Nucleus–Vacuole Junctions in *Saccharomyces cerevisiae* Are Formed Through the Direct Interaction of Vac8p with Nvj1p

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Vac8p is a vacuolar membrane protein that is required for efficient vacuole inheritance and fusion, cytosol-to-vacuole targeting, and sporulation. By analogy to other armadillo domain proteins, including β -catenin and importin α , we hypothesize that Vac8p docks various factors at the vacuole membrane. Two-hybrid and copurification assays demonstrated that Vac8p does form complexes with multiple binding partners, including Apg13p, Vab2p, and Nvj1p. Here we describe the surprising role of Vac8p–Nvj1p complexes in the formation of nucleus–vacuole (NV) junctions. Nvj1p is an integral membrane protein of the nuclear envelope and interacts with Vac8p in the cytosol through its C-terminal 40–60 amino acids (aa). Nvj1p green fluorescent protein (GFP) concentrated in small patches or rafts at sites of close contact between the nucleus and one or more vacuoles. Previously, we showed that Vac8p–GFP concentrated in intervacuole rafts, where it is likely to facilitate vacuole–vacuole fusion, and in “orphan” rafts at the edges of vacuole clusters. Orphan rafts of Vac8p red-sifted GFP (YFP) colocalize at sites of NV junctions with Nvj1p blue-sifted GFP (CFP). GFP-tagged nuclear pore complexes (NPCs) were excluded from NV junctions. In *vac8*– Δ cells, Nvj1p–GFP generally failed to concentrate into rafts and, instead, encircled the nucleus. NV junctions were absent in both *nvj1*– Δ and *vac8*– Δ cells. Overexpression of Nvj1p caused the profound proliferation of NV junctions. We conclude that Vac8p and Nvj1p are necessary components of a novel interorganelle junction apparatus.

INTRODUCTION

The yeast vacuole, like the animal lysosome, is an acidified organelle filled with soluble protein hydrolases that degrade proteins, lipids, nucleic acids, and carbohydrates (Jones *et al.*, 1997). Vacuole biogenesis occurs by several routes. Most vacuolar membrane proteins and soluble vacuolar hydrolases are initially transported into the endoplasmic reticulum (ER). The ER in *Saccharomyces cerevisiae* is limited to the nuclear envelope (NE) and ribbon-like peripheral cisternae (peripheral ER) (Preuss *et al.*, 1991; Shaywitz *et al.*, 1997). Vacuole proteins pass through the ER to the Golgi apparatus before being delivered to the vacuole by either of two known routes (reviewed in Conibear and Stevens, 1998). The carboxypeptidase Y (CPY) pathway directs vesicular cargo through a prevacuolar compartment, which is analogous to the endosome of animal cells (Vida *et al.*, 1993). The second

pathway, which delivers alkaline phosphatase (ALP) and a vacuolar T-snare (Vam3p), bypasses the prevacuolar compartment and requires the clathrin adaptor protein-related complex AP-3 (Conibear and Stevens, 1998). A third distinct route for the delivery of vacuolar proteins is the cytosol-to-vacuole targeting (Cvt) pathway, which delivers pre-aminopeptidase I (API) directly from the cytosol to the vacuole. API is sequestered in small double-membrane vesicles that ultimately fuse with the vacuole (Baba *et al.*, 1997; Kim *et al.*, 1997; Scott *et al.*, 1996, 1997; Klionsky and Ohsumi, 1999).

A major function of the vacuole is the autophagocytic degradation and recycling of cell components. Autophagy of the cytoplasm serves to recycle cell components, and is significantly induced by starvation and various metabolic signals (Jones *et al.*, 1997), during normal growth. Macroautophagy involves the formation of double-membrane autophagosomes around bulk cytoplasm and organelles (Takeshige *et al.*, 1992; Baba *et al.*, 1994). Fusion of mature autophagosomes with vacuoles releases single-membrane autophagic bodies into the vacuole lumen where they are

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degraded and their contents recycled (Takeshige *et al.*, 1992). Macroautophagy and the Cvt pathway in *S. cerevisiae* are morphologically similar processes (Baba *et al.*, 1997) that are mediated by an overlapping set of gene products (Scott *et al.*, 1996). Thus, most Cvt and autophagy mutants are defective in both API targeting and macroautophagy.

VAC8 was originally identified in a screen for genes required for the mitotic transfer of vacuoles from mother to daughter cells (Wang *et al.*, 1996). *vac8-Δ* cells are also defective in Cvt targeting and contain fragmented vacuoles (Wang *et al.*, 1998; Pan and Goldfarb, 1998; Fleckenstein *et al.*, 1998). Vacuole fragmentation is thought to indicate a defect in vacuole–vacuole fusion. Vac8p is anchored in the vacuole membrane by N-terminal myristate and palmitate moieties. Vac8p localizes over the entire surface of the vacuole (Wang *et al.*, 1998) but concentrates in rafts between vacuoles (intervacuolar rafts) and at the edges of clustered vacuoles (orphan rafts) (Pan and Goldfarb, 1998; this study). The domain structure of Vac8p is similar to a small family of proteins that includes β -catenin and karyopherin/importin α . These proteins contain central Armadillo (Arm) domains composed of ≥ 10 tandem ~ 42 -aa Arm repeats bracketed by short N- and C-terminal domains. X-ray crystal structures revealed that Arm domains fold into right-handed superhelical structures with extended grooves that serve as surfaces for binding protein cargo (Huber *et al.*, 1997; Conti *et al.*, 1998). Arm repeats are related to HEAT motifs (Malik *et al.*, 1997), which fold into polypeptides with similar structures and protein-binding characteristics (Groves *et al.*, 1999; Vetter *et al.*, 1999; Chook *et al.*, 1999; Cingolani *et al.*, 1999). In large part, α -catenin and importin α are adapters that mediate the docking of various cargo at their cognate membranes. The experiments in this article indicate that Vac8p plays an analogous role in docking proteins at the vacuole membrane. Surprisingly, we find that Vac8p interacts with Nvj1p to mediate the formation of NV junctions.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Growth Conditions

