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Yel013p (Vac8p), an armadillo repeat protein related to plakoglobin and importin α, is associated with the yeast vacuole membrane

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SUMMARY

Proteins of the *armadillo* family are involved in diverse cellular processes in higher eukaryotes. Some of them, like *armadillo*, β-catenin and plakoglobins have dual functions in intercellular junctions and signalling cascades. Others, belonging to the importin-α-subfamily are involved in NLS recognition and nuclear transport, while some members of the *armadillo* family have as yet unknown functions.

Here, we introduce the *Saccharomyces cerevisiae* protein Yel013p as a novel armadillo (*arm*) repeat protein. The ORF Yel013w was identified in the genome project on chromosome V (EMBL: U18530) and codes for an acidic protein of 578 residues with 8 central *arm*-repeats, which are closely related to the central repeat-domain of *Xenopus laevis* plakoglobin. We show that Yel013p (Vac8p) is constitutively expressed in diploid and haploid yeasts and that it is not essential for viability and growth. However, the vacuoles of mutant cells are multilobular or even fragmented into small vesicles and the processing of aminopeptidase I, representing the cytoplasm-to-vacuole transport pathway, is strongly impaired. Consistent with these observations, subcellular fractionation experiments, immunolocalization and expression of green fluorescent protein (GFP) fusion proteins revealed that Yel013p (Vac8p) is associated with the vacuolar membrane.

Our data provide evidence for the involvement of an *arm*-family member in vacuolar morphology and protein targeting to the vacuole.

Key words: Armadillo, Sporulation, Plakoglobin, Cytoplasm-to-vacuole transport

INTRODUCTION

Since the *Saccharomyces cerevisiae* genome sequencing project has been finished, a large number of open reading frames (ORFs), coding for (hypothetical) proteins is available from the databases (Cherry et al., 1997). For many of these ORFs no function is known yet, but significant sequence motifs or homologies to proteins with established roles, as well as the subcellular localization of these unknown proteins may point to potential functions. Yel013w is an open reading frame on chromosome V (EMBL U18530) with *Xenopus* plakoglobin as its closest relative in the databases. Thus, the hypothetical protein Yel013p (renamed as Vac8p by Wang et al. (1998) during the review process of this work) is a member of the rapidly growing *armadillo* multigene family which is characterized by imperfect repeats of a 42 amino acid sequence motif, the armadillo (*arm*) repeat (Peifer et al., 1994). *Arm* repeats are thought to be conserved modules involved in protein-protein interactions, similar to e.g. SH2, SH3, PH, and LIM domains (Pawson, 1995; Peifer et al., 1994). Structural data indicate that *arm* repeats are not independent folding units. A minimum number of 6 motifs seems necessary to form a highly charged groove along the *arm* domain, representing the binding sites for interacting molecules (Huber et al., 1997). Consistent with a function in mediating protein-protein interactions, members of the *arm*-family are seen at various subcellular compartments and coordinate diverse cellular processes. Based on sequence homologies several subfamilies of *arm*-repeat proteins have been identified (Gelderloos et al., 1997; Hatzfeld and Nachtsheim, 1996; Reynolds and Daniel, 1997). One subfamily comprises well characterized molecules involved in cell-cell contact formation of higher eukaryotes. Examples for this family are the *Drosophila* segment polarity gene product armadillo (Peifer and Wieschaus, 1990) and its vertebrate relatives β-catenin and plakoglobin (McCrea et al., 1991; Peifer et al., 1992). These proteins have dual functions. First, they are involved in the anchorage of the actin cytoskeleton at adherens junctions. Second, they are involved in the wingless/wnt-signalling pathways (Behrens et al., 1996; Huber et al., 1996, for review see Barth et al., 1997; Peifer, 1995).

Another important armadillo repeat subfamily comprises importin α-like proteins that are primarily involved in NLS recognition and thus in nuclear transport (Gorlich et al., 1995). Importins (also called karyopherins) are found from yeast to man. Importin α (Srp1p, karyopherin α) is the only *arm*-repeat protein that has been identified and functionally characterized in yeast so far. It is a homologue of mouse and *Drosophila* pendulin (Kussel and Frasch, 1995; Yano et al., 1994).

The yeast vacuole is a degradative organelle and the functional equivalent of lysosomes in higher eukaryotes (for recent reviews see Klionsky, 1997; Van Den Hazel et al., 1996). Protein components can be delivered to the vacuole as vesicles from the Golgi apparatus or via endocytosis (Vps pathway)(see Conibear and Stevens, 1995; Riezman, 1993). Other proteins, like aminopeptidase I, are synthesized in the cytoplasm and delivered to the vacuole via the cytoplasm-to-vacuole targeting pathway (Kim et al., 1997; Scott et al., 1997). Finally, under nutritional stress or upon sporulation, protein sequestration to the vacuole is achieved by autophagocytosis (Takeshige et al., 1992). During the cell cycle, the vacuole segregates into two compartments. One part is transferred to the emerging bud and is thus inherited in an ordered fashion, requiring spatial and temporal coordination with the cell cycle (Gomes de Mesquita et al., 1991; Weisman et al., 1990; Weisman and Wickner, 1988). Hence, this process is very different from the segregation of membrane bound organelles like Golgi apparatus or ER in vertebrates, which undergo cell-cycle-specific fragmentation and are inherited in a stochastic way (Warren and Wickner, 1996).

Considering the close relationship of Yel013p (Vac8p) with plakoglobin and other armadillo proteins over the repeat regions on the one hand, and the fact that the unicellular budding yeast does not form cell-cell contacts on the other, we were interested in the potential function and cellular localization of Yel013p. Here we show that Yel013p is constitutively expressed in diploid and haploid cells but is not essential for viability and growth. However, deletion of the gene strongly affects vacuolar morphoglogy and interferes with processing of aminopeptidase I. We demonstrate that the protein is not associated with the plasma membrane, but with the vacuole. We conclude that Yel013p is required for proper vacuole function.

MATERIALS AND METHODS

Strains, media, and genetic techniques

Yeast strains used in this study are listed in Table 1. Rich medium (YPD) and synthetic minimal medium (SD) were prepared as described (Sherman et al., 1986). Yeast mating, sporulation and tetrad analysis were performed using standard genetic techniques (Sherman et al., 1986). All yeasts were grown at 30°C if not indicated otherwise. For yeast transformations and gene disruption experiments, the lithium acetate method was used (Ito et al., 1983; Rothstein, 1991). *Escherichia coli* strain XL1-blue (Stratagene, Heidelberg) was used for bacterial manipulations and BL21(DE)pLysS (Novagen, Madison, WI) for expression of recombinant proteins. Molecular cloning techniques were performed as described (Sambrook et al., 1989).

