rabbit HRP (1:2,000; Amersham).

Microscopy. Microscopy was performed using a Nikon Eclipse 800 microscope and an ORCA2 charge-coupled device camera (Hamamatsu). ESEE Software (Inovision, Raleigh, NC) was used for image capture. Soi3-GFP images were from cells grown in log phase in minimal medium at 25°C. Quinacrine staining of yeast vacuoles was as described previously (30).

RESULTS

Overexpression of Rav1p is toxic. During the cloning of *SOI3*/*RAV1*, we observed that strains expressing *RAV1* from strong promoters exhibited poor growth (E. J. Brace and R. S. Fuller, unpublished data). To test specifically whether high levels of Rav1p expression were toxic to yeast cells, *RAV1* was placed under the galactose-inducible *GAL1* promoter. Strains expressing Rav1p from the *GAL1* promoter were able to grow on plates containing glucose but were unable to grow on plates containing galactose (Fig. 1A and B). Following a shift from sucrose to galactose, strains grew normally for approximately 10 h before exhibiting poor growth (E. J. Brace and R. S.

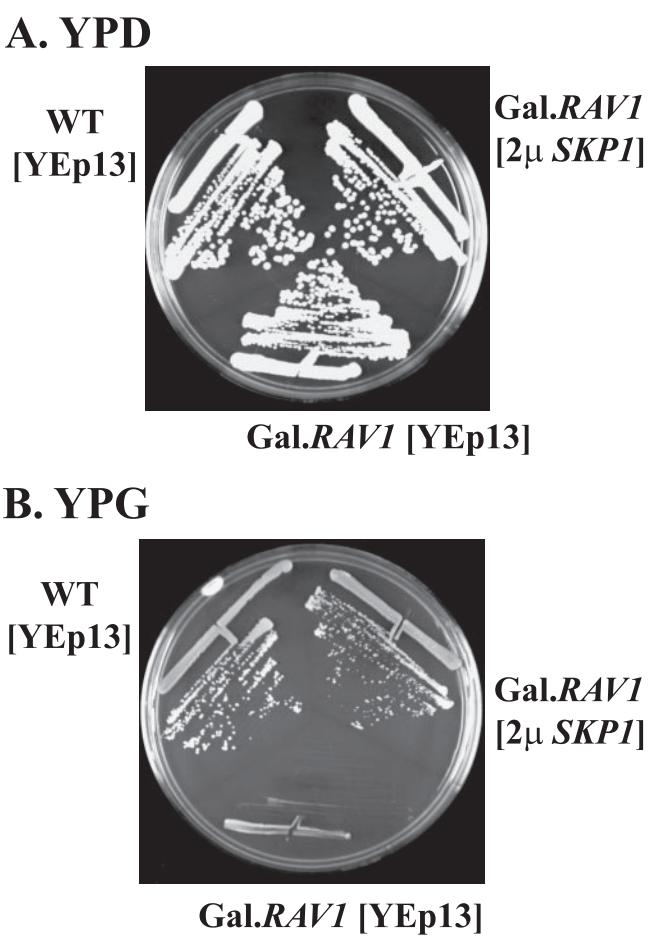

FIG. 2. Overexpression of Skp1p suppresses Rav1p toxicity. (A and B) CRY1 cells (wild type) were transformed with a YEp13 plasmid (2μm plasmid marked by *LEU2*), and EBY23 cells (integrated *GAL.RAV1*) were transformed with either the YEp13 plasmid or one of the isolated YEp13 library plasmids containing *SKP1* (YEp13-*SKP1*, "2μ *SKP1*" in figure). Cells were grown on YPD (glucose) or YPG (galactose) to demonstrate that Skp1p is a multicopy suppressor of Rav1p overexpression.

Fuller, unpublished data), eventually arresting with an elongated, multibudded phenotype (Fig. 1C).

Overexpression of Skp1p suppresses Rav1p toxicity. Rav1p is predicted to possess several WD-40 repeats that likely mediate interactions with other proteins (37). As an approach to identify possible Rav1p-interacting proteins, we attempted to isolate multicopy suppressors of the slow-growth phenotype caused by Rav1p overexpression. The *GAL1-RAV1* strain was transformed with a 2μm plasmid multicopy library and plated on galactose. Twenty-one clones that grew on galactose were selected for characterization. By sequence analysis, each contained only the *SKP1* gene in common. Figure 2 shows suppression by one of these plasmids, YEp-*SKP1*, of the toxicity of Rav1p overexpression.

Skp1p has multiple essential roles in yeast. We hypothesized that overexpression of Rav1p is toxic because excess Rav1p sequesters Skp1p, preventing Skp1p from participating in essential SCF complexes. One likely candidate is the SCF complex containing Cdc4p, SCF^{Cdc4}, because $cdc4^{ts}$ mutants arrest with a multibudded terminal phenotype similar to that seen with Rav1p overexpression (1, 16).