Yeast strains used in this study were YEF473a (*MATa*), YEF473 α (*MATa*), YEF473 (*MATa/MATa*) *trp1-Δ63 leu2-Δ1 ura3-52 his3-Δ200 lys2-801*, and L40 (*MATa*) *his-Δ200 trp1-901 leu2-3112 ade2 LYS2::lexAop₄-HIS3 URA3::lexAop₈-lacZ GAL4*. *pRK2* and *pJN40* (obtained from D. Hinkle, University of Rochester), derived from *YEplac181* and *YEplac195* (Gietz and Sugino, 1988), were used to control expression using the *CUP1* promoter (*P_{CUP1}*) (Macreadie *et al.*, 1989; Ward *et al.*, 1994). *pRK2* and *pJN40* were constructed by inserting a *Bam*HI/Klenow–*Eco*RI 443-base pair fragment encompassing the *P_{CUP1}* into a *Nar*/Klenow–*Eco*RI cut *YEplac181*. *Bam*HI and *Eco*RI sites both were reformed. *pNUP188-GFP* was created by inserting polymerase chain reaction (PCR)-amplified *NUP188* into the *Hind*III and *Cla*I sites of *pGFP-C-fus* (Neidenthal *et al.*, 1996). Yeast genes were amplified by PCR from genomic DNA, GFP from *pGFP-C-fus* (Neidenthal *et al.*, 1996), YFP/CFP from *pEYFP/pECFP* (Clontech, Palo Alto, CA), and glutathione-S-transferase (GST) was from *pGEX-2TK* (Pharmacia Biotech, Piscataway, NJ). Expand Hi-Fi DNA polymerase (Boehringer Mannheim, Indianapolis, IN) was used for all PCR amplifications. *nvj1-Δ* null cells were created by replacing *NVJ1* with a kanamycin resistance marker (*kan^r*) cassette, as described (Guldener *et al.*, 1996; Wach, 1996). A similar strategy was employed to produce *vac7-Δ* cells. A chromosomal *cNVJ1-GFP* gene fusion was similarly constructed using a PCR-amplified *NVJ1-YFP-kan^r* cassette. *kan^r* replacements were verified by PCR with

appropriate primers. Cells were cultured in standard YPD/YEPD and SCGlu media (Sherman, 1986). Nitrogen starvation medium (SD-N) contained 0.17% yeast nitrogen base without aa and without ammonium sulfate plus 2% glucose.

Diploid *nvj1-Δ* cells were obtained by mating haploid YEF473a *nvj1-Δ* + *pNJ* (URA3 marker) with YEF473 α *nvj1-Δ* + *pRK* (LEU2 marker). Diploid cells were grown in YEPA (1% bacto-yeast extract, 2% peptone, and 2% potassium acetate) for at least five generations to a titer of $\sim 1 \times 10^7$ cells/ml, were washed once in sterile ddH₂O, and were resuspended in SPM (1% potassium acetate, 0.02% raffinose, plus appropriate aa supplements at 25% of the level for complete medium). Asci began to develop within 24 h. Attempts to induce diploid *vac8-Δ* cells to sporulate included using liquid medium, as described above, and solid medium (1% potassium acetate, 0.1% bacto-yeast extract, 0.05% dextrose, appropriate aa supplements at 25% of the level for complete medium, and 2% bacto-agar) with or without prior incubation on presporulation medium (Sherman, 1986).

Glutathione Sepharose Affinity Chromatography

nvj1-Δ::kan^r cells containing *pRK-GST* or *pRK-GST-NVJ1* were used to purify GST or GST-Nvj1p using glutathione Sepharose 4B (Pharmacia). For 3 h, 0.1 mM CuSO₄ was added to early log cells (OD₆₀₀ = 0.3–0.7) in SCGlu in order to induce the expression of GST or GST-NVJ1. Total cell lysates were prepared from ~ 1 g of cells by vortexing with glass beads in 2 ml PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄) containing protease inhibitors (Sigma, St. Louis, MO). Cells were vortexed again after NP-40 was added to a final concentration of 1%. The lysate was clarified by centrifugation at $5,000 \times g$ and then at $100,000 \times g$ for 30 min at 4°C. The supernatant was diluted 10 times with PBS before it was incubated with 120 μ l of a 50% suspension of glutathione Sepharose 4B beads (Pharmacia Biotech) per gram of protein of starting cell pellet. After rocking overnight at 4°C, the beads were collected, washed three times with 10-bed volumes of PBS at 4°C, and eluted with 75 μ l of standard protein gel sample loading buffer. Immunoblots were probed with rabbit anti-Vac8p serum at 1:50,000 dilution in TST buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20) containing 0.2% nonfat dry milk and developed using ALP-conjugated secondary antibodies (1:3000) (Bio-Rad, Richmond, CA).