Cloning, plasmids and strain construction

Yeast genomic DNA was prepared from strain MRY1 as described (Struhl et al., 1979). The 1,737 bp Yel013w open reading frame (accession no. U18530), coding for Yel013p, was amplified by PCR using a 5′ primer, 5′-AAGCTTCATATGGGTTCATGTTGTAGTTG-3′ (primer 1; *Nde*I site underlined), and a 3′ primer, 5′-GAATTCGAATGTAAAAATTGTAAAATCTG-3′ (primer 2; *Eco*RI site underlined). This fragment was cloned into pGEM-T (Promega, Madison, WI) excised by *Nde*I/*Eco*RI and subcloned in pET23a (Novagen, Madison, WI), to obtain pET-Yel013 for expression in *E. coli*. BL21(DE)pLysS and for further constructs. To engineer Yel013w-disruption plasmids, pET-Yel013 was partially digested with *Sca*I cutting within the Yel013-ORF (amino acid 226) and DNA-cassettes containing either the LEU2-gene or the URA3-gene were blunt end ligated to obtain pET-Yel013::LEU2 and pET-Yel013::URA3.

To engineer a Yel013 deletion plasmid a LEU2-gene cassette was cloned with *Bam*HI/*Sal*I into pUC19 to obtain pUC-LEU2, as a first step. Then, 5′ and 3′ untranslated regions (UTRs) of Yel013w were amplified by PCR and ligated 5′ and 3′, respectively, of the LEU2-cassette, to obtain p5UTR-LEU2-3UTR. The 756 bp 5′-UTR was amplified from genomic DNA as above with the following primers: 5′ primer, 5′-GAGCTCTCAGACAGGATTGAAGC-3′ (primer 3; *Sac*I site underlined); 3′ primer, 5′-GGATCCAAC-CCATATGTTGCAAATG-3′ (primer 4; *Bam*HI site underlined). The 712 bp 3′-UTR was amplified using the following primers: 5′ primer, 5′-GTCGACTTACATTGATTTTCTTC-3′ (primer 5; *Sal*I site underlined) and 3′ primer, 5′-AAGCTTATGAGTGAACGTATTTTC-3′ (primer 6; *Hin*dIII site underlined).

To construct gene disruption strains, pET-Yel013::LEU2 and pET-Yel013::URA3, respectively, were cut with *Nde*I/*Eco*RI and transformed into the diploid strain MRY1, to obtain MRY 4 and MRY 15, respectively. Transformed diploids were sporulated and tetrads were dissected and analyzed. The tetrads showed a 2:2 segregation for the LEU2 (or the URA3) marker, and MRY-strains 7, 50 and 51 were obtained. A diploid strain with both Yel013w alleles disrupted (MRY55) was obtained by crossing MRY50 and MRY51. The identity of all constructs was analyzed by Southern blotting.

To construct gene deletion strains (Δ-strains), p5UTR-LEU2-3UTR was cut with *Sac*I/*Hin*dIII and transformed into MRY1, to obtain MRY54. Transformed diploids were sporulated and the tetrads were dissected and analyzed and MRY-strains 56 and 57 were obtained. These were mated and zygotes were isolated by micromanipulation to obtain MRY58. The identity of all constructs was verified by PCR on genomic DNA of the strains using primers 1 and 2, to show absence of the Yel013w allele and of primers 2 and 6 for correct insertion.

For expression of the Vac8p with a C-terminal GFP fusion, the Yel013 ORF was amplified by PCR from pET-Yel013p (see above) with the following primers: 5′ primer, 5′-GGACTAGTATGG-GTTCATGTTGTAGTTGC-3′ (primer 7; *Spe*I site underlined); 3′ primer, 5′-GAAGCTTATGTAAAAATTGTAAAATC-3′ (primer 8; *Hin*dIII site underlined) and cloned into the *Spe*I/*Hin*dIII site of the GFP expression vector pGFP-C-FUS (Niedenthal et al., 1996) to obtain pVac8p-GFP. For GFP expression studies, this construct was transformed into MRY58 or CEN.PK2, which has a very low autofluorescence.

Protein purification and antibody production

A 1 litre culture of *E. coli* BL21(DE)pLys S transformed with pET-Yel013 was grown at 37°C to OD600=0.5, then induced with 0.5 mM IPTG and further incubated for 3 hours. Cells were harvested, washed and resuspended in 10 ml lysis buffer (50 mM Tris, pH 8.0, 25% (w/v) sucrose, 1 mM EDTA, 0.2 mM PMSF and 10 μg/ml each of pepstatin A, aprotinin, leupeptin). Protease inhibitors were included in all steps. Lysozyme was added to 1 mg/ml and the cells were incubated for 30 minutes on ice. To degrade DNA, the suspension was adjusted to 10 mM $MgCl_2$, 1 mM $MnCl_2$ and 10 μg/ml DNaseI and incubated for 30 minutes on ice. Then, 15 ml of detergent buffer (20 mM Tris, pH 7.5, 0.2 M NaCl, 2 mM EDTA, 1% (w/v) deoxycholate and 1% Triton X-100) was added, and insoluble material was removed by centrifugation. The supernatant was then incubated for 16 hours at 4°C with Ni-chelating resin (Ni-NTA resin, Qiagen, Germany). The resin was collected, packed into a column, washed with 35 ml W1 (0.1 M sodium-phosphate, 0.01 M Tris, pH 8.0) and subsequently with 80 ml W2 (0.1 M sodium-phosphate, 0.01 M Tris, pH 6.3). Finally, the protein was eluted with 20 ml 250 mM imidazole in W2. Vac8p containing fractions were dialyzed against PBS, 14 mM mercaptoethanol.

The purified protein was used to raise antiserum in rabbits

(Eurogentec, Belgium). For some applications the serum was affinity purified on blotted recombinant protein, according to standard protocols.

Cell size determination

Cells were photographed and the length of the short axis of at least 50 budding mother cells was measured on prints. From these numbers, average values and standard deviation were calculated.

Cell fractionation experiments and membrane association of Yel013p

Cell fractionation on sucrose gradients was performed as described (Kolling and Hollenberg, 1994). Aliquots of each gradient fraction were assayed for α-mannosidase activity (vacuolar marker) as described (Kolling and Hollenberg, 1994) and analyzed by immunoblotting for the presence of the Yel013p, plasma membrane ATPase (Pma1p; plasma membrane marker) and dipeptidyl aminopeptidase A (DPAP A; Golgi marker) with peroxidase-conjugated goat anti-rabbit antibodies (Sigma) and enhanced chemoluminescence (ECL, Amersham).