The *skp1*(*Asn108Tyr*) mutant performs essential functions of Skp1p but does not bind Rav1p. To characterize specifically the role of Skp1p in Rav1p function, we screened for *skp1* mutants that provided resistance to Rav1p overexpression but could support yeast cell growth. Presumably, such mutants would fail to bind Rav1p but would retain the ability to interact with other essential F-box proteins. After PCR mutagenesis, a single *skp1* allele was identified that suppressed the toxic phenotype of Rav1p overexpression and could support growth in single copy in the absence of wild-type *SKP1*. The mutant, *skp1*(*Asn108Tyr*), was phenotypically distinct from other characterized *skp1* point mutations (*skp1-1*, *-2*, *-3*, *-11*, and *-12*) (4, 9) in that none of these suppressed Rav1p overexpression at 25°C, 30°C, or 33°C (E. J. Brace and R. S. Fuller, unpublished data).

To determine directly whether the Rav1p-Skp1p interaction was disrupted by the *skp1*(*Asn108Tyr*) mutation, the ability of Rav1p to interact with Skp1p(Asn108Tyr) was tested by coimmunoprecipitation. Protein extracts of strains expressing Rav1-HAp and either Skp1p or Skp1p(Asn108Tyr) were immunoprecipitated with anti-HA under native conditions and probed with either anti-HA or anti-Skp1 antibodies. Rav1-HA coimmunoprecipitated wild-type Skp1p, but not Skp1p(Asn108Tyr) (Fig. 3B). This result confirmed that Skp1(Asn108Tyr) did not interact with Rav1p and supports both the conclusion that toxicity of Rav1p overexpression is caused by titration of Skp1p and that the Skp1p-Rav1p interaction is direct. Furthermore, because the allele could support growth as the lone copy of *SKP1* (Fig. 3A, top), the essential interactions of Skp1p must remain intact.

The *skp1*(*Asn108Tyr*) sequence contained an AAC-to-TAC transversion in codon 108, corresponding to a Tyr-for-Asn substitution. Asn108 is conserved in human Skp1 and lies in helix five, adjacent to the F-box binding site. In the three-dimensional structure of the human Skp1-Skp2 (32), Asn108 of human Skp1 contacts residue Leu116 in the F-box domain of Skp2 (Fig. 3C) and yet is at the periphery of the interface between the two proteins. In a compilation of F-box sequences, the position equivalent to Skp2 Leu116 varies between Leu, Val, and Ile (25). Thus, Skp1p Asn108 is in a position where substitutions could selectively alter affinity for F-box proteins depending on the identity of the contacting F-box residues. Although the precise Skp1p binding domain of Rav1p has not been identified, it has been noted previously that Rav1p contains several sequences with similarity to F-box domains (34).

***skp1*(*Asn108Tyr*) does not affect V-ATPase function.** The *skp1*(*Asn108Tyr*) allele made it possible to determine whether the Skp1p-Rav1p interaction is important for V-ATPase assembly and activity. Mutations in V-ATPase subunit genes, such as *VMA2*, which encodes the B subunit of the ATPase hexamer, result in growth that is sensitive to high pH, zinc, and cobalt (3, 23) (E. J. Brace and R. S. Fuller, unpublished data). *rav1* mutants, which are defective in ATPase assembly/activation, exhibit similar phenotypes (33, 34, 36). As shown in Fig. 4A and B, whereas *vma2*Δ and *rav1*Δ strains grew at pH 5.6 but not at pH 7.5, the *skp1*(*Asn108Tyr*) strain grew well at both low and high pH. Also, unlike *rav1*Δ and *vma2*Δ mutants, the *skp1*(*Asn108Tyr*) mutant did not show sensitivity to 5 mM

