G-Protein Ligands Inhibit In Vitro Reactions of Vacuole Inheritance

Albert Haas, Barbara Conradt, and William Wickner

Molecular Biology Institute and Department of Biological Chemistry, University of California, Los Angeles, California 90024-1570; and Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755-3844

Abstract. During budding in *Saccharomyces cerevisiae*, maternal vacuole material is delivered into the growing daughter cell via tubular or vesicular structures. One of the late steps in vacuole inheritance is the fusion in the bud of vesicles derived from the maternal vacuole. This process has been reconstituted in vitro and requires isolated vacuoles, a physiological temperature, cytosolic factors, and ATP (Conradt, B., J. Shaw, T. Vida, S. Emr, and W. Wickner. 1992. *J. Cell Biol.* 119:1469–1479). We now report a simple and reliable assay to quantify vacuole-to-vacuole fusion in vitro. This assay is based on the maturation and activation of vacuole membrane-bound pro-alkaline phosphatase by vacuolar proteinase A after vacuole-to-vacuole fusion. In vitro fusion allowed maturation of 30 to 60% of pro-alkaline phosphatase. Vacuoles prepared from a mutant defective in vacuole inheritance in vivo (*vac2-1*) were inactive in this assay. Vacuole fusion in vitro required a vacuole membrane potential. Inhibition by nonhydrolyzable guanosine derivatives, mastoparans, and benzalkonium chloride suggest that GTP-hydrolyzing G proteins may play a key role in the in vitro fusion events.

DIVIDING cells use specific mechanisms to ensure correct inheritance of cytoplasmic organelles. High copy number organelles may be partitioned predominantly by random diffusion (Birky, 1983). Single or low copy number mammalian organelles such as the Golgi apparatus vesiculate, partition during mitosis, and reassemble by fusion after cytokinesis (Lucocq and Warren, 1987; Ho et al., 1989). Mammalian lysosomes are usually clustered in the perinuclear region. During cell division, these organelles disperse, and they recluster at the end of mitosis (Matteoni and Kreis, 1987).

Vacuoles, the functional equivalents in *Saccharomyces cerevisiae* of mammalian lysosomes, are low copy number organelles occupying $\sim$10–20% of the overall cell volume (Gomes de Mesquita et al., 1991; Raymond et al., 1992). Cytological and genetic studies have elucidated some of the basic principles of vacuole inheritance mechanisms in *S. cerevisiae* (Weisman et al., 1987, 1990; Weisman and Wickner, 1988; Gomes de Mesquita et al., 1991; Shaw and Wickner, 1991; Raymond et al., 1992; Weisman and Wickner, 1992). The mother cell vacuole does not fragment, but projects tubular and/or vesicular structures into the bud early in S phase. This transport ceases by the time of G2/M transition. The vesicular structures fuse in the new daughter cell to form one or a few larger vacuoles. Vacuole inheritance is blocked in the mutant *vac2-1*, while traffic from the Golgi apparatus to the vacuole is unimpaired (Shaw and Wickner, 1991). Vesiculation and fusion of vacuoles can also be observed in a cell-free vacuole inheritance reaction (Conradt et al., 1992), allowing biochemical analysis of the structural and regulatory components of the vacuole segregation machinery.

Similar in vitro assays have been developed for reconstituting transport and fusion processes, such as ER-to-Golgi transport (Beckers et al., 1987; Baker et al., 1988), transport through the Golgi stack (Balch et al., 1984), nuclear envelope assembly (Boman et al., 1992), and endosome fusion (Mayorga et al., 1989*a*). Most of these assays are either based on: (*a*) the mixing of luminal contents of such vesicles (measured by glycosylation of reporter proteins); (*b*) the formation of immune complexes (quantified after coprecipitation); or (*c*) the increase in size of fused vesicles (measured microscopically; for a review on these methods see Pryer et al., 1992). These in vitro assays have shown that GTP-hydrolyzing proteins (G-proteins[1]; Bourne et al., 1991) play an important role in intracellular transport. Guanosine 5′-*o*-(3-thiotriphosphate) (GTPγS), a non-hydrolyzable analogue of GTP, prevents G-protein cycling and inhibits transport

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Address all correspondence to W. Wickner, Department of Biochemistry, Dartmouth Medical School, 7200 Vail Building, Hanover, NH 03755-3844.

[1] *Abbreviations used in this paper*: BAC, benzalkonium chloride; CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone; CDCFDA, 5 (and 6-)carboxy-2′,7′-dichlorofluorescein diacetate; cGMP, guanosine 3′:5′-cyclic monophosphate; CPY, carboxypeptidase Y; G-protein, GTP-hydrolyzing protein; GDP, guanosine 5′-diphosphate; GDPβS, guanosine 5′-*o*-(2-thiodiphosphate); GTPβS (R_p-isomer), guanosine 5′-*o*-(3-thiotriphosphate); mas, mastoparan; *p*NPP, *para*-nitrophenyl phosphate; *vps*, vacuole protein sorting.

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from the ER to the Golgi apparatus (Baker et al., 1988; Ruohola et al., 1988; Beckers et al., 1989; Rexach and Schekman, 1991; Gruenberg and Clague, 1992; Pfeffer, 1992), transport within the Golgi stack (Melancon et al., 1987), retrograde transport from the Golgi apparatus to the ER (Tan et al., 1992), secretory vesicle formation (Leyte et al., 1992), endocytosis (Carter et al., 1993), and endosome fusion (Colombo et al., 1992). In yeast, distinct monomeric G-proteins participate in each step of vesicular transport (Sec4p, Sarlp, Yptlp, Arflp, Arf2p; for a review see Pryer et al., 1992).

Heterotrimeric ("large") G-proteins are also involved in vesicular transport in a number of mammalian systems (Stow et al., 1991; Bomsel and Mostov, 1992; Leyte et al., 1992). Mastoparan, a wasp venom 14-mer peptide, was a very useful tool in these studies. This peptide stimulates the exchange of GTP for guanosine 5′-diphosphate (GDP) on some α-subunits of heterotrimeric G-proteins by binding to their COOH termini and mimicking an agonist-activated receptor (Higashijima et al., 1988, 1990; Mousli et al., 1990; Weingarten et al., 1990; Tomita et al., 1991; Oppi et al., 1992). Thus, binding of mastoparan constitutively stimulates these heterotrimeric G-proteins, inhibiting in vitro ER-to-Golgi transport (Schwaninger et al., 1992), endocytosis (Carter et al., 1993), endosome fusion (Colombo et al., 1992), exocytosis in chromaffin cells (Vitale et al., 1993), and apical transport in epithelial cells (Pimplikar and Simons, 1993; for a recent review see Bomsel and Mostov, 1992). Mastoparans have not yet been used to study trafficking processes in yeast.

Rapid, quantitative assays are needed for the purification of proteins involved in vacuole inheritance and to define reagents which interfere with the underlying processes (e.g., G-protein inhibitors). We now report such an in vitro assay for at least some of the late steps in vacuole inheritance, vacuole-to-vacuole fusion. This assay has been used to identify reagents which interfere with inter-vacuole fusion and to stage the fusion reaction (see accompanying paper; Conradt et al., 1994).