The membrane association of Yel013p was probed by extracting Yel013p enriched membranes at pH 7.6, pH 11, 1.5 M NaCl and 0.5% Triton X-100 basically as described and by digesting vacuole enriched fractions with proteinase K in the absence and presence of Triton X-100 as described (Klionsky and Emr, 1989).

Immunoblotting of crude extracts

Exponentially growing cells were harvested by centrifugation, suspended to 20 OD units/ml in 10 mM Tris, pH 7.6, 10 mM EDTA, 1 mM DTT, 0.2 mM PMSF and 10 μg/ml each of pepstatin A, aprotinin, leupeptin and disrupted by shaking with glass beads. Extracts where boiled in sample buffer and 0.15 OD units/lane were analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and probed using antibodies directed against carboxypeptidase Y (10A5-B5, Molecular Probes, Eugene, OR) as described by the manufacturer, against API as described (Harding et al., 1995; Klionsky et al., 1992) and against Yel013p using a 1:5,000 dilution of our antibody (see above).

Fluorescence microscopy

For analysis of GFP fusion protein expression, MRY65 was grown in synthetic minimal medium without uracil and without methionine as described (Niedenthal et al., 1996), to induce expression of Yel013p-GFP. Cells were directly spotted on coverslips and GFP-fusion proteins were visualized in vivo as described. All preparations were examined with a Zeiss Axiophot microscope equipped with epifluorescence and photographed on Kodak Tri-X-Pan. Alternatively, images were recorded with a CCD camera (VarioCam, PCO Computer Optics GmbH, Kehlheim, Germany) and processed using Adobe Photoshop™.

Electron microscopy

To prepare spheroplasts, cells were suspended in 0.05 M EDTA, pH 7.5, for 10 minutes at room temperature and then in 0.1 M EDTA, pH 9.0 and 0.3 M β-mercaptoethanol. After 15 minutes at room temperature the cells were resuspended in 1 M sorbitol, 0.1 M EDTA, pH 7.5. After 5 minutes 0.25 mg zymolyase (20,000 units/g) was added, the incubation continued for 10-15 minutes at 37°C and spheroplast formation was monitored by light microscopy.

Embedding of spheroplasts

Spheroplasts were pelleted and fixed in 0.2% glutaraldehyde and 1% formaldehyde in 1 M sorbitol, 0.1 M EDTA, pH 9.5 for 1 hour on ice. After quenching free aldehyde groups, samples were embedded according to the progressive lowering of temperature protocol (PLT-method). Dehydration was done in a graded series of ethanol: 10%,

Table 1. Yeast strains

Strain	Genotype	Source
DBY1104	*MAT***a** ade2-1 cdc46ts	D. Botstein
DBY1705	*MAT*α leu2-3,112, lys2-801, ura3-52	D. Botstein
KFY92	*MAT***a** his1	K. U. Fröhlich
KFY93	*MAT*α his1	K. U. Fröhlich
KFY19 (MRY42)	*MAT*α ade2-1, leu2-3,112, lys2-801, ura3-52	K. U. Fröhlich DBY1104XDBY1705
CEN.PK2	*MAT***a**/α ura3-52/ura3-52, trp1-289/trp1-289, leu2-3, 112/leu2-3,112, his3-Δ1/his3-Δ1	K. D. Entian
KFY20 (MRY41)	*MAT***a** ade2-1, leu2-3,112, lys2-801, ura3-52	K. U. Fröhlich DBY1104XDBY1705
MRY1	*MAT***a**/α ade2-1/ade2-1, leu2-3,112/leu2-3,112, lys2-801/lys2-801, ura3-52/ura3-52	KFY19×KFY20 (this study)
MRY4	*MAT***a**/α VAC8/vac8::LEU2, ade2-1/ade2-1, leu2-3,112/leu2-3,112, lys2-801/lys2-801, ura3-52/ura3-52	This study
MRY15	*MAT***a**/α VAC8/vac8::URA3, ade2-1/ade2-1,leu2-3,112/leu2-3,112, lys2-801/lys2-801, ura3-52/ura3-52	This study
MRY7	*MAT*α vac8::LEU2, ade2-1, leu2-3,112, lys2-801, ura3-52	This study (segregant from MRY4)
MRY50	*MAT***a** vac8::URA3, ade2-1, leu2-3,112, lys2-801, ura3-52	This study (segregant from MRY15)
MRY51	*MAT*α vac8::URA3, ade2-1, leu2-3,112, lys2-801, ura3-52	This study (segregant from MRY15)
MRY54	*MAT***a**/α VAC8/Δvac8::LEU2, ade2-1/ade2-1, leu2-3,112/leu2-3,112 lys2-801/lys2-801, ura3-52/ura3-52	This study
MRY55	*MAT***a**/α vac8::LEU2/vac8::URA3, ade2-1/ade2-1,leu2-3,112/leu2-3,112, lys2-801/lys2-801, ura3-52/ura3-52	This study MRY7×MRY50
MRY56	*MAT***a** Δvac8::LEU2, ade2-1, leu2-3,112, lys2-801,ura3-52	This study (segregant from MRY54)
MRY57	*MAT*α Δvac8::LEU2, ade2-1, leu2-3,112, lys2-801,ura3-52	This study (segregant from MRY54)
MRY58	*MAT***a**/α Δvac8::LEU2/Δvac8::LEU2, ade2-1/ade2-1, leu2-3,112/leu2-3,112, lys2-801/lys2-801, ura3-52/ura3-52	This study (MRY57×MRY58)
MRY64	CEN.PK2 transformed with pVac8p-GFP	This study
MRY65	MRY58 transformed with pVac8p-GFP	This study

30% ethanol in ice for 30 minutes; 50% ethanol at −20°C for 30 minutes; 70%, 90% and 100% ethanol at −35°C for 30 minutes. After a final dehydration step (100% ethanol for 1 hour) the samples were infiltrated with the hydrophilic Lowicryl K4M methacrylate resin as follows: 1 part ethanol/1 part K4M resin overnight, 1 part ethanol/2 parts K4M resin for 8 hours, pure K4M resin for 2 days with several changes. Polymerization of the resin was done with UV-light (366 nm) for 1 day at −35°C, and an additional two days at room temperature after trimming the samples.