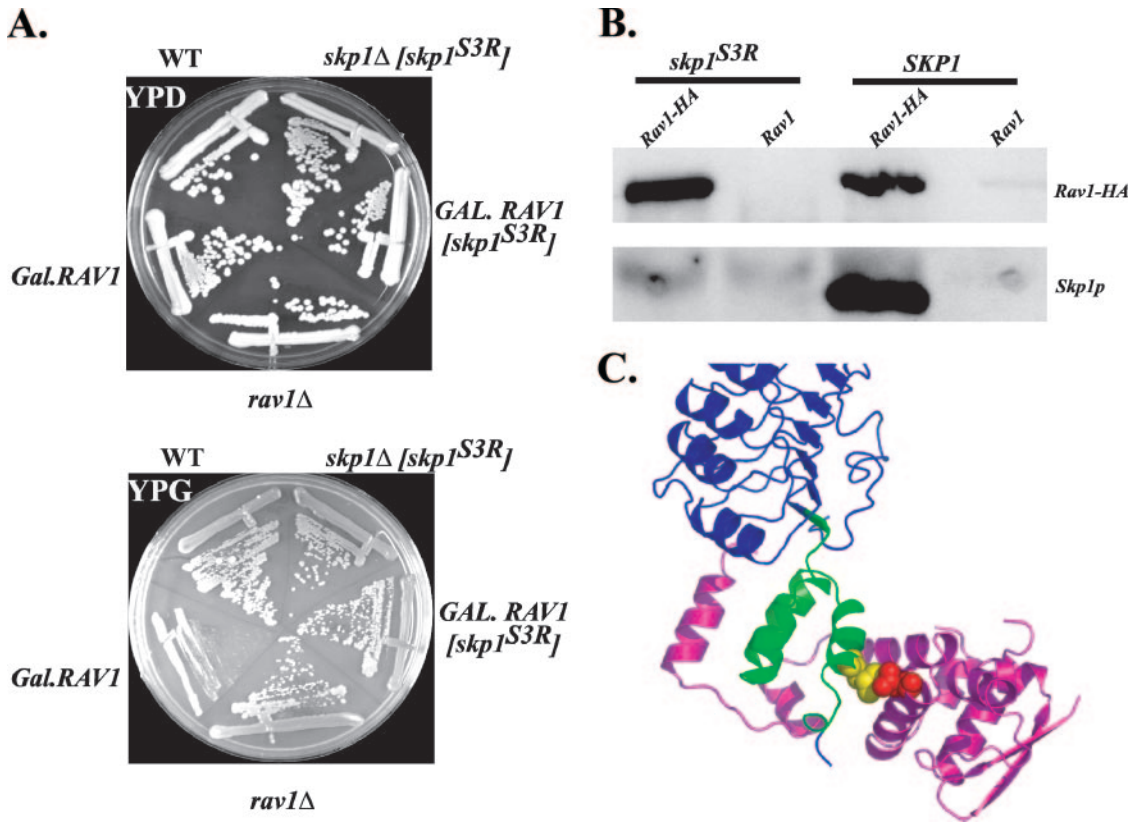

FIG. 3. Skp1p(Asn108Tyr) performs essential functions of Skp1p but does not bind Rav1p. A *skp1* mutant [*skp1*(*Asn108Tyr*)] was identified through a genetic screen that suppressed the toxicity of Rav1p overexpression. (A) CRY1 (wild type), EBY23 (*GAL.RAV1*), GSY11-1A (*rav1*Δ), EBY23 [p*skp1*(*Asn108Tyr*)], and EBY116-6 (*skp1*Δ) [p*skp1*(*Asn108Tyr*)] were streaked out on YPD (glucose) and YPG (galactose) plates, demonstrating that *skp1*(*Asn108Tyr*) can both suppress Rav1p overexpression and exist as the sole copy of Skp1p in the cell. (B) Rav1p does not coimmunoprecipitate Skp1p(Asn108Tyr). Strains LPY01-4 {*rav1*Δ *skp1*Δ [p*skp1*(*Asn108Tyr*)]} and LPY02-4 (*rav1*Δ *skp1*Δ [pCB6]) were transformed with p413.ADH.*RAV1*.HA.HIS6 or p413.ADH.*RAV1*. The strains were grown at 25°C, and 10 ODs of S13 extract from yeast spheroplasts were immunoprecipitated under native conditions with 12CA5 antibody (anti-HA). Immunoprecipitations were separated on SDS-PAGE and transferred to nitrocellulose where they were probed with anti-HA (12CA5) or anti-Skp1 antibody to characterize the extent of Rav1p-Skp1 interactions. (C) The crystal structure of the mammalian Skp1-Skp2 complex is depicted with the *skp1*(*Asn108Tyr*) mutation $Asn_{108}Tyr$ modeled (Skp1, magenta; leucine-rich repeats of Skp2, blue; F-box of Skp2, green; Skp2-Leu116, yellow; Skp1-Asn108, red) (32). Graphic representation was made using MacPyMol (DeLano Scientific, LLC).

$ZnCl_2$ or 750 μM $CoCl_2$ (Fig. 4C and D). V-ATPase and *rav1*Δ mutants are defective for vacuolar accumulation of the fluorescent dye quinacrine (33, 36). In contrast, the *skp1*(*Asn108Tyr*) strain exhibited normal quinacrine accumulation (Fig. 4E). These results demonstrate that binding of Skp1p to Rav1p is not required for V-ATPase assembly/activation.