Materials and Methods

Yeast Strains and Media

S. cerevisiae K91-1A (unpublished data) *MAT a pho8::pAL134 phol3::pPH13 ura3 lysl* was a generous gift of Dr. Y. Kaneko (Institute for Fermentation, Osaka, Japan). Further *S. cerevisiae* strains used were: BJ3505 (*MATa pep4::HIS3 prbl-Δl.6R HIS3 lys2-208 trpl-Δ101 ura3-52 gal2 canl*; Jones et al., 1982), DKY6281 (*MATα leu2-3 leu2-112 ura3-52 his3-Δ200 trpl-Δ901 lys2-801 suc2-Δ9 pho8::TRPl*; kindly supplied by Dr. D. Klionsky, University of California, Davis, CA), ABYS1 (*MATα pral prbl prcl cpsl ade*; Achstetter et al., 1984), GPY449 (*MATα leu2-3,112 ura 3-52 his4 trpl canl^R PEP4::LEU2*), JSY114 (*VACl$^+$ vac2-1 ade2-201 ura3-52 his 4-519 leu2-112 leu2-3 PEP4::URA3*), JSY115 (*VACl$^+$ vac2-1 ade2-201 ura3-52 his 4-519 leu2-112 leu2-3 PHO8::LEU2*), JSY116 (*MATa leu2-3,112 ura3-52 his4 trpl canlR PHO8::LEU2*) (JSY114, 115, and 116 were from Dr. J. Shaw, University of Utah, Salt Lake City, UT). YEPD (20 g bactopeptone, 10 g yeast extract [Difco Laboratories, Inc., Detroit, MI], 20 g dextrose; each per liter) was used as a rich growth medium in all experiments.

Materials

TriX-pan 400 and TMAXp3200 films were used for microscopic photography. Samples were loaded in 6 mm wells of immunofluorescence slides (teflon-coated; Polyscience, Niles, IL) and stained with 5 (and 6-) carboxy-2′,7′-dichlorofluorescein diacetate (CDCFDA) from Molecular Probes Inc. (Eugene, OR). Oxalyticase was from Enzogenetics (Corvallis, OR), DEAE dextran, Ficoll 400, ATP, and GTP from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), creatine phosphokinase, creatine phosphate, and guanosine 5′-*o*-(2-thiotriphosphate), (R_p-isomer) (GTPβS [R_p-isomer]) were from Boehringer Mannheim Corp. (Indianapolis, IN), and the ECL-chemiluminescence development kit from Amersham Corp. (Arlington Heights, IL). Protein concentrations were determined using the Bio-Rad protein assay reagent kit (Richmond, CA) using bovine serum albumin as a standard. Sigma Chemical Co. (St. Louis, MO) provided GTPγS, guanosine 5′-*o*-(z-thiophosphate) (GDPβS), benzalkonium chloride (C_{12-14}), neomycin sulfate, *p*-nitrophenyl phosphate (*p*NPP)/TRIS salt, Triton X-100, DTT, carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), and PMSF. Bafilomycin A_1 was kindly provided by Dr. K. Altendorf (Universität Osnabrück, Osnabrück, Germany). The anti-PHO8 serum used in Western blot analysis was kindly provided by Dr. T. Stevens (University of Oregon, Eugene, OR).

Mastoparans

Highly pure mastoparans (mas) (>95%) were purchased either from Peninsula Laboratories Inc. (Belmont, CA; mas, INLKALAALAKKIL-NH_2; mas X, INWKGIAAMAKKLL-NH_2; masl7, INLKALAALAKALL-NH_2), or were custom synthesized and prepurified (>90%) by BioSynthesis Inc. (Lewisville, TX; mas-PEP2, LKIALNLKALIAAK-NH_2), or were generous gifts from Dr. Tsutomu Higashijima (University of Texas, Dallas, TX; mas7[Glu4;Glu11], INLQALALAALAQALL-NH_2; purity >98%) or from Dr. Cristina Oppi (Istituto Guido Donegani, Rome, Italy; mas-PEP7, LNAKLKAIALALIK-NH_2; mas-PEP8, NILALAKALIKALK-NH_2).

Preparation of Soluble Yeast Fraction (Cytosol)

Cytosol was prepared on a small scale according to Conradt et al. (1992). Large scale preparations were done from fermenter material, using K91-1A cells (1 kg wet weight) grown overnight to an OD_{600} of 4.0–5.0 in YPD (25 mg/l kanamycin sulfate) at 30°C. The cells were suspended in 2 liters of ice cold water, centrifuged for 5 min at 5,000 *g*, resuspended in 150 ml of lysis buffer (0.3 M sorbitol, 150 mM KAc, 20 mM Pipes/KOH, pH 6.8, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF), and disrupted batchwise in a "beat beater" (Biospec Products, Bartlesville, OK; 50% (vol/vol) resuspended cells, 50% (vol/vol) chilled acid-washed glass beads, 0.5-mm diam) by consecutive cycles of 30-s disruption and 1-min cooling on ice. The combined lysates were spun at 8,000 rpm in a JA-14 rotor (Beckman Instruments, Palo Alto, CA) for 20 min at 4°C. The supernatants were combined and spun in a Ti60 rotor (Beckman Instruments) at 58,000 rpm for 120 min. The resulting supernatants (140 ml, 40–50 mg/ml) were collected, aliquoted into microfuge tubes, frozen in liquid nitrogen, and stored at −70°C.

Isolation of Vacuoles

Vacuoles were isolated as described in Conradt et al. (1992), except that $MgCl_2$ was omitted before flotation. Each vacuole preparation was analyzed microscopically and consisted of nonclustered vacuoles, and some lipid bodies which in yeast are typically associated with vacuoles.

In Vitro Analyses with Isolated Vacuoles

The protein concentration was determined from the vacuole samples isolated daily from the strains BJ3505 and DKY6281, as described above. The vacuole preparations were adjusted with 0% Ficoll buffer (10 mM Pipes/KOH, pH 6.8, 0.2 M sorbitol) to 0.25 mg protein/ml. Standard reactions in 1.5 ml microfuge tubes contained 10 μl of each of the two vacuole preparations (0.25 mg/ml), 2.2 μl of 10× salt buffer (100 mM Pipes/KOH, pH 6.8, 1 M sorbitol, 50 mM $MgCl_2$, 1 M KCl, 0.5 M KOAc), 3.5 μl of a 10× ATP-regenerating system (400 mM creatine phosphate, 20 mg/ml creatine phosphokinase, 10 mM MgATP), 1× reaction buffer (20 mM Pipes/KOH, pH 6.8, 0.3 M sorbitol, 100 mM KCl, 50 mM KOAc, 5 mM $MgCl_2$), and cytosol (2.2 mg protein/ml). The reaction mixtures were adjusted to a total volume of 35 μl using 1× reaction buffer and incubated at 25°C for 120 min (or as indicated). After completion of the reaction, the tubes were prewarmed at 30°C for 2 min and an alkaline phosphatase activity assay was performed (Mitchell et al., 1981). 465-μl assay reaction solution (250 mM Tris/Cl, pH 8.0, 0.4% Triton X-100, 10 mM $MgCl_2$, 1 mM *p*NPP) was added to each sample. The samples were incubated for 5 min at 30°C, the reactions terminated by the addition of 500 μl 1 M glycine/KOH, pH 11.0, and the A_{400} of the samples (0–0.8) were determined and corrected for the background (8–10%) due to separate incubations of vacuoles and cytosol. 1 U of activity was defined as the production of 1 μMol *p*-nitrophenol/min/μg BJ3505-vacuolar protein (vacuoles lacking proteinase A but containing proPHO8) or *vac2pep4* vacuoles, respectively (see Fig. 4).