Two-Hybrid Screen

An *Eco*RI–*Bam*HI restriction fragment encoding VAC8 was inserted into the bait plasmid pBTM116 (Bartel and Fields, 1995) to produce pBTM116-VAC8. L40 cells (Vojtek *et al.*, 1993) harboring pBTM116-VAC8 were transformed with a yeast cDNA library (87002; American Type Culture Collection, Manassas, VA) by the lithium acetate procedure (Schiestl and Gietz, 1989). Approximately 10^5 transformants were pooled and replated at 1:1000 on plates supplemented with 3 mM 3-amino-1,2,4-triazole (3-AT) (Sigma). Extracts from cells that grew on –His, + 3-AT media were assayed for β -galactosidase activity, as described, using L40 cells containing pBTM116-VAC8, and a plasmid encoding only the *GAL4* activation domain was used as a negative control (Bartel and Fields, 1995). The regions of Nvj1p interacting with Vac8p were identified by PCR-amplifying portions of *NVJ1* and cloning the PCR products into pGAD424 (Clontech). The resulting plasmids were transformed into L40 cells containing pBTM116-VAC8.

Cell Fractionation

Cells of 600 ml weight containing either *pRK-Nvj1p-GFP* or *pRK-GFP-NVJ1* were grown to an OD₆₀₀ of 0.45 and were supplemented for 2.75 h with 0.1 mM CuSO₄. Cells were pelleted and resuspended in 2 ml PBS containing protease inhibitors (Boehringer Mannheim). An equal volume of glass beads was added, and the cells were broken by vortexing at 4°C. Suspensions were cleared by centrifugation.

gation at $500 \times g$ for 15 min. Each supernatant was divided into four aliquots, adjusted to 1 ml with PBS, and centrifuged at $100,000 \times g$ at 4°C for 30 min. S100 supernatants were collected leaving P100 pellets, which were resuspended in 1 ml PBS, 1% NP-40 in PBS, 1 M NaCl in PBS, or 0.1 mM Na_2CO_3 (pH 11.5). Suspensions then were centrifuged at $100,000 \times g$. The resulting supernatants were collected, and the pellets solubilized in 1 ml protein gel sample loading buffer. Equal amounts of extracts from $\sim 1.4 \text{ OD}_{600}$ equivalents ($\sim 1.5 \text{ ml}$ of original culture) were used for immunoblotting, as described above. Anti-GFP antiserum (Clontech) was used at 1:20,000, and the Vac8p antibody was used at 1:50,000.

Analysis of API processing

Midlog-phase cells (2×10^7) were incubated in 4 ml SD-N (nitrogen starvation conditions) or 4 ml YEPD for 4 h at 30°C . Cells were collected, resuspended in $400 \mu\text{l}$ of 50 mM sodium phosphate, 20 mM MES, pH 7.0, 1% SDS, 3 M urea, 1 mM sodium azide, and 2 mM phenylmethanesulfonate, and lysed with 0.15-g glass beads by vortexing $3 \times 1 \text{ min}$. After the addition of SDS sample buffer, lysates were spun at $14,000 \times g$ for 10 min and extracts from $\sim 0.5\text{-OD}$ equivalents were analyzed by SDS-PAGE. API was detected by immunoblotting using a rabbit anti-API polyclonal antiserum (1/10,000 dilution, gift of D. Klionsky), followed by HRP-conjugated goat-anti-rabbit secondary antibody (Zymed, San Francisco, CA) and chemiluminescent detection (ECL, Amersham, Arlington Heights, IL).

Anti-Vac8p Antibodies

A *Bam*HI–*Eco*RI restriction fragment encoding Vac8p was inserted into the bacterial GST fusion expression vector pGEX-2TK (Pharmacia). The GST–Vac8p fusion protein was affinity purified from BLR *Escherichia coli* cells (Novagen, Madison, WI) using glutathione Sepharose 4B (Pharmacia). Full-length Vac8p was released from GST by thrombin (Sigma). Polyclonal antibodies were prepared by Pocono Rabbit Farm & Laboratory (Canadensis, PA).

Microscopy

A Nikon fluorescence microscope equipped with a SPlan 100 objective (NA 1.25), an Image1 (Universal Imaging, West Chester, PA) controlled CCD camera, and filter sets for YFP and CFP (sets 41028 and 31044; Chroma Technology, Brattleboro, VT) were used to capture CFP and YFP images. Confocal microscopy was performed on a Leica TCS NT microscope (Zeiss, Thornwood, NY) equipped with UV, Ar, Kr/Ar, and He/Ne lasers. Images were processed using MetaMorph (Universal Imaging) or Photoshop 5.0 (Adobe Systems, San Jose, CA). FM4–64 staining of vacuoles was performed as previously described (Pan and Goldfarb, 1998).

Cells were prepared for electron microscopy basically as described (Banta *et al.*, 1988). Briefly, logarithmically growing cells were harvested by centrifugation and were fixed in 3% glutaraldehyde in buffer A (170 mM KH_2PO_4 , 0.1 mM MgCl_2 , and 30 mM sodium citrate, at pH 5.8) for 2 h at 22°C . Cells were washed once in buffer A, resuspended in a minimal volume of buffer A containing 1 M sorbitol and zymolyase-100T (ICN Biomedicals, Costa Mesa, CA), and incubated for 2 h at 30°C . Spheroplasts were washed two times in buffer A before post fixation with osmium and embedding in Spurr's resin.

RESULTS

Nvj1p interacts with Vac8p

The yeast two-hybrid screen was used to identify putative Vac8p binding partners. Vac8p fused to the *lexA* DNA-binding domain was used to screen a yeast cDNA library fused to the *GAL4* activation domain (Bartel and Fields, 1995; Vojtek and Hollenberg, 1995). From $\sim 10^5$ two-hybrid

clones, partial cDNA fragments encoding five different putative Vac8p binding proteins were isolated multiple times. The interactions of Vac8p with three of these proteins, Apg13p, Vab2p, and Nvj1p, were further characterized. *APG13* was originally identified in a screen for genes required for viability in starvation medium (Tsukada and Ohsumi, 1993) and was subsequently shown to be required for autophagy (Funakoshi *et al.*, 1997). *VAB2* (Vac8 binding protein 2) is encoded by an uncharacterized ORF (YEL005c) with no similarity to known proteins. The third, encoded by the uncharacterized locus YHR195w, was named Nvj1p for its role in NV junctions (see below).