***skp1*(*Asn108Tyr*) and *rav1*Δ alter Kex2p trafficking in distinct ways.** *rav1*Δ mutants exhibit defects in the trafficking of Kex2p between the early endosome and PVC (34). The effect of *rav1*Δ or other mutations on Kex2p localization can be monitored in vivo using the "onset of impotence" assay (27). Mislocalization of Kex2p disrupts processing of pro-α-factor and thus impairs production of the peptide mating pheromone α-factor, which is required for mating. When Kex2p expression, under control of the glucose-repressible *GAL1* promoter, is shut off by shifting cells to glucose medium, the rate of loss of mating competence over time reflects the loss of preexisting enzyme from the pro-α-factor processing compartment (Fig. 5A). A $Y_{713}A$ mutation in TLS1 in the Kex2p cytosolic tail causes more rapid loss of mating competence because of failure to retrieve Kex2p from the PVC to the TGN. Loss of TLS2 function results in more rapid loss of mating competence because of increased transport to the PVC (6).

In the onset of impotence assay, the effects of the *skp1*(*Asn108Tyr*) mutation were distinct from effects of the *rav1*Δ mutation with all forms of Kex2p tested (Fig. 5B). Whereas the *rav1*Δ mutation suppressed effects of both the TLS1 mutation alone and in combination with the TLS2 mutation, the *skp1*(*Asn108Tyr*) mutation did not. The *skp1*(*Asn108Tyr*) mutation did have effects in the assay, causing more rapid loss of mating competence compared to the wild-type strain background with wild-type (by 10 h) (Fig. 5B, top panel) and TLS2 mutant (by 6 h) (Fig. 5B, third panel) Kex2p and had a slight effect on TLS1 mutant Kex2p (Fig. 5B, second panel). These effects of the *skp1*(*Asn108Tyr*) allele were not due to general defects in mating because the *skp1*(*Asn108Tyr*) mutant demonstrated mating indistinct from wild-type strains in a quantitative mating assay (E. J. Brace and R. S. Fuller, unpublished data). Paradoxically, *skp1*(*Asn108Tyr*) improved mating over time with the $TLS1^-$ $TLS2^-$ double mutant (Fig. 5B, bottom