When cytosolic preparations from strains other than K91-1A were used, the cytosolic alkaline phosphatase activities (particularly of the *PHO13* gene product; Kaneko et al., 1989) had to be removed prior to the alkaline phosphatase activity determination. This was accomplished by two sedimentation steps after fusion reactions were completed. Samples were placed on ice, and 300 μl of ice-cold "washing" buffer (40 mM Tris-Cl, pH 8.0) was added. The samples were shaken on a vortex mixer at highest setting for 15 s, chilled on ice, and shaken for another 15 s. Membranous material was sedimented in a microfuge at 4°C and 14,000 rpm for 15 min. Pellets were resuspended in 300 μl of ice-cold washing buffer and the vortex steps were repeated. After another centrifugation at 14,000 rpm for 15 min, the supernatants were discarded and the pellets resuspended in 35 μl 1× reaction buffer including 2% Triton X-100 (see above). Phosphatase activity was assayed as above.

To separate vacuoles from added reagents, a short microfuge centrifugation was performed (45 to 90 s at 10,000 rpm, 4°C), the supernatant was discarded, and the pellet gently resuspended in 1× reaction buffer.

To reduce ATP-dependent, cytosol-independent fusion background, vacuoles from *S. cerevisiae* (BJ3505 and DKY6281, mixed 1:1, 0.25 mg protein/ml) were preincubated (where indicated) in a 200 μl volume with 1× reaction buffer at 30°C for 10 min, and then collected by a microfuge spin at 10,000 rpm for 80 s. The supernatant was discarded, the pellet gently resuspended in 1× reaction buffer (final concentration 0.23 mg protein/ml) and used in fusion reactions.

For microscopic in vitro assays, 1 μl CDCFDA (from a 1 mM CDCFDA [10% DMSO] stock solution) was added per standard reaction. Small aliquots were removed at the indicated time and analyzed by fluorescence microscopy (see Materials and Methods; and Conradt et al., 1992).

Immunoblot analysis of proPHO8 maturation was performed as previously described for proCPY maturation (Conradt et al., 1992). The polyclonal antiserum was used at a 1:5,000 dilution. 2× sample buffer contained 2% SDS, 50 mM Tris-Cl (pH 6.8), 10% glycerol, and 0.01% bromphenol blue.

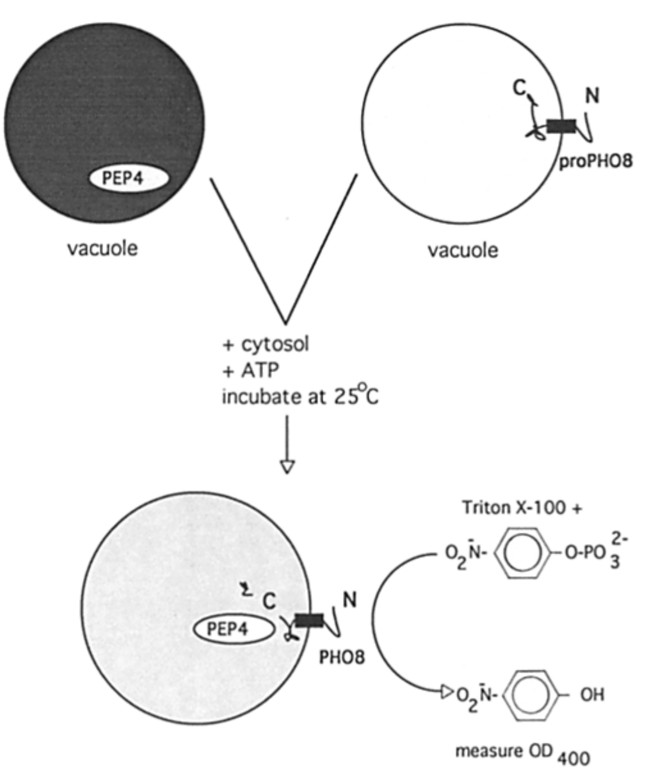


Figure 1. Scheme of the quantitating vacuole-to-vacuole fusion assay. For details see text.

Results

Outline of the In Vitro Fusion-Quantification Assay

Vacuoles of *S. cerevisiae* possess a membrane-bound alkaline phosphatase (*PHO8* gene product) with its catalytic domain oriented into the vacuole lumen (Kaneko et al., 1987; Klionsky and Emr, 1989; Klionsky et al., 1990). Activation of PHO8 enzymatic activity requires functional vacuolar proteinase A (*PEP4* gene product; Klionsky et al., 1990) which cleaves the COOH terminus of proPHO8 (Ammerer et al., 1986; Klionsky and Emr, 1989). Thus, vacuoles lacking either PEP4 or proPHO8 have only marginal alkaline phosphatase activity (Jones et al., 1982; Klionsky and Emr, 1989).

In the in vitro vacuole-to-vacuole fusion reactions (Fig. 1), vacuoles isolated from *pho8* and *pep4* strains were incubated at 25°C with cytosol, ATP, and an ATP-regenerating system. Under these conditions, the mixing of vacuolar membranes and luminal contents during and after fusion (Conradt et al., 1992) brings the proPHO8 substrate and PEP4 protease together, which results in the maturation of proPHO8 (apparent M_r 76,000) to its enzymatically active form (apparent M_r 72,000; Klionsky and Emr, 1989; Raymond et al., 1992). After solubilization of the vacuolar membrane, alkaline phosphatase activity is quantified spectrophotometrically using *p*NPP as a chromogenic substrate. To avoid a high background from cytosolic phosphatases, a double deletion mutant in *PHO8* and *PHO13* (the latter encoding a cytosolic *p*NPP-specific phosphatase; Kaneko et al., 1989) was used as a source of cytosol. Only trace alkaline phosphatase activities were detected in such preparations.

Maturation of proPHO8 Reflects Inter-vacuole Fusion

The average vacuole diameter, determined by fluorescence microscopy, increased during incubation in a standard reaction (Fig. 2 *A*; Conradt et al., 1992). This increase in size was correlated with increasing PHO8 maturation (Fig. 2 *B*) and a concurrent increase in alkaline phosphatase activity (Fig. 2 *C*). These results indicated that vacuole fusion occurs in our in vitro reaction.

ProPHO8 maturation requires physiological temperature, cytosol, ATP, and both types of vacuoles (Fig. 3; also Conradt et al., 1992). The small background of cytosol-independent, ATP-dependent proPHO8 maturation (Fig. 3, lane *3*) could be removed by incubating both vacuole types together in 1× reaction buffer (10 min, 30°C) with a subsequent reisolation of vacuoles by sedimentation (data not shown). We suggest that this treatment removed fusion-promoting (cytosolic) factors which had still been bound to the vacuoles. Trypsin-treated cytosol did not promote fusion in vitro, and no fusion could be observed in standard fusion reactions to which Triton X-100 (0.01%) was added, or when vacuoles had been pretreated in an ultrasonic waterbath (Conradt et al., 1992, data not shown), indicating that maturation of proPHO8 occurred only in intact vacuoles.