Immuno labelling protocol

Ultrathin sections (approx. 100 nm) were cut with glass knives and collected onto Formvar coated 300 mesh nickel grids. Without air-drying the grids were placed, section side down, onto drops of the affinity purified Yel013p-specific antibody (4 μg/ml IgG final concentration) and incubated at 4°C overnight. After washing with PBS (100 mM phosphate buffer, 0.15 M NaCl, pH 6.9) grids were placed onto drops of 10 nm Protein A-gold complexes (1:100 dilution of the stock solution, British Biocell, Great Britain) for 1 hour at room temperature. Grids were washed with PBS containing 0.01% Tween-20, and with water and air dried. Counter staining was performed with 4% aqueous uranyl acetate for 5 minutes. After washing with water, the grids were air-dried and examined in a Zeiss EM 910 transmission electron microscope with an acceleration voltage of 80 kV and at calibrated magnifications.

Freeze-fracturing of yeast cells

1 μl samples of growing yeast cells were placed onto copper freezing pins (Balzers, Liechtenstein) and frozen in a nitrogen slush. Frozen samples were transfered into a cryo-transfer unit (Oxford, Model HF1500, Wiesbaden, Germany) directly attached to a Zeiss field-emission-scanning electron microscope (FESEM) DSM 982 Gemini. Samples were freeze-fractured with a blade, etched for 10-30 seconds at −100°C, sputter coated with gold-palladium (thickness approx. 2 nm, Denton Magnetron Sputter Coater, USA) and subsequently transferred onto a cryo-desk in the FESEM and observed at −150°C with an acceleration voltage of 2 kV.

RESULTS

Yel013p is a plakoglobin related protein

Yel013w was identified by searching the *Saccharomyces* genome for sequences with homologies to proteins from adherens junctions in animal cells. During the review process of this work, the gene was also identified through complementation of the vacuole inheritance mutant *vac8-1* (Wang et al., 1998). Accordingly, we refer to Yel013p as Vac8p throughout this report. Vac8p has a calculated molecular mass of 63,207 Da and an IP of 4.86. As noted previously (Wang et al., 1998), Vac8p exhibits significant homologies to members of the armadillo (arm) repeat family. The arm-proteins are characterized by the presence of a 42 amino acid repeating sequence that may be involved in protein-protein interactions. A sequence similarity analysis of the repeat regions of various *arm*-proteins is shown in Fig. 1A. Vac8p is only 21-25% identical (45-49% similar) to other family members and thus displays a similar degree of relationship to both the importin (mouse-pendulin or yeast-Srp1p) and the catenin-subgroup (plakoglobin, β-catenin, armadillo). Fig. 1B gives an alignment of the 8 complete and one incomplete arm-repeats identified on the basis of the consensus sequence given by Hatzfeld et al. (1994) and the ISREC ProfileScan server (http://www.ch.embnet.org/software/PFSCAN_form.html).

A

	Vac8p	Srp1p	pendulin	plakoglobin	β-catenin	armadillo
Vac8p	-------	**25.6**	**23.5**	**22.1**	**22.6**	**21.5**
Srp1p	48.6	-------	**52.5**	**22.7**	**22.6**	**21.1**
pendulin	45.7	67.0	-------	**23.4**	**26.6**	**25.1**
plakoglobin	44.5	46.9	48.1	-------	**77.0**	**70.4**
β-catenin	45.4	46.8	48.2	88.6	-------	**79.2**
armadillo	44.5	45.8	48.1	81.8	88.3	-------

B

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                 L       N       V
                 VQA K   S       AT    T H A     SEAI
Consensus:       GGIPELVRLL+-R++-DE++-LL+AA+GVLRNLS++++++-NKLAIR
YEAST:     86    VLEP-ILILLQ-SQDPQIQV-AACAALG-LGNLAV--NNENKLLIVEM    125
          127    GLEP-LI--NQ-MMGDNVEV-QCNAV-GCITNLATRDDNKHKIATS      166
          168    ALIP-LTKLAK-SKHIRVQRNATGALLNMTHSEE----NRKELVNA      207
          208    GAVPVLVSLL-SST--DPDVQYYCTT--ALSNIAVDEANRKKLA        246
          251    RLVSKLVSLMD-SPSS----RVKCQATLALRNLASDTSYQLEIVRA      291
          292    GGLPHLVKLIQ-SDSIPL-V-LASVA--CIRNISIHPLNEGLIVDA      332
          333    GFLKPLVRLLDYKDS--EEIQCH-AV-STLRNLAASSEK-NRKEFFESG   376
          377    GAVEKCKELALDSPVSVQSEISAC---FAILALADVS----KLDLLEAN   417
          421    ALIPMTFSQNQEVSGN--------AAAA-LANLCSRVNNYTKII
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C

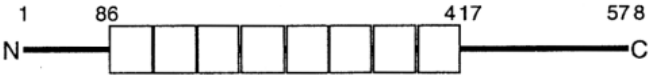

Fig. 1. Analysis of the Vac8p sequence. (A) The repeat regions of the proteins were compared using the BLAST algorithm. Values in the upper part of the table (printed in bold) are percentage sequence identity, values in the lower part are percentage sequence similarity. The extent of sequence identity and similarity was determined according to the method of Needleman and Wunsch (1970). Accession numbers: Vac8p (U18530), Srp1p (M75849; yeast), pendulin (U12270; mouse), plakoglobin (M95593; *Xenopus*), β-catenin (M77013; *Xenopus*), armadillo (X54468; *Drosophila*). (B) Identification and alignment of the arm-repeat sequences of Vac8p with the *arm*-repeat consensus sequence according to the method of Hatzfeld et al. (1994) and Peifer et al. (1994). Numbers refer to the position of each repeat within the Vac8p amino acid sequence. (C) Schematic representation of the protein domains. Boxes represent *arm*-repeats. Numbers refer to positions in the amino acid sequence.

Two additional arm repeats have been identified by Wang et al. (1998) based on a different consensus sequence. As shown schematically in Fig. 1C, the central repeat region is flanked by short N- and C-terminal regions. No homologies to other proteins were found in the database for these flanking regions.