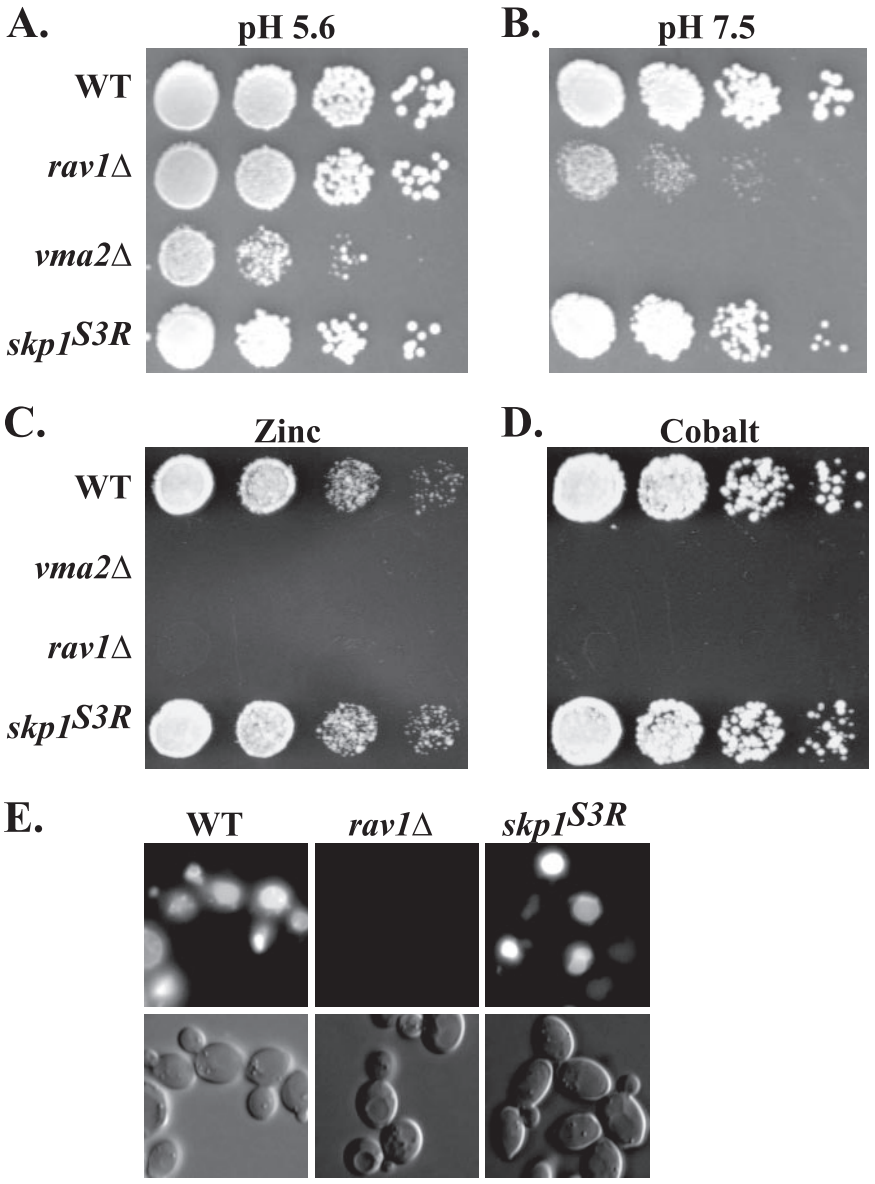

FIG. 4. *skp1*(*Asn108Tyr*) does not affect V-ATPase function. (A to D) Serial dilutions of strains CRY2 (wild type), GSY11-2A (*rav1*Δ), EBY72-2 (*vma2*Δ), and EBY116-6 [*skp1*Δ p*skp1*(*Asn108Tyr*)] were pronged onto the indicated media and incubated for 2 days. (A and B) YPAD plates, pH 5.6 or pH 7.5, grown at 37°C; (C) YPAD with 5 mM $ZnCl_2$ (pH 5.6) grown at 30°C; (D) YPAD with 750 μM $CoCl_2$ at 30°C. (E) Strains CRY2 (wild type), GSY11-2A (*rav1*Δ), and EBY116-6 [*skp1*Δ p*skp1*(*Asn108Tyr*)] were tested for their ability to accumulate quinacrine in their vacuoles. Cells were allowed to accumulate quinacrine for 10 min at 30°C and cooled on ice for 5 min. Cells were then visualized immediately by differential interference contrast and fluorescence microscopy.

panel). Although the effects of *skp1*(*Asn108Tyr*) in this assay are complex, the key result, that the *skp1*(*Asn108Tyr*) and *rav1*Δ mutants behave differently, is completely consistent with the conclusion reached above that the Skp1p-Rav1p interaction is not required for the function of Rav1p in V-ATPase assembly/activation.

Of note, the *skp1*(*Asn108Tyr*) mutation did not disrupt either the trafficking of the vacuolar protein sorting receptor, Vps10p, as measured by secretion of carboxypeptidase Y or endocytosis, as measured by FM4-64 uptake (E. J. Brace and R. S. Fuller, unpublished results). This points out one similarity between the *rav1*Δ and *skp1*(*Asn108Tyr*) mutations: both affect Kex2p but not Vps10p trafficking. This may reflect the fact that Kex2p reaches the PVC both by direct TGN-PVC transport and by trafficking through the early endosome, whereas Vps10p follows only the direct pathway (34).

Rav1-GFP is recruited to membranes in *skp1*(*Asn108Tyr*) and *doa4*Δ mutants. In yeast cells, Rav1p is present both in a soluble pool and as a peripheral protein associated with a high-density membrane compartment suggestive of early endosomes (34). The bulk of Rav1-GFP fluorescence was cytosolic, but image deconvolution made it possible to identify discrete Rav1-GFP puncta (34). The *skp1*(*Asn108Tyr*) mutant was examined to determine whether Skp1p affects recruitment