The VAC2 Gene Product Is Required for In Vitro Vacuole-to-Vacuole Fusion

Vac2-1 is a temperature-sensitive mutant which fails to deliver vacuolar material to the daughter cell in vivo at the nonpermissive temperature but which seems unaffected in the

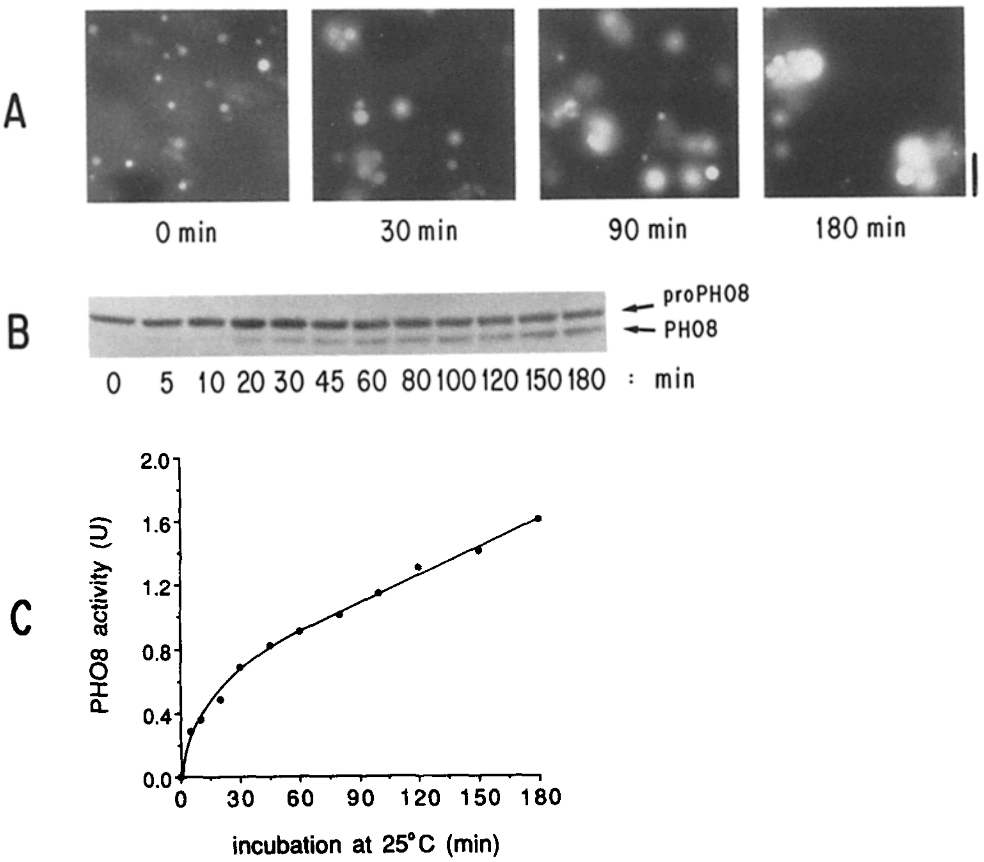
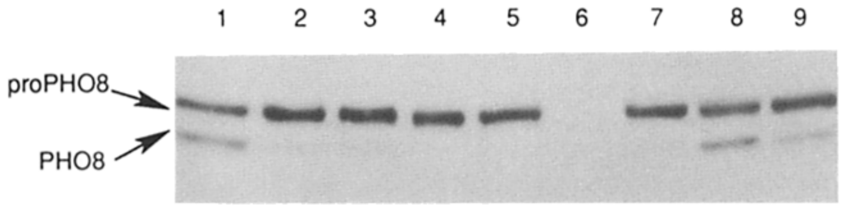


Figure 2. Kinetics of in vitro vacuole-to-vacuole fusion. All data stem from the same experiment. Isolated vacuoles were incubated in a large scale fusion assay which consisted of a multiple volume of the standard assay. Samples in 12 ml Falcon tubes containing the reaction mixture without added ATP were transferred from ice to 25°C and prewarmed for 90 s. ATP and its regenerating system were added, the tube was gently shaken, and immediately the "0-min samples" were removed. At the indicated timepoints, aliquots were removed and microphotographed (*A*) or placed on ice (*B* and *C*). (*A*) Vacuoles were labeled with the specifically vacuole-staining reagent, CDCFDA (see Materials and Methods). (*B*) Anti-PHO8 Western blot. After completion of the reaction (*180 min*), one volume (35 μl) 2× sample buffer was added to the samples and the samples were heated for 2 min at 95°C. One third of each sample (for each timepoint) was separated in a 10% acrylamide/SDS gel, the sample proteins were electrotransferred onto PVDF membranes (Bio-Rad Laboratories) and probed with an anti-PHO8 serum. (*C*) After completion of the reaction, duplicate samples (35-μl each) were analyzed for alkaline phosphatase activities. Bar, 2.9 μm.

inheritance of other organelles (Shaw and Wickner, 1991). *Vac2-1*-derived vacuoles fragment in vitro and fail to fuse during even prolonged incubation in the presence of ATP and cytosol from wild-type parent strains (Conradt et al., 1992). In an alkaline phosphatase maturation assay with vacuoles isolated from *vac2-1 pep4* and *vac2-1 pho8*, respectively, together with cytosol from a wild-type strain, virtually no fusion could be detected. Vacuoles isolated from the respective parent strains, however, fuse normally under these conditions (Fig. 4). When *vac2-1 pho8*- or *vac2-1 pep4*-derived



	1	2	3	4	5	6	7	8	9
Cytosol	+	+	-	-	+	+	+	+	+
ATP	+	-	+	-	+	+	+	+	+
BJ3505 vacuoles	+	+	+	+	+	-	+	+	+
DKY6281 vacuoles	+	+	+	+	-	+	+	+	+
Mastoparan (μM)	-	-	-	-	-	-	-	2.0	12.0
Temperature	25°C						0°C	25°C	
Alkaline phosphatase (U)	1.87	0.26	0.46	0.09	0.13	0.09	0.53	1.54	0.98

Figure 3. Requirements for alkaline phosphatase maturation in vitro. Standard reactions with all components (lane *1*), or lacking components (lanes *2*-*6*), or placed on ice (lane *7*), or with added inhibitor (lanes *8* and *9*) were incubated in triplicate. For each sample type, alkaline phosphatase activities were determined from duplicates (the mean of which is shown here). The remaining samples were used for Western blot-analysis as in *B*. The alkaline phosphatase activities shown in this figure have not been corrected for the enzymatic activity background produced by the endogenous alkaline phosphatase activities of cytosol and vacuoles (0.07 U).

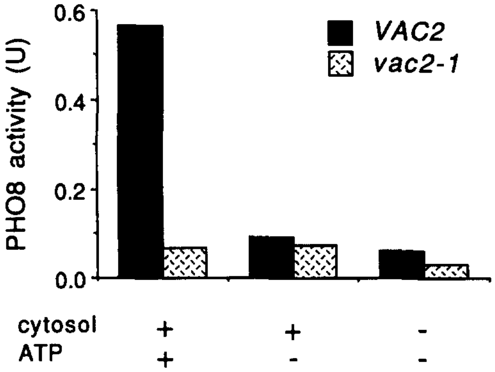


Figure 4. Vacuoles isolated from *vac2-1* cells do not fuse. Vacuoles were isolated from *vac2-1* mutant cells and parent strain cells (*VAC2*) grown at 23°C. *vac2-1/pho8* and *VAC2/pho8*-derived vacuoles were incubated with *vac2-1/pep4* and *VAC2/pep4*-derived vacuoles, respectively. The final volume per sample was 60 μl, vacuoles were present at a final concentration of 0.09 mg protein/ml, cytosol from *S. cerevisiae* ABYS1 was added to a final concentration of 2.0 mg protein/ml, and an ATP-regenerating system was used (same composition as in the standard assay). After completion of the reaction (120 min, 23°C), ABYS1-cytosol was removed from the vacuoles by a spin in a microfuge (15 min, 14,000 rpm, 4°C). The resulting vacuole membrane pellets were resuspended in 35 μl of a 1× reaction buffer/1% (wt/vol) Triton X-100 solution (per sample) and the alkaline phosphatase activities were determined as in the standard reaction. The alkaline phosphatase activities shown here refer to a buffer blank (the background of alkaline phosphatase activity intrinsic to wild-type vacuoles and cytosol is 0.12 U).

vacuoles were mixed with vacuoles from *VAC2 pep4* or *VAC2 pho8*, respectively, intermediate fusion activities were measured (data not shown). This defect in fusion of *vac2-1*-derived vacuoles was even pronounced at a permissive temperature which allows the formation of segregation structures in semi-intact cells (Conradt et al., 1992). Since wild-type cytosol was used in these experiments, the *vac2-1* defect must be associated wih the vacuole itself. We conclude that our in vitro fusion reactions reflect physiological vacuole-to-vacuole fusion.