The two hybrid interactions were corroborated by the copurification of Vac8p with the three putative binding partners. When whole-cell extracts expressing GST fusions of the binding partners, or GST alone, were incubated with glutathione Sepharose beads, significant amounts of Vac8p coeluted with GST–Nvj1p (Figure 1A), GST–Apg13p (Figure 1B), and GST–Vab2p (Figure 1C). In each case, the GST-fusion proteins appeared as silver-stained bands (Figure 1, A to C, left panels). Vac8p was detected on immunoblots using anti-Vac8p antibodies (Figure 1, A to C, right panels). These interactions were specific since Vac8p was absent when extracts from cells expressing GST alone were treated in parallel (Figure 1, A to C). In addition, Nvj1p with a C-terminal GFP tag copurified by glutathione affinity chromatography with Vac8p–GST (our unpublished results). Therefore, both N-terminal (GST–Nvj1p) and C-terminal (Nvj1p–GFP) fusions retain the capacity to associate with Vac8p. Although we have presumed that the two-hybrid and copurification results indicate a direct interaction between Vac8p and the three putative binding partners, it remains a remote possibility that one or more of the interactions are indirect. In this study, we have focused on the interaction between Vac8p and Nvj1p. Possible implications regarding the interactions of Apg13p and Vab2p with Vac8p are presented in the Discussion section.

Nvj1p Fractionates as an Integral Membrane Protein

Nvj1p does not show significant sequence similarity to known proteins. A Kyte–Doolittle hydrophobicity plot and sequence analysis suggested that Nvj1p contains an N-terminal signal peptide ($\text{M}_1\text{TRPPLVRGIFSLGLSVAVLKGVEK}_{25}$) and a single membrane-spanning domain ($\text{V}_{96}\text{LILLISFLLPIAWTVL}_{112}$) (Figure 2A). The putative signal peptide is typical in that it contains a basic residue followed by a core of hydrophobic residues (Watson, 1984). Evidence that Nvj1p is indeed an integral membrane protein was obtained by biochemical fractionation. A reporter protein containing GFP fused to the C-terminus of Nvj1p (Nvj1p–GFP) was used for this purpose in order to facilitate its detection by anti-GFP antibodies. Results presented below validate the use of Nvj1p–GFP as a functional reporter. A whole-cell lysate from cells expressing Nvj1p–GFP was centrifuged to separate soluble and insoluble fractions. Immunoblot analysis of these fractions showed that Nvj1p–GFP fractionated with the particulate membrane fraction (Figure 2B). Membrane fraction-associated Nvj1p–GFP was resistant to extraction by high salt and high pH but was solubilized by nonionic detergent (NP-40) treatment (Figure 2B). Vac8p, which is anchored into the vacuole mem-

Figure 1. Glutathione-Sepharose affinity copurification of Vac8p with three binding partners. Proteins from cell extracts (c), wash solutions (w), and eluates (e) were separated by SDS-PAGE and were analyzed by silver staining (left panels) and immunoblot using anti-Vac8p antibodies (right panels). (A) Copurification of Vac8p with GST-Nvj1p. (B) Copurification of Vac8p with GST-Apg13p. (C) Copurification of Vac8p with GST-Vab2p. Labeled bands correspond to GST (27 kDa), GST-Nvj1p (63.4 kDa), and Vac8p (63.2 kDa).

brane by myristate and palmitate residues, also behaved as an integral membrane protein (Figure 2B). Nvj1p with an N-terminal GFP tag also fractionated as an integral membrane protein, even though its signal peptide is blocked.

C-Terminus of Nvj1p Is Sufficient and Necessary for Binding Vac8p

The combination of an N-terminal signal peptide and a single internal membrane-spanning domain predicts that

B

Association of Nvj1p-GFP and GFP-Nvj1p with membrane

Figure 2. Nvj1p is an integral membrane protein. (A) Hydrophobicity plot and map of Nvj1p. The map shows the location of the putative N-terminal signal peptide (M_1 TRPPLVRGIFSLGLSVAVLKGVK $_{25}$), the significant hydrophobic region (V_{96} LILLSFLLPIAWTVL $_{112}$), and the portions of *NVJ1* that were isolated in independent two-hybrid clones (aa 93–321 and 119–321). (B) Nvj1p fractionates as an integral membrane protein. Panel 1: A whole cell extract from cells expressing Nvj1p-GFP was separated into supernatant (S100) and membrane (P100) fraction by centrifugation. P100 was subsequently treated as described in Material and Methods (S, supernatant; P, pellet). The presence of Nvj1p in each fraction was assayed by immunoblot using anti-GFP antibodies. Panel 2: Extract from cells expressing GFP-Nvj1p and Vac8p were treated as described in panel 1.

Figure 3. Two hybrid mapping of the C-terminal domain of Nvj1p that interacts with Vac8p.