Vac8p is not essential for viability and growth

To investigate whether *VAC8* is an essential gene, the open reading frame (ORF) was deleted in diploid cells as described in Materials and Methods. All four meiotic products were viable in agreement with Wang et al. (1998), however, we did

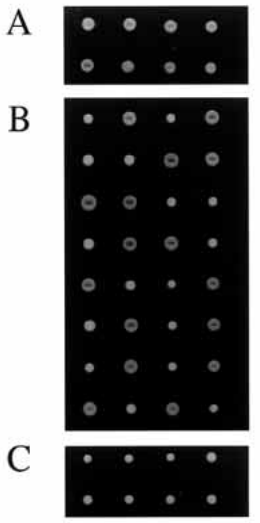

Fig. 2. Tetrad analysis of (A) wt-cells (MRY1), (B) heterozygous cells with one disrupted Yel013 allele (MRY4) and (C) homozygous cells with both Yel013w alleles disrupted (MRY55).

not find that the *vac8Δ* cells grew more slowly, in contrast with the previously reported results. The doubling times were not significantly different in rich medium; both strains had doubling times of approximately 90 minutes at 30°C and 25 hours at 4°C. In contrast, we found that the germinants from the sporulated null strains consistently produced smaller colonies (Fig. 2). Similarly, we found that the mutant cells were considerably smaller than wt cells; the average diameters were 3.2±0.6 μm and 5.2±0.6 μm, respectively. Consistent with this finding, mutant cells formed smaller colonies than the

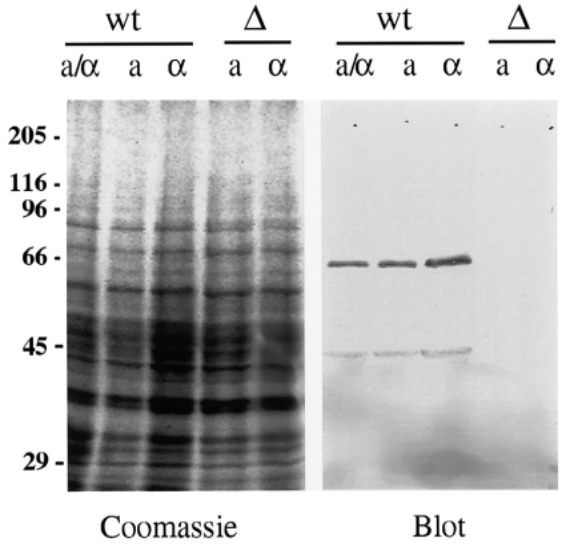

Fig. 3. Expression of endogenous Yel013p. Extracts from diploid (**a**/α) wild-type cells and of haploid (**a** and α, respectively) wild-type (wt) and gene deletion strains (Δ), were analyzed by SDS-PAGE (Coomassie) and immunoblotting (Blot), for the presence of Yel013p. Note, that Yel013p is found in all types of wt cells, but not in the gene deletion strains.

Fig. 4. Freeze-fracture electron microscopic images of wild-type (a) and MRY58 Vac8p-deficient yeast (b,c). V is vacuole. Bars, 2.5 μm.

wt-cells, when 10-fold dilutions of log-phase cells were spotted onto YPD agar plates (not shown).

Vac8p is expressed in diploid and haploid cells

With antibodies raised against recombinant Vac8p produced in *E. coli*, a protein of about 63 kDa was detected in diploid and haploid wt-cells of both mating types, but not in the gene deletion mutants (Fig. 3). In all wt-samples a minor band at about 40 kDa was also detected which was not seen in the mutants, suggesting that it is a proteolytic product of Vac8p. Thus, we conclude that the antibody does not crossreact with other armadillo-repeat proteins in yeast.

Vac8p-deficient cells display altered vacuole morphology

In agreement with the data of Wang et al. (1998) we found that the vacuole in *vac8Δ* cells displays an abnormal morphology. We extended the analysis by examining these cells through electron microscopy. In contrast to wt cells, the vacuole in *vac8* mutant cells was frequently multilobular or fragmented into several small vesicles (Fig. 4).

Vac8p is associated with the vacuole membrane

Velocity sedimentation coupled with indirect immunofluorescence indicated that Vac8p may be associated with the vacuole membrane (Wang et al., 1998). We have carried out a careful subcellular fractionation using equilibrium gradient centrifugation to examine the location of Vac8p. Cell extracts obtained from the wild-type strain (MRY1) were separated on 20-50% sucrose gradients. Each fraction was assayed for the presence of the vacuolar enzyme α-mannosidase by measuring enzyme activities (Fig. 5A), and, by immunoblotting after SDS-PAGE (Fig. 5B), for the presence of Vac8p, the plasma membrane ATPase (Pma1p) and the Golgi marker protein dipeptidyl-aminopeptidase A (DPAP A). Vac8p co-distributed with the activity of α-mannosidase, with a peak in fractions 6 and 7, while the maxima of Pma1p and of DPAP A were seen in fractions 5/6 (higher density fractions) and fractions 8/9 (lower density fractions), respectively. The vacuolar localization of Vac8p was corroborated by immunofluorescence studies showing

that Vac8p colocalizes with the vacuolar H^+-ATPase (Fig. 6). In wt-cells the vacuole is stained as a ring-like structure by Vac8p specific as well as by H^+-ATPase specific antibodies, whereas in mutant cells, the Vac8p directed antibody does not stain any structure. Altogether these data indicate that at least most of Vac8p is associated with the vacuolar membrane, in agreement with Wang et al. (1998). To address the questions whether Vac8p, which lacks a transmembrane consensus sequence, is a peripheral membrane protein and whether it is exposed on the luminal or cytoplasmic face of the vacuolar membrane, we first tried to solublize the protein from the membrane under various conditions. While the protein is easily and completely solubilized by Triton X-100, we find that a significant part but not all the Vac8p can be removed from the membrane following prolonged treatment with 1.5 M NaCl or at pH 11 (Fig. 5C), arguing that Vac8p is not associated with the vacuolar membrane solely via protein-protein interactions. Second, mild osmotic lysis of spheroplasts rendered Vac8p sensitive to proteolysis with proteinase K in the absence of Triton, suggesting it is most likely exposed on the cytoplasmic face of the membrane (Fig. 5D).