Inhibitors of In Vitro Vacuole Fusion Reactions

Neomycin is an aminoglycoside antibiotic which is known to bind strongly to phosphatidylinositol-polyphosphates, particularly to the diphosphate form (Lodhi et al., 1979; Prentki et al., 1986) preventing their further metabolism (Marche et al., 1983; Carney et al., 1985; Gabev et al., 1985). Neomycin blocks inter-vacuole fusion in our assay at a relatively low concentration (0.2 mM; Fig. 5 *A*). Both CCCP, which uncouples the proton gradient across vacuolar membranes (Anraku et al., 1989) and bafilomycin A_1, which inhibits the generation of such a proton gradient (Bowman et al., 1989; Klionsky et al., 1990) also inhibited vacuole fusion in vitro (Fig. 5, *B* and *C*). Microscopy confirmed that these inhibitory effects correlated with a significant decrease in fusion frequency (Fig. 6, *D* and *E*). Vacuoles in a standard fusion reaction increased in size in an ATP-dependent manner (Fig. 6 *A*) relative to a control reaction with Pipes/KOH as the sole salt (Fig. 6 *B*), while addition of neomycin, bafilomycin A_1, or CCCP blocked vacuole fusion but allowed vacuole aggregation (Fig. 6, *C–E*). None of these compounds inhibited catalysis by mature alkaline phosphatase, as determined by measuring proPHO8-maturation in the presence of these compounds by immunoblot analysis and comparison with the data obtained in the alklaine phosphatase activity measurements.

In Vitro Vacuole-to-Vacuole Fusion Is Inhibited by Non-hydrolyzable Guanosine Nucleotides

GTPγS reduced proPHO8 maturation by 50% at 0.4 mM, and by 90% (maximal inhibition) at 1.5 mM (Table I). Microscopically, vacuoles were observed to cluster in the presence of GTPγS, but fusion was largely inhibited (Fig. 6 *F*). Nonhydrolyzable GTPβS and GDPβS also clearly inhibited the reaction (Table I). Neither hydrolyzable GTP, GDP, nor guanosine 3';5'-cyclic monophosphate (cGMP) affected the reaction at the concentrations tested (Table I).

G-protein-activating Mastoparan Inhibits In Vitro Vacuole Fusion and Is Antagonized by BAC

Mastoparan, a 14-amino acyl wasp venom peptide (Hirai et al., 1979), activates some heterotrimeric G-proteins by stimulating a GTP-for-GDP exchange on their α-subunits (Higashijima et al., 1988, 1990; Weingarten et al., 1990). In the presence of mastoparan the in vitro fusion reaction was inhibited (see Fig. 8 *A*). The mastoparan derivatives masX and mas7 (Higashijima et al., 1990) and synthetic mastoparan-derived peptide 8 (Oppi et al., 1992), previously shown to also be potent G-protein activators, inhibited inter-vacuole fusion with approximately the same efficiency as mastoparan. The synthetic mastoparan-derivative mas17 (Higashijima et al., 1990) and the mastoparan-derived peptides 2 and 7 (Oppi et al., 1992; data not shown for peptide 7), all of which do not stimulate G-proteins in vitro, inhibited fusion slightly and then only at high concentrations

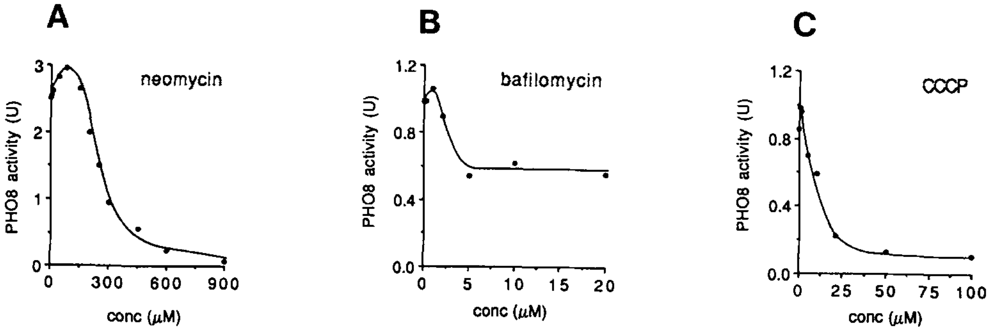


Figure 5. Effects of some reagents on the in vitro vacuole-to-vacuole fusion frequency. Neomycin (*A*), bafilomycin A_1 (*B*), and CCCP (*C*) were added to standard reactions at the indicated final concentrations. Fusion was performed and analyzed as indicated for the standard assay in the Materials and Methods section.

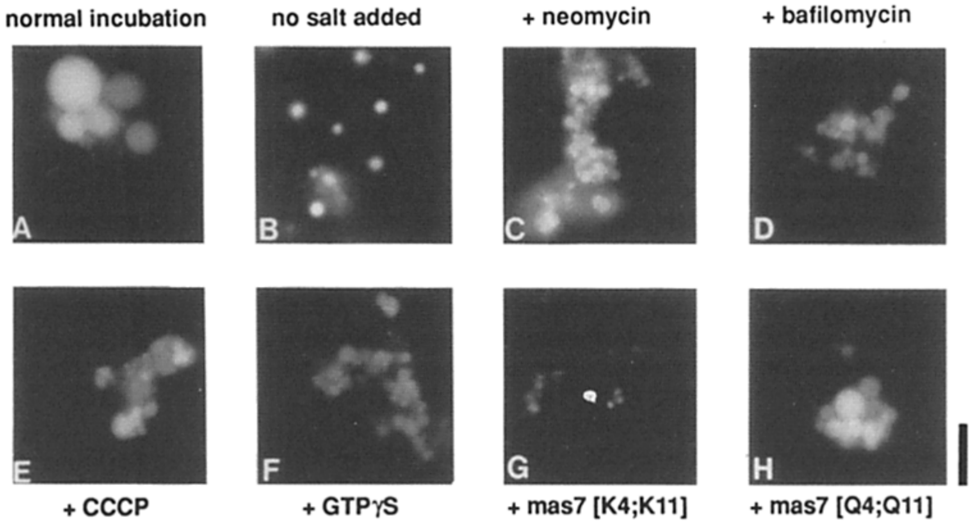


Figure 6. Microphotographs of standard fusion samples containing various inhibitors of the fusion reactions. Vacuoles were labeled with CDCFDA and standard vacuole fusion reactions were incubated for 120 min as described in the Materials and Methods section. (*A*) Standard fusion reaction, (*B*) fusion reaction lacking KCl, KOAc, and $MgCl_2$, but containing all other components, (*C*) neomycin (0.6 mM), (*D*) bafilomycin A_1 (20 μM), (*E*) CCCP (20 μM), (*F*) GTPγS (1.0 mM), (*G*) mastoparan 7 (20 μM), (*H*) mastoparan 7 [Q4;Q11] (20 μM). Bar, 2.9 μm.