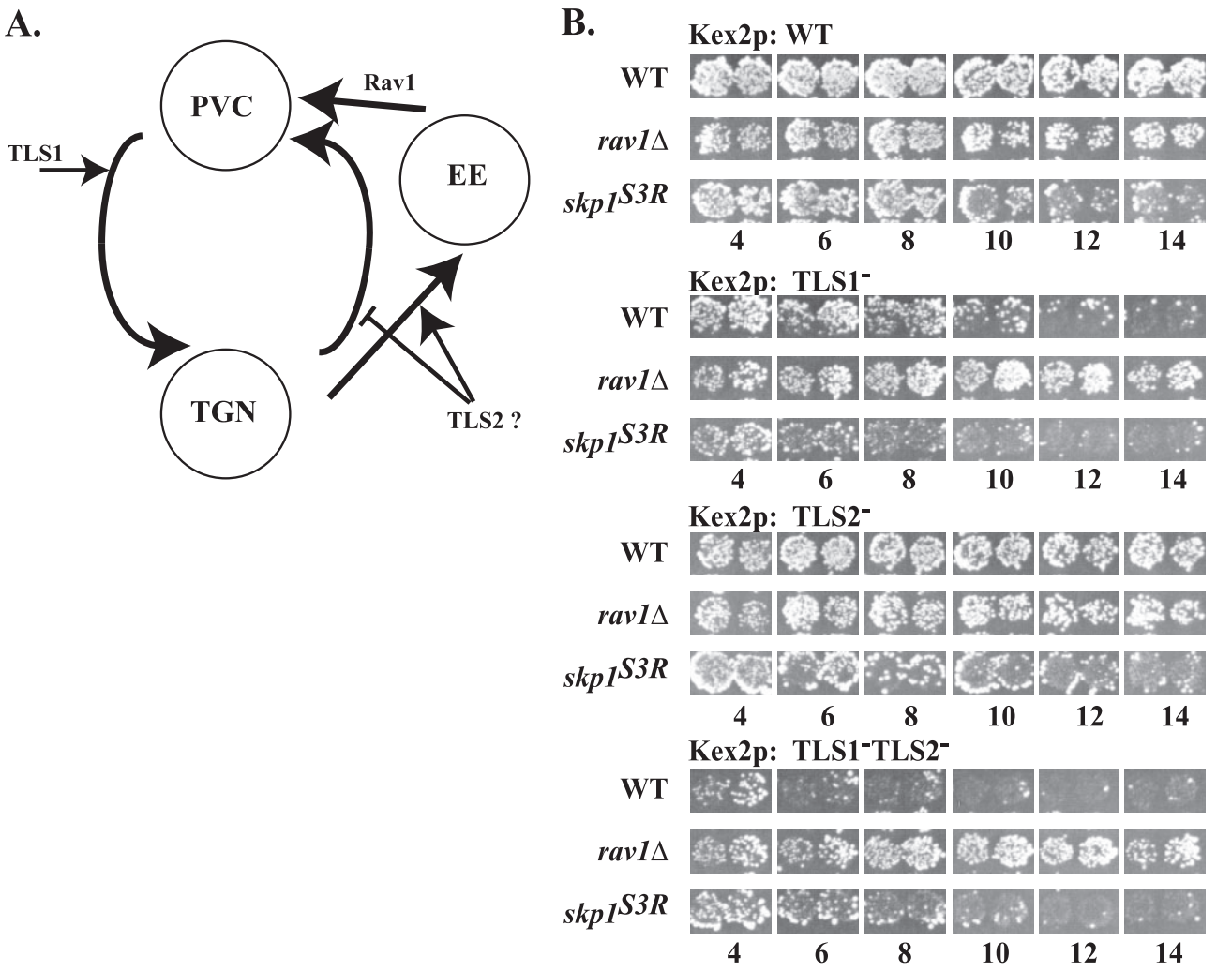

FIG. 5. *Skp1p* affects trafficking of Kex2p. (A) Model of TLS1 and TLS2 function in Kex2p trafficking between the TGN and endosomal system. (B) Strains KRY18-1A (*kex2*Δ), GSY11-r1 (*kex2*Δ *rav1*Δ), and EBY127-2 [*kex2*Δ *skp1*Δ p*skp1*(*Asn108Tyr*)] were transformed with pCWKX20 (*GAL1-KEX2*; wild type [WT]), pCWKX21 (*GAL1-KEX2* $Y_{713}A$; $TLS1^-$), pCWKX20-I718tail (*GAL1-KEX2-I718* tail; $TLS2^-$), and pCWKX21-I718tail (*GAL1-KEX2* $Y_{713}A$-I718 tail; $TLS1^-$ $TLS2^-$). Strains were grown at 25°C, shifted from galactose to glucose medium for the indicated times, and assessed for mating competence as described previously (27).

of Rav1-GFP to membranes. Surprisingly, in the *skp1* (*Asn108Tyr*) mutant, Rav1-GFP localized more extensively to puncta that were visualized even without the aid of image deconvolution (Fig. 6A). To determine if total Rav1p protein levels were altered in the *skp1*(*Asn108Tyr*) or *doa4*Δ mutants, Rav1-GFP levels were determined by Western blotting (L. P. Parkinson and R. S. Fuller, unpublished results). Rav1-GFPp levels were unchanged in the *skp1*(*Asn108Tyr*) mutant but were slightly lower in the *doa4*Δ mutant. The latter effect may account for the lower cytosolic Rav1-GFP fluorescence seen in the *doa4*Δ strain. These data suggest that Rav1-GFP accumulation on membranes is not due to increased levels of Rav1-GFP protein but is due to altered localization of the protein. Sucrose step gradients were used to provide an alternative way of assessing Rav1-GFP localization to membranes in the *skp1*(*Asn108Tyr*) background. S13 fractions (13,000 × *g* supernatant) of yeast extracts were further fractionated by equilibrium centrifugation at 200,000 × *g* using a two-step sucrose gradient. Samples were tested for the accumulation of Rav1-GFP at the 15%–60% sucrose interface that separates membrane fractions from large protein complexes that pellet and cytosolic proteins that sediment slowly and fractionate above the 15% step. Rav1-GFP more readily accumulated at the 15%–60% interface of the gradient in a *skp1*(*Asn108Tyr*) mutant, suggesting that the mutant is defective in releasing Rav1p from membranes (Fig. 6B).