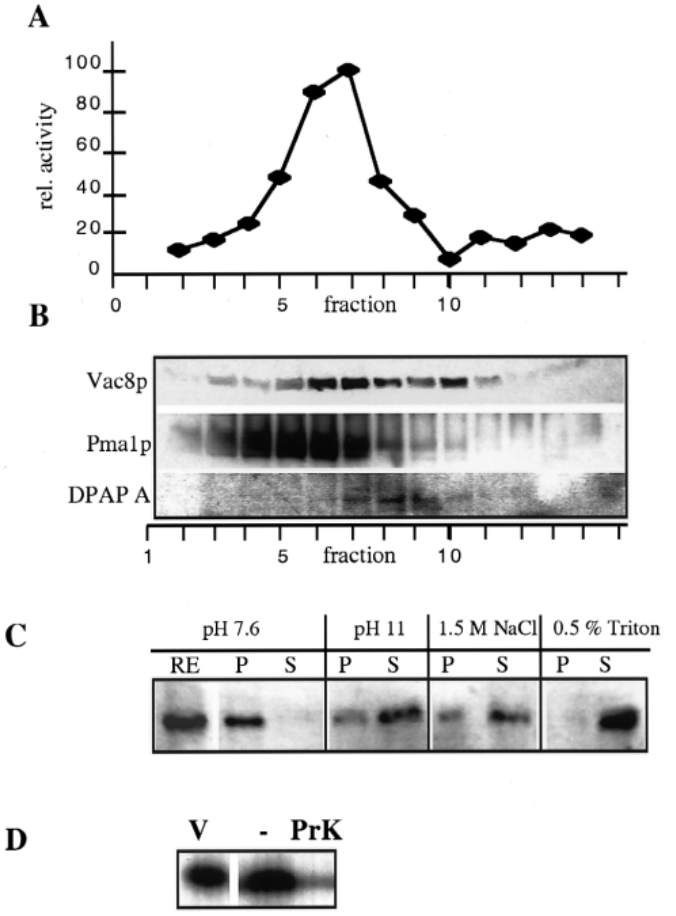

Fig. 5. Membrane association of Vac8p. (A,B) Cell fractionation on sucrose gradients. A crude extract of MRY1 was fractionated by density-gradient centrifugation. Aliquots of each gradient fraction were assayed for α-mannosidase activity (A) and immunoblotted after SDS-PAGE (B) for the presence of Vac8p, the plasma membrane ATPase (Pma1p) and the Golgi marker dipeptidyl-aminopeptidase (DPAP A), as indicated. (C) Crude extracts (RE) of MRY1-wt cells were adjusted to pH 7.6 and separated into soluble (S) and membrane fraction (P) by centrifugation at 100,000 ***g***. Pellets were extracted either at pH 11, or with 1.5 M salt or after the addition of 0.5% Triton X-100. Extracts were then again separated into pellet and supernatant as above. All fractions were separated by SDS-PAGE and analyzed by immunoblotting for Vac8p. (D) Vacuole enriched fractions (V) obtained by mild osmotic lysis of spheroplasts were treated with (PrK) or without (-) proteinase K under identical conditions in the absence of Triton X-100 and probed for the presence of intact Vac8p by immunoblotting.

Expression of a Vac8p-GFP fusion protein restores the Cvt pathway

To add further evidence to the cell fractionation and immunolocalization studies, we expressed Vac8p as a GFP fusion protein in the double deletion mutant (MRY65). The *vac8* mutant is defective in vacuole inheritance and is the only characterized *vac* mutant that displays a defect in the cytoplasm to vacuole targeting (Cvt) pathway (Wang et al., 1996, 1998). The *vac8* mutant accumulates the precursor form of aminopeptidase I (API) (Fig. 7A). The Vac8p-GFP hybrid complements the API defect of *vac8*, indicating that the fusion protein is functionally active (Fig. 7B). We determined the average expression level of the fusion to be somewhat lower than the level of endogenous Vac8p in wt cells (data not shown). Upon the examination of cells expressing the Vac8p-GFP fusion protein, the construct was always seen at the vacuolar membrane (Fig. 8), as can be seen by comparing the GFP pictures (Fig. 8a-d) with the phase pictures (Fig. 8a′-d′). In most cases, the vacuole appeared as a single globular organelle (Fig. 8a,b), but in some cells the vacuole was surrounded by many small GFP-labelled vesicles (Fig. 8c) or

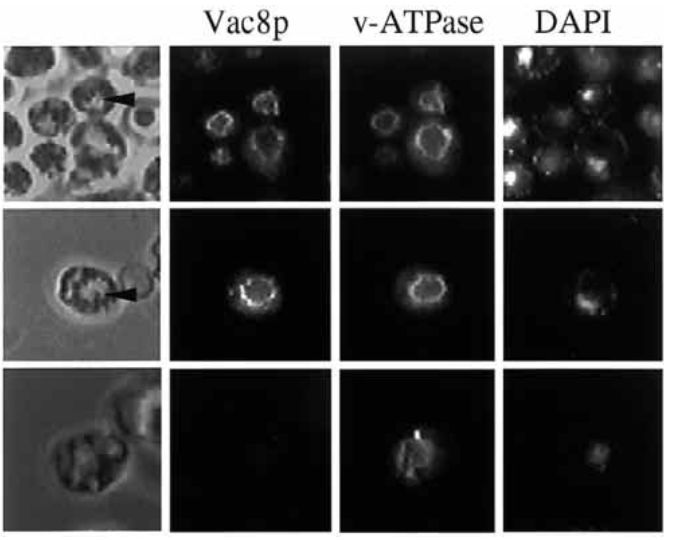

Fig. 6. Immunofluorescence analysis of Vac8p localization. Wt-cells (rows a and b) and *vac8*-deletion mutants (row c) were triple stained with a Vac8p specific serum followed by a FITC-conjugated secondary antibody (second column); a monoclonal antibody specific for the 60 kDa subunit of the vacuolar H^+-ATPase followed by a TRITC-conjugated secondary antibody (third column); and with DAPI to stain DNA (fourth column). Corresponding phase contrast pictures are shown in the first column. Arrowheads point to typical vacuoles.

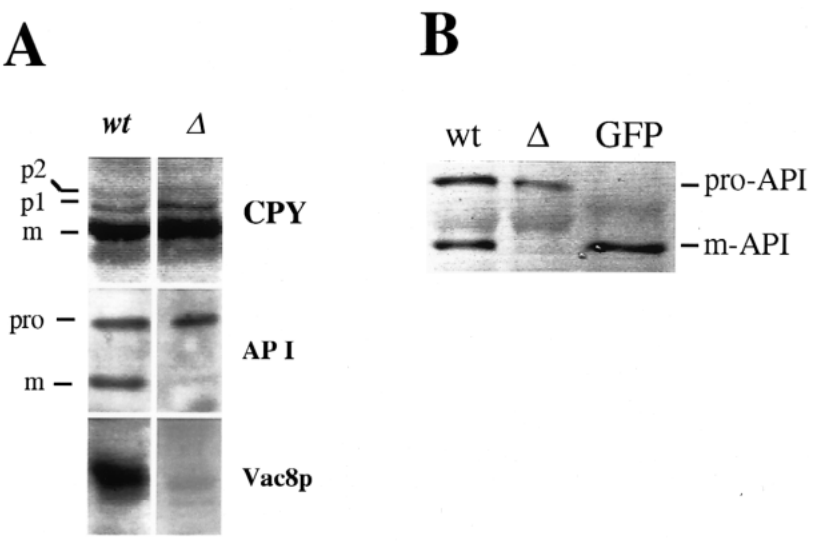

Fig. 7. Processing of aminopeptidase I (API) and carboxypeptidase Y (CPY). (A) Crude extracts from exponentially growing wild-type (*wt*) and mutant cells (Δ) were probed for the presence of CPY, API and Vac8p, respectively, as indicated. The positions of mature and precursor forms of CPY are indicated (m, p1 and p2), as well as the positions of pro-API (pro) and mature API (m). Note that no mature API is found in the mutant. (B) Extracts from wt-cells (wt), from the gene-deletion strain MRY58 (Δ) and from MRY58 expressing Vac8p-GFP (GFP) were analyzed for the presence of API by immunoblotting. Note that API is completely processed to the mature form (m-API) in the strain expressing Vac8p-GFP.

was fragmented into many small vesicles (Fig. 8d). As a control, GFP was expressed without a Vac8p fusion. In such cells, a uniform green fluorescence excluding the vacuole was observed as described earlier (Niedenthal et al., 1996).