(Fig. 7 *A*). Mas7[Q4;Q11] is a mas7 derivative in which two lysyl residues have been replaced by glutamyl residues. This derivative forms an α-helix upon binding to membranes and is as potent as mas7 in its membrane-binding properties; however, it does not stimulate G-proteins (Higashijima, T., personal communication). Mas7[Q4;Q11] did not inhibit inter-vacuole fusion at concentrations up to 50 μM, but did inhibit at a very high concentration (100 μM; Fig. 7 *A*). In summary, these data indicate that the inhibition of fusion by mas7 (at 20 μM) is probably not due to membrane-destabilizing effects. To determine whether the effects of mastoparans are still due to their interactions with vacuole membranes, vacuoles were briefly preincubated with various concentrations of mastoparan and reisolated by centrifugation. These vacuoles were inhibited for fusion (Fig. 7 *B*). Preincubation of cytosol with up to 100 μM mastoparan (10 min, 25°C) did not significantly influence its fusiogenic activity (Fig. 7 *C*), supporting the suggestion that mastoparans act on the vacuole membrane rather than on a cytosolic factor.

Table I. Effects of Various Guanosine Nucleotides on the In Vitro Fusion Reactions

Reagent	Final concentration	Percent inhibition (SD)
	mM	
GTPγS	0.2	33 (5.1)
	0.4	53 (3.6)
	0.8	69 (2.1)
	1.5	89 (3.6)
GDPβS	0.5	27 (10)
	1.0	39 (18)
	2.0	61 (15)
GTPβS (R_p-isomer)	0.5	23 (5.9)
	1.0	42 (2.6)
	2.0	61 (6.7)
GTP	1.0	10 (13)
	2.0	14 (13)
GDP	1.0	−3 (10)
	2.0	6 (8.2)
cGMP	2.0	9 (4.4)

The vacuoles used in these experiments had been subjected to a preincubation step (see Materials and Methods) to reduce ATP-dependent, cytosol-independent fusion. Each reagent was tested in duplicate in all experiments. Lithium, which is present as a counterion in most GTP-derivative preparations, did not influence the fusion reactions. Data from three to five independent experiments were used to calculate each standard deviation.

Mastoparan-mediated inhibition of alkaline phosphatase activity was not due to a direct inhibition in the alkaline phosphatase catalytic activity (Fig. 3, lanes *8* and *9*; this feature has also been confirmed for all other inhibitors used in this study; data not shown). Furthermore, in a standard reaction, fusion-inhibiting mastoparans caused vacuole fragmentation (Fig. 6 *G*), whereas Mas7[Q4;Q11] did not (Fig. 6 *H*). This "fragmentation" might be interpreted as a constitutive formation of segregation structures, forming vesicles which do not have the ability to fuse. In summary, those mastoparan derivatives which activate G-proteins inhibited fusion, while those homologs which do not influence G-protein activity did not influence vacuole fusion.

Benzalkonium chloride (BAC) is a hydrophobic quarternary amine which stimulates GTPase activity and nucleotide exchange of purified G_i and which antagonizes the stimulatory effect of mastoparan on the GTPase activity of purified G_o (Higashijima et al., 1990; Mousli et al., 1990; Fischer et al., 1993). By this action, it reverses the effects of mastoparan on exocytosis in chromaffin cells (Vitale et al., 1993) and the effects of other cationic compounds on histamine release from mast cells (Read and Kiefer, 1979; Mousli et al., 1990). BAC itself inhibited inter-vacuolar fusion (Fig. 8) with maximal effects at 10 μg/ml. Strikingly, BAC antagonized the inhibition of vacuole-to-vacuole fusion caused by mastoparan (at a concentration of 20 μM), again with a maximal effect seen at 10 μg/ml. The same BAC concentration is needed to reverse mastoparan effect on purified G_o proteins (Higashijima et al., 1990). Triton X-100 was used as a detergent control for BAC (Read and Kiefer, 1979). It did not inhibit vacuole fusion, even at the highest concentration tested (30 μg/ml), and did not influence the effects of mastoparan on fusion (Fig. 8).

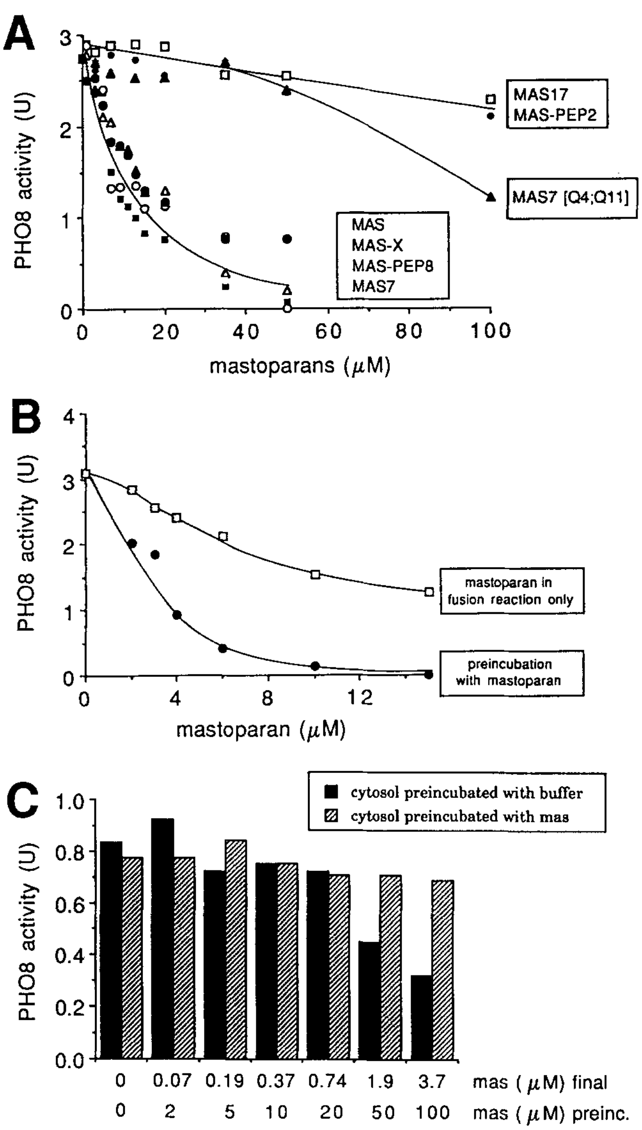


Figure 7. Effects of various mastoparan derivatives on the in vitro vacuole-to-vacuole fusion efficiency. (*A*) Effects on fusion by addition to the standard reaction of mastoparan-peptide 2 (MAS-PEP2, closed small circules), mastoparan 17 (MAS17, □), mastoparan (MAS, open triangles), mastoparan X (MAS X, ■), and mastoparan-peptide 8 (MAS-PEP8, ○), mastoparan 7 (MAS7, ●) and the mastoparan 7 derivative, MAS7[Q7;Q11] (▲). For the amino acid sequences of the mastoparan derivatives, see the Materials and Methods. (*B*) Preincubations of vacuoles with mastoparan before the fusion assay. Vacuoles from strains BJ3505 and DKY6281 were diluted to 0.25 mg/ml protein with vacuole isolation buffer (250 mM sorbitol, 10 mM Pipes/KOH, pH 6.8). These preparations were mixed and 1/10 final volume of 10× reaction buffer (see Materials and Methods), and various mastoparan concentrations in 1× reaction buffer were added. The mixtures were transferred to a 25°C waterbath for 5 min, placed again on ice, and then centrifuged (microfuge, 80 s, 10,000 rpm, 4°C). Supernatants were discarded and the pellets gently resuspended in reaction buffer and used in standard fusion reactions (●). All vacuolar protein was recovered in the pellet under these conditions and the resuspended vacuoles were not fragmented (as judged by microscopy). In parallel, vacuoles were pretreated as above, but with reaction buffer only. To the latter samples only, mastoparan was added to the indicated final concentrations in standard fusion reactions (□). (*C*) Cytosol was preincubated with various concentrations of mastoparan (0 to 100 μM, in 1× reaction buffer) at 25°C for 10 min. Cytosol was diluted 26.5-fold into standard reactions (▨). In parallel, cytosol was preincubated with reaction buffer only and added to standard reactions which received the same amount of mastoparan that was introduced by mastoparan-preincubated cytosol into the other samples (the final concentrations in fusion reactions were between 0 and 3.8 μM; ■). All data are averages of two parallel fusion reactions and the "0" data again represent two parallel cytosol preincubations with reaction buffer. Fusion was performed for 120 min at 25°C and alkaline phosphatase activities were determined as described in Materials and Methods.