Nvj1p is a type I integral membrane protein with its C-terminal domain exposed to the cytosol where it could interact with Vac8p. Consistent with this hypothesis, the four independently isolated *NVJ1* two-hybrid clones all contained the C-terminal portion of *NVJ1* but not the N-terminal domain upstream of the membrane-spanning sequence (Figure 2A). The putative Vac8p binding domain of Nvj1p was further delineated using a two-hybrid interaction assay. Based on relative growth rates on 3-AT plates and LacZ expression levels, the C-terminal 40 aa of Nvj1p (aa 281–321) were sufficient to mediate a minimal two-hybrid interaction with Vac8p; however, an additional 20 aa were necessary for a strong interaction (Figure 3). The deletion of the C-terminal 60 aa of Nvj1p abolished the two-hybrid interaction, demonstrating that these residues are also necessary for Vac8p binding. The two-hybrid assay results were corroborated by showing that a GFP reporter protein containing only the C-terminal 40 aa of Nvj1p (aa 281–321) partially localized to the vacuole membrane (our unpublished results).

NVJ1 Is Not Required for Growth or Vacuole Biogenesis

A complete disruption of *NVJ1* was generated by replacing the entire ORF with a *kan^r* cassette (Guldener *et al.*, 1996; Wach, 1996). *nvj1* Δ cells grew normally at 30 and 37°C. *vac8* Δ cells exhibit a number of characteristic defects. Because Nvj1p associates with Vac8p, we asked whether *nvj1* Δ cells also exhibited these phenotypes. *vac8* Δ cells are defective in Cvt sorting but are normal for both CPY and ALP pathways (Wang *et al.*, 1998). As shown in Figure 4, both *vac8* Δ and *pep4* Δ control cell extracts contained significant amounts of unprocessed API (~50 and 90% respectively). In contrast, parental wt and *nvj1* Δ cells contained, for the main part, fully processed API. PrA (Pep4p) is required for the proteolytic activation of PrB, which, together with PrA, activates many vacuolar hydrolases, including API after their targeting to the vacuole (Jones *et al.*, 1997). Thus, *pep4* Δ cells import but do not process preAPI. Following the induction of autophagy by shifting the cells into a nitrogen-limited medium (SD-N), only *pep4* Δ cells contained unprocessed API (Figure 4). We note that using the

Figure 4. API processing is normal in *nvj1-Δ* cells. Midlog-phase cells were incubated for 4 h in rich (YEPD) or nitrogen starvation (SD-N) medium. Cell lysates were prepared and processed for immunoblotting with anti-API antibody. The positions of precursor and mature API are indicated.

vac8-Δ strain constructed by Wang *et al.*, (1998), which is in a different genetic background, a virtually complete API processing defect in YEPD medium was observed, consistent with previous studies (Wang *et al.*, 1998), and a moderate defect (~ 50 unprocessed) in SD-N medium (our unpublished results). We attribute these variations in API processing to strain differences and conclude that Vac8p probably plays a role in the kinetic efficiency of both Cvt sorting and autophagy. A role for Vac8p in autophagy also is suggested by its interaction with Apg13p, and the finding that *vac8-Δ* cells exhibited a loss of viability in SD-N medium (our unpublished results). The key result here is that *nvj1-Δ* cells had no significant defect in the *VAC8*-dependent Cvt of AP1. *nvj1-Δ* cells were also normal for CPY and ALP targeting (our unpublished results).

vac8-Δ cells exhibit abnormally large numbers of small vacuoles and are defective in vacuole inheritance (Wang *et al.*, 1998; Pan and Goldfarb, 1998) and sporulation (our unpublished results, see Material and Methods). *nvj1-Δ* cells were tested for these defects. The numbers of vacuoles per cell and the efficiency of vacuole inheritance (see below) and sporulation were normal in *nvj1-Δ* cells. Therefore, although there is strong evidence that Vac8p and Nvj1p form a complex *in vivo*, *nvj1-Δ* cells displayed none of the defects that have been described for *vac8-Δ* cells.

Evidence for a physiologically significant interaction between Vac8p and Nvj1p was obtained by studying the effects of overexpressing *NVJ1* on vacuolar inheritance. The efficiency of vacuolar inheritance is normally expressed as the percentage of daughter cells that receive FM4-64-stained vacuoles from their mothers (Vida and Emr, 1995; Wang *et al.*, 1998). The efficiency of vacuolar inheritance dropped from $96.9 \pm 1.1\%$ in wt cells to $6.2 \pm 1.3\%$ in *vac8-Δ* cells. In contrast, the deletion of *NVJ1* did not affect the efficiency of vacuolar inheritance ($95.9 \pm 0.6\%$). This result indicates that the role of the Vac8p–Nvj1p interaction is unrelated to the role of Vac8p in vacuolar inheritance. Interestingly, however, the overexpression of *NVJ1* in wt cells decreased the efficiency of vacuolar inheritance more than twofold to $40.8\% \pm 0.7$. Overexpression of *NVJ1*-GFP had a similar effect ($39.1 \pm 3.6\%$). Thus *NVJ1* overexpression has an inhibitory effect on vacuole inheritance. Because the deletion of *NVJ1* did not affect vacuolar inheritance, the inhibitory effect of *NVJ1* overexpression is likely to be indirect.

Possibly, the pool of Vac8p that is normally employed in vacuolar inheritance is partially sequestered by excess Nvj1p. Thus, the overexpression of *NVJ1* would indirectly effect vacuole inheritance by sequestering Vac8p. If true, then the coordinate overexpression of both *VAC8* and *NVJ1* should replete the Vac8p pool and suppress the transfer defect caused by the overexpression of *NVJ1*. The vacuolar inheritance defect of *NVJ1* overexpressing cells was, in fact, partially suppressed from an efficiency of 40.8% to $63.7 \pm 14\%$ in wt cells by the coordinate overexpression of both genes.