Localization of Vac8p by immunogold electron micoscopy

We were not able to detect endogenous Vac8p in wt cells (CEN.PK2) by immuno-EM (Fig. 9a). However, since we had shown above that GFP-Vac8p fully complements the Cvt pathway defect and that the protein is localized along the vacuole membrane on the light microscopic level, we used a CEN.PK2 strain expressing Vac8p-GFP (MRY64) in addition to the endogenous Vac8p for immuno-EM (Fig. 9b-e). In these cells we could readily detect Vac8p, possibly due to the higher total amount of Vac8p. As expected, the protein was found along the vacuolar membrane. It often seemed to be enriched at sites of vacuole/vacuole contact (arrowheads in c and e) and around small vesicles pinching off into the vacuolar lumen (d).

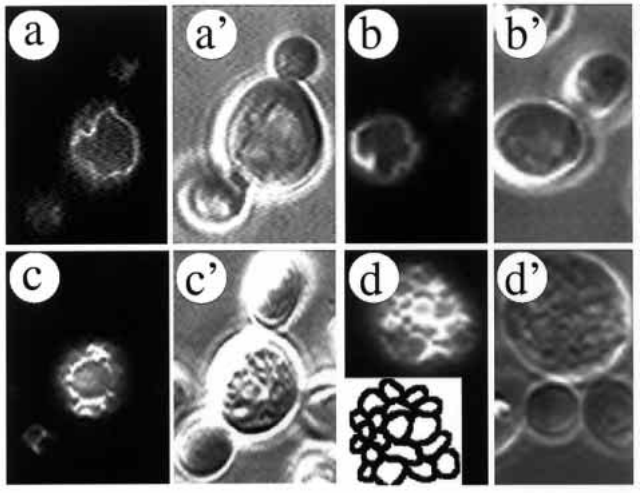

Fig. 8. Localization of Vac8p-GFP fusion protein.Vac8p-GFP was expressed in *vac8Δ* cells (MRY 58) and examined by fluorescence microscopy as described in Materials and Methods. GFP fluorescence pictures are shown in a-d together with the corresponding phase contrast pictures (a′-d′). The GFP-fluorescence pattern in d has been re-drawn for clarity (inset in d).

DISCUSSION

In this study, we describe Vac8p, initially identified as an open reading frame in the yeast genome sequencing project. The core region of the molecule (residues 86-417) consists of at least 8 complete *arm*-repeats, displaying significant homologies to *Xenopus* plakoglobin and other members of the *armadillo* multigene family, whereas no significant homologies to other proteins were found in the N- and C-terminal flanking regions. Therefore, after yeast importin α, also named Srp1p or karyopherin α (Kussel and Frasch, 1995), Vac8p is the second yeast armadillo protein to be characterized. Recently, the same protein was identified by Wang et al. (1998) through complementation of the *vac8-1* mutant which displays a defect in vacuole inheritance.

We were initially interested in Vac8p due to its apparent homology to plakoglobin, a protein involved in both, cell-cell junction formation and junction related signalling in higher eucaryotes (Franke et al., 1989; Gelderloos et al., 1997; Behrens et al., 1997). Sequences with homologies to other cell junction-related structural proteins are not present in the yeast genome, in accordance with the fact that yeast does not form tissues. However, many proteins conserved between yeast and mammals have been shown to play fundamental roles (Botstein et al., 1997) in the regulation of the actin cytoskeleton. Homologous proteins involved in actin organization in yeast and man are for instance, Sac6p/fimbrin (Adams et al., 1989), Cof1p/cofilin (Moon et al., 1993), or bee1p/Wiskott-Aldrich syndrome protein (Li, 1997). Hence, we regarded Vac8p a candidate protein, potentially involved in the interaction of actin with the yeast plasma membrane (Ayscough and Drubin, 1996; Welch et al., 1994).

To elucidate the potential function of Vac8p, we investigated the subcellular localization of the protein and constructed gene