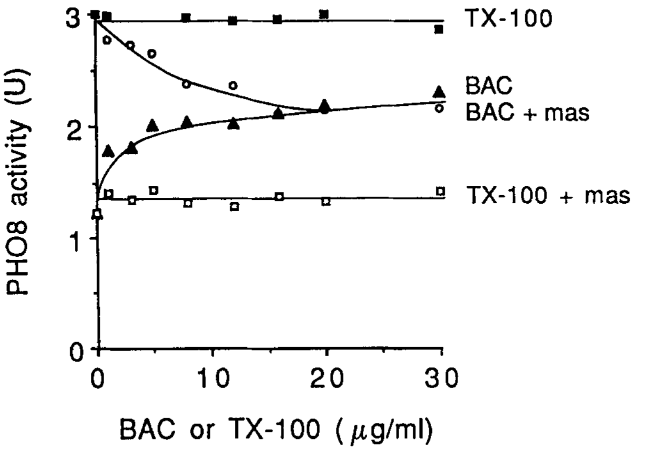


Figure 8. Effects of mastoparan and BAC on inter-vacuole fusion. Standard fusion assays were incubated with the indicated concentrations (μg/ml) of BAC or Triton X-100 alone (○ and ■, respectively), or with the indicated concentration of BAC plus 20 μM mastoparan (▲), or Triton X-100 plus 20 μM mastoparan (□).

Discussion

The choice of proPHO8 as the substrate protein for maturation by PEP4 after vacuole fusion has several important advantages over, e.g., procarboxypeptidase Y: (*a*) proPHO8 is a membrane protein (Klionsky and Emr, 1989) and thus can be isolated with vacuole membranes, even under conditions which lead to vacuole lysis. Vacuole membrane isolation after in vitro fusion allows cytosol peparations from various yeast mutants (e.g., *vac*, *vps*) to be used in this assay system (i.e., from cells with high levels of cytosolic alkaline phosphatase activities). (*b*) PHO8 is the only known vacuole membrane protein whose enzymatic activity is dependent on cleavage by PEP4 (Klionsky et al., 1990). (*c*) Cytosol can be prepared from a mutant yeast strain with very low alkaline phosphatase activity, making cytosol removal unnecessary after standard fusion reactions (see Materials and Methods section). (*d*) ProPHO8 and other vacuole membrane proteins are delivered to the vacuole in vivo by transport pathways which are different from those for the vacuole luminal proteins carboxypeptidase Y (CPY), PEP4, or proteinase B (Klionsky and Emr, 1989). Thus, proPHO8-transport is insensitive to numerous vacuole protein sorting (*vps*) mutations. In contrast, transport of CPY or proteinase B is *VPS* dependent (Klionsky and Emr, 1989; Raymond et al., 1992). As most of the vacuole biogenesis mutants mislocalize CPY to the cell surface (Klionsky et al., 1990; Weisman and

Wickner, 1991), proPHO8 is more likely to be properly localized to the vacuole and thus available for the in vitro fusion assay for each newly identified vacuole segregation mutant: (*e*) transport of proPHO8 from the Golgi apparatus to the vacuole and proPHO8 maturation are not strictly dependent upon vacuole acidification (Klionsky and Emr, 1989; Klionsky et al., 1990). This allows separate studies of the roles of acidification and of an electrochemical potential in vacuole fusion in vitro (Klionsky et al., 1990).

By using a vacuole inheritance mutant (*vac2-1*), we have shown that the in vitro reactions presented here reflect in vivo inheritance processes. Furthermore, another nonallelic vacuole inheritance mutant (*vac5*) is also defective in vacuole-to-vacuole fusion in vitro (Nicolson, T., L. Weisman, G. Payne, and W. Wickner, unpublished data).

The proPHO8-maturation assay has been used to define some effects of biochemical reagents on inter-vacuole fusion. Reagents which block the H^+-translocating ATPase or uncouple the proton gradient across the vacuole membrane inhibit inter-vacuole fusion in vitro. This was not merely due to a diminished maturation of proPHO8 by PEP4 in a more alkaline environment. Our microscopic observations show that CCCP and bafilomycin A_1 allow vacuole aggregation but prevent fusion. Furthermore, recent studies showed that normal precursor protein maturation can occur in yeast vacuoles with an elevated pH (Yamashiro et al., 1990). Thus, a loss in acidification and/or a drop in the electrochemical potential across the membrane inhibited fusion frequency. This may be due to a reduced binding of cytosolic factors to vacuole membranes, similar to the decreased binding of ADP-ribosylation factor to bafilomycin-treated microsomal membranes (Zeuzem et al., 1992). Clague et al. (1994) showed that the formation of a vesicular intermediate between early and late endosomes (endosomal carrier vesicles) is impaired by inactivation of the endosomal ATPase via bafilomycin A_1. Thus, further studies are needed to investigate whether the formation of segregation structures is impaired in bafilomycin-treated samples. Inhibition of fusion by neomycin may reflect the involvement of phosphatidyl inositol-polyphosphatase. A role for the inositol-signaling pathway has recently been shown in vitro during nuclear vesicle fusion (Sullivan et al., 1993), and similar processes might play a role in vacuole fusion processes (see also Burgoyne, 1994).

Inhibition of vesicular transport processes by GTPγS is a hallmark of GTP-binding and hydrolyzing (G-) proteins in intracellular trafficking events (Gruenberg and Clague, 1992; Pfeffer, 1992; Pryer et al., 1992; Rothman and Orci, 1992). We have performed GTP-binding blots (Vater et al., 1992) which have shown that there are at least five different "small" GTP-binding proteins present in our vacuole isolates (data not shown). We observed a considerable inibition of inter-vacuole fusion by GTPγS (70% at 0.8 mM, and 90% at 1.5 mM). These inhibitory concentrations are clearly higher than those necessary to block vesicular transport in some other in vitro systems (Mayorga et al., 1989*b*; Rexach and Schekman, 1991; Leyte et al., 1992; Carter et al., 1993), but are approximately the same concentrations necessary for the inhibition of in vitro vesicle fusion during nuclear envelope assembly in Xenopus eggs (Boman et al., 1992) and retrograde transport from the Golgi region to the ER in human hepatoma cells (Tan et al., 1992). Interestingly, Taylor et al. (1992) found that GTPγS did not inhibit their in vitro intra-Golgi transport reactions although a role for small G-proteins had clearly been established. These authors suggested that this was due to the fact that GTPγS might not have been bound to the respective G-protein(s). Thus, in our fusion system, replacement of bound GDP by GTPγS might, in the absence of a GTP-exchange factor (Boguski and McCormick, 1993), be necessary to inhibit the fusion reactions (i.e., activation of G-proteins, not GTP hydrolysis by activated G-proteins, would be necessary for fusion inhibition). In this case, high GTPγS concentrations may favor a spontaneous exchange reaction over rebinding of previously bound GDP. Guanine nucleotide–dissociation inhibitors (Boguski and McCormick, 1993) may interfere as well.