Although Rav1p-Skp1p complexes do not contain other components typically found in SCF complexes (Cdc32p, Rbx1p, and Cdc53p) (33), it remains possible that ubiquitin plays a role in Rav1p function or localization. *DOA4*, which encodes a deubiquitinating enzyme, is localized to endosomes and is involved in the multivesicular body pathway (2). Mutations in *DOA4* result in lower levels of free ubiquitin in the cells, which often leads to the impairment of cellular functions requiring ubiquitin (39). Therefore, Rav1-GFP localization was tested in a *doa4*Δ mutant to determine if Rav1p localiza-

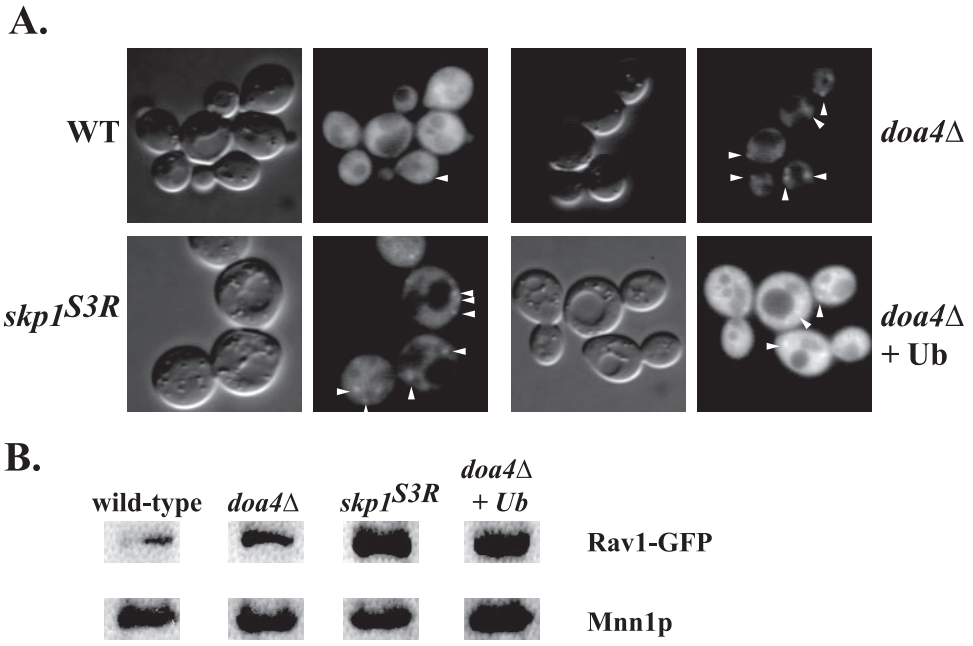

FIG. 6. Rav1-GFP is localized to membranes in mutants. (A) Strains GSY11-2A (*rav1*Δ), EBY116-4 [*rav1*Δ *skp1*Δ *skp1*(*Asn108Tyr*)], EBY79-12 (*rav1*Δ *doa4*Δ), and EBY79-12 (YEp96; wild-type [WT] ubiquitin) were transformed with p416.ADH.*RAV1*-GFP. For simplicity, the *rav1*Δ is not marked on the figure, since it is covered by the *RAV1* plasmid. Strains were grown at 25°C in minimal media (including 100 μM $CuSO_4$ for the ubiquitin experiment) and maintained in log phase. Differential interference contrast and fluorescence microscopy were performed using an E800 Nikon microscope and ISEE digital imaging software. All images were obtained with the same exposure times and gain. Punctate staining can be seen in each of the mutants pictured (white arrowheads). (B) Rav1-GFP accumulates in the membrane fraction. Strains GSY11-1A (*rav1*Δ), EBY116-4 [*rav1*Δ *skp1*Δ *pskp1*(*Asn108Tyr*)], EBY79-11 (*rav1*Δ *doa4*Δ), and EBY79-11 (*rav1*Δ *doa4*Δ pYEp96) were transformed with p416ADH.*RAV1-G*FP. S13 was created from 125 ODs of yeast spheroplasts and loaded on top of a sucrose step gradient consisting of 15% and 60% layers. To measure Rav1-GFP localization to membranes, membrane fractions were collected at the 15 to 60% interface and separated by SDS-PAGE before being transferred to nitrocellulose. Rav1-GFP was not visible in whole-cell extracts because of apparent degradation. The nitrocellulose was probed with anti-GFP or anti-Mnn1p, which was used as a loading control.

tion depended on ubiquitin. Indeed, Rav1-GFP demonstrated increased membrane localization in a *doa4*Δ mutant, as shown both by fluorescence microscopy and by the sucrose step gradient fractionation (Fig. 6A and B). However, ubiquitin overexpression did not rescue the effect of the *doa4*Δ mutant, suggesting that Doa4p may be directly involved in Rav1p localization.

DISCUSSION

We isolated the *SKP1* gene as a multicopy suppressor of the toxicity caused by Rav1p overexpression. Because Skp1p is essential, we designed a screen to identify a novel *SKP1* allele that would specifically abrogate the Rav1p-Skp1 interaction but would not disrupt other, essential Skp1p interactions. This approach allowed us to identify a single point mutation in Skp1p that was adjacent to the F-box binding domain and that blocked the Rav1p-Skp1p interaction but maintained essential protein-protein interactions of Skp1p. This separation of function allele, *skp1*(*Asn108Tyr*), allowed us to characterize the role of the Rav1p-Skp1p interaction in Rav1p function and localization.