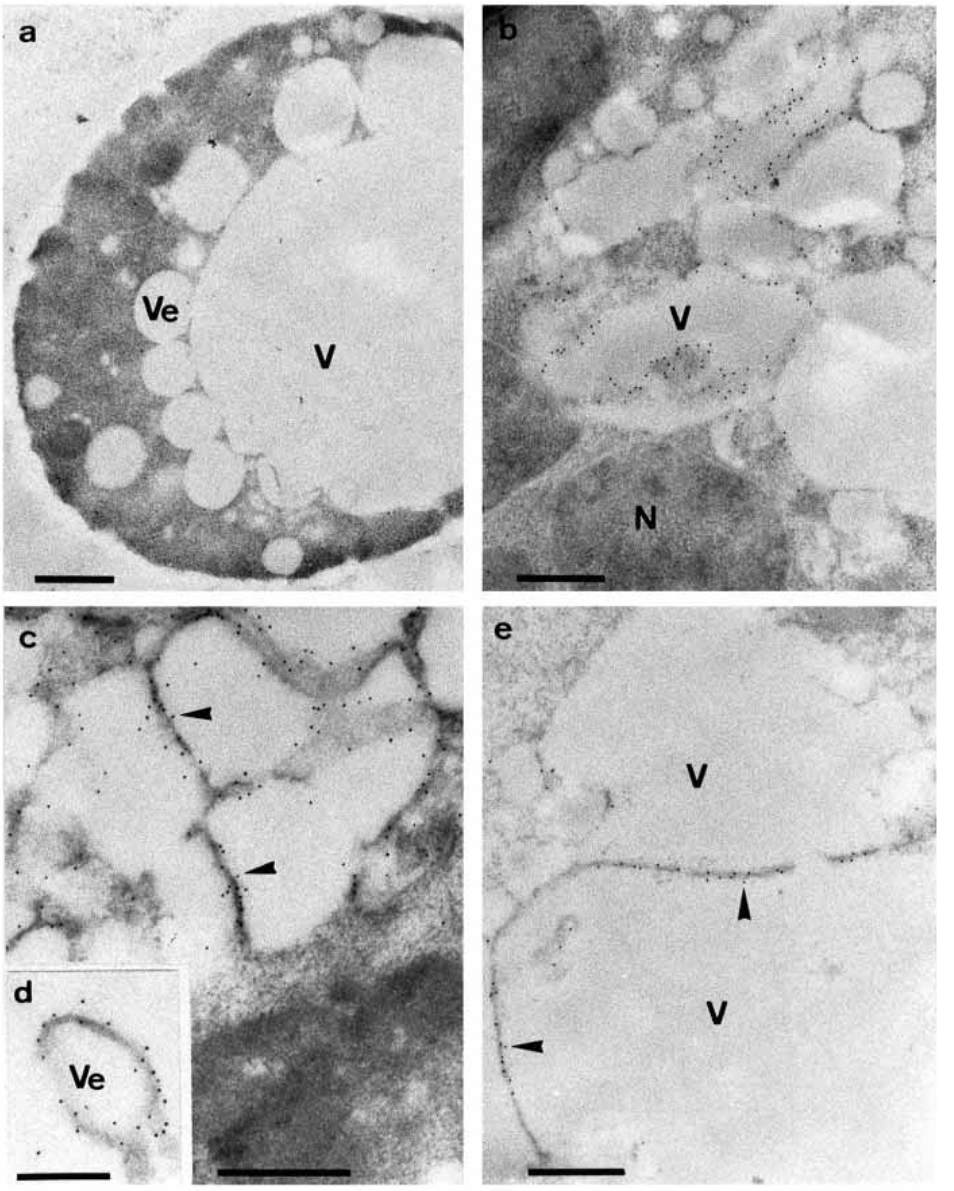

Fig. 9. Immunogold electron microscopy. Cells were immuno-gold labelled using affinity-purified Vac8p directed antibodies. Endogenous Vac8p in the wild-type strain (CEN.PK2) is below the detection level (a). In the CEN.PK2-derived strain overexpressing Vac8p-GFP (MRY64) the protein is detected along vacuolar membranes and vesicles (b-d). Arrowheads point to membrane regions with an apparent enrichment of Vac8p. Vesicles (Ve), vacuoles (V) and the nucleus (N) are marked. Bars: 0.5 μm (a-c,e); 0.25 μm (d).

disruption/deletion mutants and strains overexpressing Vac8p as a GFP-fusion protein, to characterize phenotypic changes. While we did not detect association of Vac8p with the yeast cytoskeleton, nor any changes in the morphology and distribution of actin patches (not shown), we found that both, endogenous Vac8p, as well as exogenously expressed Vac8p-GFP fusion protein associated with the vacuolar membrane. This is in agreement with our finding that endogenous Vac8p codistributes with the vacuolar marker enzyme α-mannosidase in cell fractionation studies on sucrose gradients. Treatment of membrane fractions with proteinase K demonstrated that the protein is exposed on the cytoplasmic face of the vacuolar membrane. The protein is readily extracted from the membranes by Triton X-100, but even vigorous treatment at pH 11 or with 1.5 M salt results in solubilization of only part of the protein. During the review process of this work, this finding was attributed to the fact that Vac8p is myristoylated and palmitoylated and thus lipid-anchored to the membrane (Wang et al., 1998). In accordance with a vacuolar localization, strong effects on vacuolar morphology were seen by light- and immuno-electron microscopy in mutant cells that do not express Vac8p. In such cells the vacuole appeared multilobed or fragmented into small vesicles. Thus, Vac8p appears to be involved in the maintenance of a normal vacuolar morphology.

An analysis of protein delivery to the vacuole revealed that the processing of aminopeptidase I (API), which represents the cytoplasm-to-vacuole targeting (Cvt) pathway is impaired in the *vac8/YelΔ13w* mutants resulting in the accumulation of the cytoplasmic precursor form of API. However, the processing and transport of carboxypeptidase Y, a protein delivered to the vacuole via the secretory pathway, is unaffected. The defect in the Cvt-pathway could be completely repaired with the Vac8p-GFP construct. Immuno-EM revealed that this fusion protein was often enriched around the membrane of vesicles apparently pinching off into the vacuolar lumen and along membranes separating vacuolar vesicles.

The yeast vacuole is a multifunctional equivalent of lysosomes in higher eukaryotes. Its physiological functions comprise hydrolytic breakdown of macromolecules, pH- and osmoregulation, as well as storage of amino acids and polyphosphates (for recent reviews see Klionsky et al., 1990; Van Den Hazel et al., 1996). As discussed by Van Den Hazel et al. (1996), vacuolar proteolysis is of highest importance upon sporulation and generally under nutritional stress conditions. This is corroborated by the observation that homozygous *pep4* diploids, which lack the protease PrA at the top of a vacuole activation cascade for many hydrolases (Ammerer et al., 1986; Woolford et al., 1986), do not sporulate (Zubenko and Jones, 1981). Hence, mutations interfering with the vacuole are likely to influence sporulation. This assumption is supported by our findings: while mating efficiencies of *vac8* mutant cells are identical with the wild-type, sporulation efficiency and the formation of normal asci containing 3-4 spores is about tenfold lower (not shown).

In a screen by Wang et al. (1996) a series of new mutants was described defective in vacuole partitioning and targeted transport of proteins to the vacuole. This work allowed the authors to define three classes of non-*vps* (vacuolar protein-sorting) mutants, based on the vacuole morphology. Class I mutants of this screen are characterized by multilobed vacuoles, which were also seen in our mutant, while Class II is defined by round and Class III by enlarged vacuoles with scission defects. As described recently (Wang et al., 1998), the class I defect of *vac8-1* is complemented by the protein which is the subject of the present study.

Another recent report demonstrated that there is also a close link between the actin cytoskeleton and the vacuole: Mutants in actin, myosin and profilin were found defective in vacuole inheritance (Hill et al., 1996). Wang et al. (1998) showed that Vac8p contained in crude extracts co-sediments with exogenously added F-actin. With this in mind, the homology of Vac8p to plakoglobin, which is associated with cadherin-dependent microfilament anchorage to the plasma membrane in higher eukaryotes, seems appealing and we could show an interaction of purified recombinant Vac8p with purified mouse α-catenin in spot-overlays (not shown). Although α-catenin is not found in yeast, this emphasizes the similarity with plakoglobin and the potential role of Vac8p in vacuole/F-actin interaction, as discussed by Wang et al. (1998).

Further studies will have to address the precise function of Vac8p, to elucidate the molecular mechanisms in vacuole scaffolding, linkage of vacuolar components to the cytoskeleton, and protein targeting to the vacuole.

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