Vac8p Recruits Nvj1p into NV Junctions

To investigate the localization of Nvj1p expressed from its own promoter, GFP was integrated into the chromosome in frame with the C-terminal codons of *NVJ1* to create *cNVJ1*-GFP. When cells were grown to midlog, *cNVJ1*-GFP localized to short stripes or patches (Figure 5A, panels a and b). In stationary-phase cells, *cNVJ1*-GFP stripes had expanded in many cells into arcs that were sandwiched between FM4-64-stained vacuole clusters and Hoechst-stained nuclei (Figure 5A, panels c and d). The increase at higher cell densities in the length (and intensity) of *cNVJ1*-GFP stripes is consistent with gene array analysis, which showed that *NVJ1* mRNA levels rose two- to threefold after the diauxic shift (DeRisi *et al.*, 1997). When viewed en face, stripes appear as patches. We conclude that Nvj1p-GFP concentrates in rafts in the NE.

The dependence of Nvj1p localization on *VAC8* was determined using a plasmid copy of *NVJ1*-GFP expressed from the P_{CUP1} . When cells containing this plasmid were grown in a medium containing small amounts of copper ($\sim 0.25 \mu\text{M}$ Cu^{2+}), P_{CUP1} -*NVJ1*-GFP expression levels were held at a minimum and the localization of Nvj1p-GFP resembled that of *cNVJ1*-GFP (Figure 5B). In *vac8-Δ* cells, Nvj1p-GFP generally failed to concentrate in rafts (Figure 5B, panels d and e) and, instead, delocalized over the entire surface of the Hoechst-stained nuclei (Figure 5B, panel f). Especially at higher cell densities, some rafting of Nvj1p-GFP in *vac8-Δ* cells was observed. Therefore, binding to Vac8p appears to facilitate Nvj1p-GFP rafting, which still can occur at a low frequency in its absence. These results are consistent with the hypothesis that the pool of Nvj1p in the NE is recruited into rafts predominantly through direct interaction with Vac8p. The lower frequency occurrence of Vac8p-independent rafting suggests that Nvj1p may coassociate with itself. Alternatively, factor(s) beside Vac8p may facilitate rafting of Nvj1p.

Following the induction of P_{CUP1} -*NVJ1*-GFP expression, elevated levels of Nvj1p-GFP concentrated in expanded rafts (see below) but at very high levels spilled out of its rafts and spread over the surface of the NE (see Figure 6C). Therefore, the amount of Nvj1p-GFP that can accumulate in rafts is determined by a limiting factor. The endogenous level of Nvj1p is normally maintained at levels sufficiently below this limit to allow low levels of ectopically expressed Nvj1p-GFP to concentrate in rafts. When expressed at levels that exceed this limit, excess Nvj1p-GFP is free to distribute randomly over the surface of the nucleus, presumably by diffusing in the plane of the NE. It is noteworthy that when expressed at fully induced levels, Nvj1p-GFP still did not spread into peripheral ER mem-

Figure 5. Nvj1p-GFP localizes to a specialized region of the NE in a *VAC8*-dependent manner. (A) Localization of cNvj1p-GFP in wt cells grown to an OD₆₀₀ of 2.8 in SCglu (panels a and b), and in cells grown to an OD₆₀₀ of 10 in YPD and costained for DNA (Hoechst) (panel c) and vacuoles (FM4–64) (panel d). (B) Nvj1p-GFP in wt (*VAC8*⁺) (panels a and b) and *vac8*-Δ cells (panels d and e) grown in SC medium to OD₆₀₀ ~0.1–0.3. Localization of Nvj1p-GFP in wt and *vac8*-Δ cells stained for DNA (Hoechst) and vacuoles (FM4–64) (panels c and f). Plasmid-borne Nvj1p-GFP was expressed uninduced from the *CUIP1* promoter.

branes. Thus, it is apparent that Nvj1p is selectively sorted to the NE and, subsequently, concentrated in rafts.

The localization of Vac8p in intervacuole rafts suggests that it may play a structural or regulatory role in homotypic vacuole docking and/or fusion. This notion is supported by the observation that *vac8*-Δ cells contain fragmented vacuoles (Pan and Goldfarb, 1998; Wang *et al.*, 1998; Fleckenstein *et al.*, 1998). The relationship between intervacuole and orphan rafts of Vac8p-GFP was studied in *vac7*-Δ cells, which contain a single enlarged vacuole (Bonangelino *et al.*, 1997; Bryant *et al.*, 1998; Gary *et al.*, 1998). As shown in Figure 6A, Vac8p-GFP in *vac7*-Δ

cells concentrated in single perinuclear rafts adjacent to Hoechst-stained nuclei. As in wt cells, Nvj1p-GFP localized to a single perinuclear patch in *vac7*-Δ cells when expressed at low levels (Figure 6B) and spread over the surface of the NE when overexpressed (Figure 6C). Therefore, orphan rafts of Vac8p-GFP occur independently from intervacuole rafts. In summary, intervacuole Vac8p-GFP rafts occur where vacuoles abut vacuoles, and orphan Vac8p-GFP rafts occur where vacuoles abut perinuclear rafts of Nvj1p-GFP.

The colocalization of rafts of Vac8p and Nvj1p to NV contact sites was achieved by fusing the two proteins to

Figure 6. Localization of Vac8p-GFP and Nvj1p-GFP to single perinuclear rafts in *vac7-Δ* cells. Vac8p-GFP localization (A), Nvj1p-GFP localization before (B) and after (C) the induction of expression by 0.1 mM CuSO₄. Phase contrast (panels a, e, and i), GFP (panels b, f, and j), Hoechst (panels c, g, and k), and overlay of phase contrast and Hoechst images (panels d, h, and l). Bar: 5 μm.

different spectrally shifted GFP variants. Blue-shifted CFP (cyan) was fused to *NVJ1* (Nvj1p-CFP) and red-shifted YFP (yellow) was fused to *VAC8* (Vac8p-YFP). Vac8p-YFP decorated the entire vacuole surface and, as with Vac8p-GFP, accumulated in intervacuole and orphan rafts (Figure 7). Nvj1p-CFP localized to single rafts that colocalized exclusively with Vac8p-YFP orphan rafts at the edges of nuclei (Figure 7). In conclusion, orphan rafts of Vac8p-GFP colocalize with perinuclear rafts of Nvj1p-GFP.