Surprisingly, the *skp1*(*Asn108Tyr*) mutant exhibited phenotypes that were distinct from those of both *rav1*Δ and *vma2*Δ mutants, suggesting that the Skp1p-Rav1p interaction is not essential for the function of Rav1p in V-ATPase assembly/activation. Unlike *rav1*Δ and *vma2*Δ mutants, the *skp1*(*Asn108Tyr*) mutant exhibited no defects in quinacrine accumulation in the vacuole or in growth on zinc-containing, cobalt-containing, or high-pH media. In a previous study, the temperature-sensitive *skp1-12* mutant exhibited growth that was sensitive to high pH as well as a defect in vacuolar quinacrine accumulation (33). However, *skp1-12* did not suppress Rav1p overexpression (E. J. Brace and R. S. Fuller, unpublished data), suggesting that the Skp1p-Rav1p interaction is not disrupted by *skp1-12*. The effects of the *skp1-12* mutation may therefore be indirect, resulting from the nonspecific nature of the allele. *skp1-12* was identified as a mutant that caused temperature sensitivity for growth and which, therefore, disrupted at least one essential Skp1p function (4). In contrast, the *skp1*(*Asn108Tyr*) mutant specifically disrupts the Skp1p-Rav1p interaction but leaves intact the Skp1p interactions that are essential for growth. Synthesis of these findings suggests that the *skp1-12* mutation may affect pH sensitivity and quinacrine accumulation not by loss of the specific Skp1p-Rav1p interaction but by loss of one or more other Skp1p interactions. We conclude, therefore, that the Skp1p-Rav1p interaction is not required for V-ATPase assembly/activation. A second line of evidence that Skp1p function is distinct from that of Rav1p is the fact that although the *skp1*(*Asn108Tyr*) mutation did alter Kex2p localization, as judged by the onset of impotence assay, the effects were quite different than those of the *rav1*Δ mutation. The effects of the

skp1(*Asn108Tyr*) allele may be a consequence of alterations in Rav1p localization.

Whereas *skp1*(*Asn108Tyr*) did not affect Rav1p function in V-ATPase assembly and activation, it did have a clear effect on Rav1p localization. In contrast to wild-type cells, in the *skp1*(*Asn108Tyr*) mutant, Rav1-GFP was readily apparent in cytoplasmic puncta even without image deconvolution, presumably reflecting both an increase in membrane localization and a decrease in the cytosolic pool. Increased membrane localization was confirmed by biochemical fractionation. Interestingly, although Rav1-GFP showed increased localization to membranes in the *skp1*(*Asn108Tyr*) mutant, microscopic examination showed that sites of localization corresponded to punctate organelles rather than to vacuolar membranes. This is consistent with the proposed role for the RAVE complex in V-ATPase assembly/activation at the early endosome (34). A link between the roles of Rav1p in trafficking and V-ATPase activity is suggested by the fact that bafilomycin A1, a potent and specific inhibitor of V-ATPase, disrupts traffic between the early and late endosome in mammalian cells (8). Mutations in V-ATPase cause defects in the internalization of the fluorescent dye FM4-64 from the plasma membrane to the vacuole in yeast (26). Additionally, V-ATPase mutants exhibit defects in Kex2p trafficking (E. J. Brace and R. S. Fuller, unpublished data).

Loss of Doa4p also resulted in increased membrane association and punctate localization of Rav1-GFP. Because this effect was not overcome by ubiquitin overexpression, it was not an indirect result of ubiquitin depletion in the cells. These results suggest that both the Skp1p interaction with Rav1p and a deubiquitination event mediated by Doa4p are required for release of Rav1p from endosomal membranes. Although the RAVE complex has been classified as a non-SCF type Skp1p complex because of the absence of Cdc53p, Rbx1p, and Cdc34p (33), an intriguing but highly speculative possibility is that Skp1p may participate transiently in a ubiqitination reaction that is important for release of the RAVE complex from endosomal membranes. In this view, Skp1p and Doa4p might be required for a cycle of ubiquitination and deubiquitination that regulate Rav1p association with the endosomal compartment.

There are two other known Skp1p-containing non-SCF complexes in yeast, the Rcy1p-Skp1p complex (12) and the kinetochore complex CBF3 (9). Notably, the F-box protein Rcy1p functions at the early endosome to regulate recycling of internalized proteins from the cell surface (12, 20, 40). Mutations in *RCY1* have recently been shown to affect Kex2p trafficking (7). Rav1p and Rcy1p may govern distinct pathways out of the early endosome. Thus, it is plausible that Skp1p may function as a regulator of both processes.

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