Supplementary material

Vac1p coordinates Rab and phosphatidylinositol 3-kinase signaling in SNARE-dependent vesicle docking/fusion at the endosome

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Supplementary materials and methods

CPY sorting analysis

The construction of *vac1* mutant alleles is described in [S1]. The GDP-locked form of *VPS21*, *vps21* (S21L), was cloned into a yeast CEN expression vector (pRS415). Four suppressor genes, full-length *VPS9*, *PEP12*, *VPS45* and *SEC19* (pRS423), were subcloned into 2μ expression vectors. All CPY-sorting analyses for this study were conducted in the SEY6210 *vac*1Δ::*URA3* yeast strain. Yeast cultures were radiolabeled using previously published procedures [S2]. Immunoprecipitations of CPY were performed using the method of Klionsky *et al*. [S3].

Two-hybrid analysis

The genes indicated in Table 1 were subcloned into either bait (pGBT9) or prey (pGADGH) vectors (Clontech). Constructs were introduced into a reporter strain (HF7c, Clontech), and tested for association by growth on plate media lacking histidine and supplemented by 1 mM 3-amino-2,4-triazole, an inhibitor of the *HIS3* gene product. Binding of bait and prey proteins causes transcription of the *HIS3* gene product and allows for growth on such media. After several days at 30°C, plates were scored for growth and placed into four groups, from +++ to –, depending on growth rate.

Co-precipitation analysis

A mutant form of *VPS21*, either *vps21* (Q66L) or *vps21* (S21L), was subcloned in-frame 3′ to a Protein-A domain in multicopy 2μ vectors (pRS424), and transformed into a wild-type strain (SEY6210). Two Protein-A domains were subcloned into the *Pac*1/*Bst*EII sites at the 3′ end of the full-length *VAC1* gene and the result subcloned into both CEN and multicopy 2μ yeast expression vectors (pRS415 and pRS425, respectively). Cultures containing the Protein-A-tagged constructs were grown to mid-log phase (0.5–0.8 OD_{600}). Approximately 200 OD_{600} units of cells were converted to spheroplasts and resuspended at 20 OD_{600}/ml in 150 mM KOAc, 5 mM MgOAc, 20 mM HEPES pH 8.0 with protease inhibitors. Cells were lysed by dounce homogenization, and lysates were centrifuged for 5 min at 300 × *g*. Digitonin was added to 1%, and lysates were shaken on ice for 10 min, and spun for 10 min at 13,000 × *g*. The supernatants were chromatographed on columns packed with 300 μl IgG–Sepharose (Pharmacia). The columns were washed three times with 1 ml 150 mM KOAc, 5 mM MgOAc, 20 mM HEPES pH 8.0, 0.05% digitonin, and once with 1 ml 5 mM ammonium acetate pH 5.0. Bound proteins were eluted with 1 ml 0.5 M acetic acid pH 3.4. Following TCA precipitation, 50 OD_{600} equivalents of each sample was subjected to SDS–PAGE and western blotting, with antisera raised against Vac1p [S1], Vps9p [S4] and Vps45p [S5]. Enhanced chemiluminescence was used to visualize immunoblots (Amersham). Quantitation of the Vps9p result was performed on a Macintosh computer using the public domain NIH Image program.

References

S1. Burd C, Peterson M, Cowles C, Emr S: A Novel Sec18p/NSF-dependent complex required for Golgi-to-endosome transport in yeast. *Mol Biol Cell* 1997, 8:1089-1104.

S2. Horazdovsky BF, Emr SD: The VPS16 gene product associates with a sedimentable protein complex and is essential for vacuolar protein sorting in yeast. *J Biol Chem* 1993, 268:4953-4962.

S3. Klionsky DJ, Emr SD: Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. *EMBO J* 1989, 8:2241-2250.

S4. Burd CG, Mustol PA, Schu PV, Emr SD: A yeast protein related to a mammalian Ras-binding protein, Vps9p, is required for localization of vacuolar proteins. *Mol Cell Biol* 1996, 16:2369-2377.

S5. Cowles C, Emr S, Horazdovsky B: Mutations in the VPS45 gene, a SEC1 homologue, result in vacuolar protein sorting defects and accumulation of membrane vesicles. *J Cell Sci* 1994, 107:3449-3459.