Effect of *NVJ1* Expression on NV Junction Morphology

Using the electron microscope (TEM), we attempted to demonstrate a causal relationship between the expression of *NVJ1* and the formation of physical junctions between the nucleus and vacuole (NV junctions). Thin sections of cells were scored for the presence of at least one area of contact between the nucleus and a vacuole. Only those thin sections exhibiting both vacuoles and a nucleus were scored. Sixty-

two percent (71 of 115) of thin sections of wt cells exhibited NV contacts (Figure 8, panel a) and only 5% (6 of 113) of *nvj1-Δ* thin sections exhibited NV contacts (Figure 8, panel b). The rare *NVJ1*-independent NV contacts may represent instances where the two organelles were simply pressed together. NV contacts were also mostly absent from thin sections of *vac8-Δ* cells (our unpublished results, see below) indicating that both partners are required for the formation of NV junctions.

The overexpression of *NVJ1* in wt cells had a striking effect on the frequency and morphology of NV contacts. *NVJ1* overexpression increased the frequency of NV contacts to 97% of thin sections (146 of 151). Both the numbers of separate contacts per cell and the extensiveness of individual junctions were increased (Figure 8, panel c). The overexpression of Nvj1p-GFP also increased the frequency of NV contacts to 95% (99 of 104) and had virtually the identical effects that elevated levels of native Nvj1p had on the morphology of NV junctions (Figure 8, panel d). These effects

Figure 7. Partial colocalization of Vac8p and Nvj1p. Nvj1p-CFP (blue-shifted GFP) and Vac8p-YFP (red-shifted GFP) were localized in the same cells. Monochromatic images of Nvj1p-CFP (a) and Vac8p-YFP (b), and color overlay images over a DIC mask of Nvj1p-CFP and Vac8p-YFP (c), and Nvj1p-CFP, Hoechst, and DIC (d). In panel c, yellow occurs where Nvj1p-CFP and Vac8p-YFP overlap.

indicate that the total area of NV junctions in a cell is normally limited by *NVJ1*, and not *VAC8*, expression. Consistent with this conclusion, the overexpression of P_{GAL1} -*VAC8* in cells expressing cNvj1p-GFP altered neither the morphology of cNvj1p-GFP localization nor increased the area of NV junctions (our unpublished results). In addition, these results provide compelling evidence that Nvj1p-GFP is an active and valid reporter of Nvj1p function.

Nuclear Pore Complexes Are Excluded From NV Junctions

Severs *et al.* (1976) noted that pore complexes were absent from regions of the NE that were adjacent vacuole membranes. We sought to confirm this observation by studying the localization of Nup188p-GFP in *nup188-Δ* cells. Nup188p is an abundant nonessential nucleoporin that assembles with other nucleoporins in symmetrical structures located on both the cytoplasmic and nuclear faces of the pore complex (Nehrbass *et al.*, 1996; Rout *et al.*, 2000). As shown in Figure 9, Nup188p-GFP localized to the NE of *nup188-Δ* cells. Significantly, Nup188p-GFP staining was absent in contact sites between nuclei and vacuoles. These results

suggest that NPCs are normally either actively or passively excluded from NV junctions.

DISCUSSION

In this study, we define the role of Vac8p and Nvj1p in the formation of NV junctions. The discovery that the yeast nucleus and vacuole are physically linked by protein–protein interactions was foreshadowed by published observations. Foremost among these is the commonplace occurrence in published TEM images of extensive contact sites between the nuclei and vacuoles of vegetatively growing (Severs *et al.*, 1976; Heath *et al.*, 1995) and sporulating *S. cerevisiae* cells (Lynn and Magee, 1970). Second, Severs *et al.* (1976) noted that NPCs were absent from NV contact sites. This observation implies that some form of contact or communication, either direct or indirect, occurs between the two organelles. Finally, Koning *et al.* (1993) reported that the two organelles often move together in a manner that is consistent with their being attached.

Vac8p and Nvj1p Mediate NV Junctions

Four lines of evidence support the hypothesis that Vac8p and Nvj1p interact with one another to form NV junctions. First, two-hybrid and copurification assays demonstrate that Vac8p and Nvj1p form specific and stable complexes. Second, the specific topologies of Vac8p and Nvj1p within their respective membranes provide for their interaction at the interface between the nucleus and vacuole. Thus, Vac8p is posttranslationally anchored in the cytoplasmic face of the vacuole membrane by N-terminal myristate and palmitate residues, and Nvj1p is an integral membrane protein of the NE whose C-terminal domain extends into the cytosol. As predicted by this model, the cytosolic C-terminal 40–60 aa of Nvj1p are necessary and sufficient to interact with Vac8p. Third, Vac8p-GFP and Nvj1p-GFP accumulate in membrane rafts that coincide with contact sites between vacuoles and nuclei. The accumulation of Vac8p-GFP and Nvj1p-GFP in membrane rafts is dependent on the expression and proper localization of its binding partner. Thus, Nvj1p-GFP accumulated in single NE rafts in *VAC8* cells but delocalized over the entire surface of the NE in *vac8-Δ* cells. Conversely, Vac8p-GFP orphan rafts were absent in *nvj1-Δ* cells (X. Pan, unpublished results). Because rafts of Nvj1p-GFP still occur at a low frequency in *vac8-Δ* cells, especially at high cell densities, Nvj1p may self-assemble or there may be factors in addition to Vac8p that facilitate Nvj1p rafting. Fourth, the appearance in electron micrographs of close contacts between nuclei and vacuoles was dependent on the expression of Vac8p and Nvj1p. Particularly convincing was the finding that elevated levels of Nvj1p and Nvj1p-GFP caused a marked increase in numbers and total surface area of NV junctions. This indicates that Vac8p can be recruited into NV junctions from around the vacuole when Nvj1p levels are naturally or ectopically increased. In cells expressing very high levels of Nvj1p, the available Vac8p pool can be sequestered into NV junctions so efficiently as to cause a defect in Vac8p-dependent vacuole inheritance.

The use of Nvj1p-GFP as a valid reporter is supported by functional criteria. First, Nvj1p-GFP selectively binds and copurifies with Vac8p. Second, the overexpression of Nvj1p and Nvj1p-GFP had identical effects on the efficiency of vacuolar

Figure 8. NVJ1 expression levels control the occurrence and morphology of NV junctions. (A) wt cells + empty vector; (B) *nvj1*-Δ cells + empty vector; (C) wt cells + $P_{CUP1-NVJ1}$; (D) wt cells + $P_{CUP1-NVJ1-GFP}$. Overexpression from the *CUP1* promoter was achieved by adding 0.1 mM $CuSO_4$ to growth media for 3 h. Cells were subsequently fixed and prepared for TEM as described in Material and Methods.

inheritance. Third, Nvj1p-GFP successfully recruited Vac8p-GFP into NV junctions in *nvj1*-Δ cells (our unpublished results). Fourth, the ectopic overexpression of Nvj1p and Nvj1p-GFP had identical effects on NV junction morphology.

Previous structure–function studies on mitochondria-associated ER membranes provides some precedent for the kinds of processes that might occur at NV junctions. For example, specific steps in the biosynthesis of glycosylphosphatidylinositols, which serve to anchor proteins in membranes, occur at sites of contact between mitochondria and ER (reviewed in Vidugiriene *et al.*, 1999). Mitochondria–ER contact sites also facilitate the transfer of lipids between ER and mitochondria in yeast (Achleitner *et al.*, 1999) and may participate in the regulation of cytosolic Ca^{2+} (Vidugiriene *et al.*, 1999). In yeast, the vacuole rather than the ER is the major repository of cellular Ca^{2+} (Jones *et al.*, 1997; Strayle *et al.*, 1999). Finally, the recycling of nuclear components by vacuolar hydrolases could be facilitated by a form of microautophagy within the context of NV junctions (Dunn, 1994; Sakai *et al.*, 1998).

Vac8p Docks Multiple Factors at the Vacuole Membrane

In addition to defining the role of Vac8p in NV junction formation, these investigations suggest that Vac8p plays

other roles in vacuole-associated processes. Three lines of evidence support this hypothesis. First, *vac8*-Δ cells display several phenotypes that are absent in *nvj1*-Δ cells, including defects in vacuole inheritance, Cvt, sporulation, and vacuole fragmentation. Second, the pattern of Vac8p-GFP localization on the surface of the vacuole suggests multiple functions. Whereas Nvj1p-GFP localizes exclusively to a single membrane patch on the surface of the NE, Vac8p localizes over the entire surface of the vacuole (Wang *et al.*, 1998) and in at least two types of rafts, vacuole–vacuole and orphan (Pan and Goldfarb, 1998; this study). As shown here, orphan rafts correspond to NV junctions. The function of intervacular rafts is not known, however, they are likely to be related to the vacuole fragmentation phenotype of *vac8*-Δ cells. In fact, Wang *et al.* (1998) suggested that Vac8p has a role in vacuole fusion.

A third line of evidence is that Vac8p binds at least two other proteins, Vab2p and Apg13p, whose characteristics suggest that they function at the vacuole membrane. In addition to the interaction of Vab2p with Vac8p, Vab2p interacts by two-hybrid assay with the vacuolar ATPase subunit Vma8p (Roberts and Goldfarb, unpublished results). Thus, Vac8p may participate in the assembly or regulation of the vacuolar ATPase. Another Vac8p-binding protein, Apg13p, was originally identified as being required for

Figure 9. Nup188p-GFP-tagged NPCs are excluded from NV junctions. Pore complexes in *nup188-Δ* cells were labeled with Nup188p-GFP. Vacuoles were stained with FM4-64, and chromatin with Hoechst (Materials and Methods). Examples of four cells that exhibited large NV junctions are shown. Upper panels: Overlays of GFP-Nup188p and DIC images. Middle panels: GFP-Nup188p. Lower panels: Overlays of GFP-Nup188p, FM4-64, and Hoechst.

efficient autophagy (Funakoshi *et al.*, 1997). Because autophagy and Cvt are mediated by many of the same factors, the requirement for Vac8p in Cvt and autophagy may result from its interaction with Apg13p. Both *vac8-Δ* and *apg13-Δ* cells, but not *nvj1-Δ* cells, exhibit decreased viability in SD-N medium (our unpublished results). Thus, it is likely that Vac8p is required for efficient Cvt and autophagy. Neither VAB2 nor Apg13 are required for NV junction formation (Pan and Goldfarb, unpublished observations). Together, these results support the hypothesis that Vac8p mediates independent vacuole-specific processes, including the formation of NV junctions through its interaction with different binding partners. This study provides support for the hypothesis that the primary function of Arm domain proteins such as Vac8p, importin α , and β -catenin is to dock binding partners at their cognate membranes.

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