The inhibition of vacuole fusion by GTPβS (R_p-isomer) is remarkable. GTPβS has been used to probe the stereospecificity of GTP-binding sites in G-proteins. Most G-proteins have a much lower affinity for GTPβS than for GTPγS or GTP (e.g., 1/10,000 the affinity in transducin, Yamanaka et al., 1985; 1/15 the affinity in c-Ha-*ras*, Tucker et al., 1986; Jones et al., 1990; Paris and Eckstein, 1992). In our system, only approximately twice as much GTPβS as GTPγS is needed for the same inhibition. Thus, GTPβS might be a useful probe to identify the G-protein(s) involved in vacuole-to-vacuole fusion. The lack of GDP-mediated inhibition of fusion, together with a clear GDPβS-dependent inhibition, could be explained by the presence of cytosolic diphosphonucleotide kinase which catalyzes the transfer of γ-phosphate from ATP (which is constantly present in our reactions at a concentration of 1 mM) to GDP, thus creating G-protein–activating GTP. The same phenomenon has been described for G-protein–dependent vesicle formation from yeast ER (Barlowe et al., 1993). GDPβS, in contrast to GDP, is not phosphorylated and may compete with GDP and, particularly, GTP for the binding to G-proteins. At 2 mM, GDPβS may lock most G-proteins in their inactive state, thus, inhibiting fusion reactions.

Inhibition of fusion by GTP derivatives alone is, however, not sufficient proof for the involvement of G-proteins in this reaction. Other reagents which are relatively specific for certain (mammalian) G-protein subclasses could not be used in our studies: aluminum fluoride strongly activates mammalian heterotrimeric G-proteins by mimicking the *ortho*-phosphate on bound GDP (Bigay et al., 1987). However, in our reaction fluoride itself was inhibitory (data not shown). Pertussis toxin, which inhibits mammalian heterotrimeric G-proteins (Kopf and Woolkalis, 1991), does not function in yeast; the three so far published fungal G-protein α-subunit sequences (Dietzel and Kurjan, 1987; Nakafuku et al., 1987, 1988; Obara et al., 1991) lack the cysteine residue which, in some mammalian α-subunits, is the target for ADP ribosylation (McDonald, 1992).

The use of mastoparan and its derivatives, however, has been fruitful. Though mastoparan has also been reported to activate purified phospholipase C (Wallace and Carter, 1989), nucleotidases (Bomsel and Mostov, 1992), and to bind strongly to calmodulin (Malenik and Anderson, 1983), many trafficking events are selectively inhibited by the G-protein–stimulating activity of mastoparans (as shown by the prevention of mastoparan effects by adding antibodies specifically directed against certain α-subunit COOH termini; Carter et al., 1993; Pimplikar and Simons, 1993; Vitale et al., 1993). Our results show that mastoparan strongly inhibited inter-vacuole fusion at concentrations almost identical to those needed either for fusion inhibition in these other in vitro systems or for the activation of mammalian G-proteins in vitro (where the concentration for half-maximal inhibition was 10 μM; Higashijima et al., 1990). A mastoparan derivative, mas7[Q4;Q11], was only inhibitory at a very high concentration (100 μM), whereas mas7 [K4;K11] was inhibitory in the low micromolar range. The [Q4;Q11] derivative binds as strongly to lipid bilayers as the [K4;K11] variant (as determined by circular dichroism spectra), although it clearly does not activate G-proteins (Higashijima, T., personal communication; the lysine residues in mastoparan have previously been shown to be critical for the activation of G-proteins; Higashijima et al., 1990; Sukumar and Higashijima, 1992). Furthermore, there was only a weak influence of 20 μM Mas7[Q4;Q11] on the increase in size of vacuoles in a microscopy fusion assay. However, 20 μM Mas7 led to vacuole fragmentation. These data strongly suggest that inhibition of fusion by 20 μM mas7 was not due to membrane perturbation but due to an interaction of mas7 with a membrane component. This agrees with the observation that preincubation of cytosol with up to 100 μM mastoparan did not inhibit its fusion-promoting activity. Thus, small rho-like proteins (Koch et al., 1991) or other cytosolic factors are not directly involved in the inhibition of fusion by mastoparan. Mas7[Q4;Q11] did not relieve the inhibition caused by mas7 effects when both compounds were added together (data not shown), suggesting that mas7[Q4;Q11] does not bind to the receptor-binding site of the putative target G-protein. Mas7[Q4;Q11] is the best available control for excluding membrane-disturbing side effects: Mas17, which is commonly used for this purpose, does not assume an α-helical structure in the presence of membrane and thus binds to them only weakly (Higashijima et al., 1990). Two other Mas derivatives, the Mas-derived peptides 2 and 7 (Oppi et al., 1992), which do not stimulate G-proteins in vitro, were also inactive in the fusion assay.

BAC antagonized the mastoparan-induced inhibition of fusion. Both mastoparan and BAC have only negligible detergent effects at the concentrations which show clear effects in our assay of vacuole fusion (Katsu et al., 1990; Vaara, 1992). When applied together they show less inhibition of fusion than mastoparan alone, suggesting that there are specific targets for these compounds in this system. The detergent Triton X-100 did not influence fusion frequency when added at the same concentrations at which BAC inhibited. Recently, it has been shown that BAC antagonizes the stimulation of purified G_i protein by mastoparan, whereas mastoparan-induced stimulation of purified G_o protein remains unaffected (Higashijima et al., 1990). On the other hand, BAC alone stimulates the activity of purified G_o but not of G_i (Higashijima et al., 1990), and inhibits G_o-regulated exocytosis in chromaffin cells (Vitale et al., 1983). The BAC concentrations necessary to observe similar effects in these systems were again about the same as those needed in our in vitro system.

Considering, (*a*) the inhibition of fusion by mastoparan derivatives which are active towards isolated G-proteins (but not by those that are inactive towards G-proteins); (*b*) the fact that mastoparan acts on the vacuoles rather than the cytosol; (*c*) fusion inhibition by BAC; (*d*) the antagonizing effect of BAC on mastoparan-induced fusion-inhibition, and (*e*) the inhibition of fusion by GTPγS, GTPβS, and GDPβS (but not by GTP, GDP, or cGMP), we suggest the following hypothesis. There may be vacuolar membrane proteins which react in the same manner as G_i and G_o (no such classification currently exists for yeast G-proteins). These proteins may normally stimulate or inhibit fusion but, once constitutively activated by mastoparan or nonhydrolyzable GTP analogues, they inhibit fusion. In fact, inhibition of intracellular trafficking events by mastoparans has been shown to be either due to G_i (Stow et al., 1991; Pimplikar and Simons, 1993; Wilson et al., 1993), or G_o (Vitale et al., 1993). The interaction of two different types of heterotrimeric G-proteins has already been shown for other trafficking systems (Colombo et al., 1992; Leyte et al., 1992; Pimplikar and Simons, 1993). It is also possible that a single vacuolar protein which combines features of both G_o and G_i proteins regulates vacuole fusion. Heretofore, neither mastoparan nor BAC have been used in yeast in vitro models, nor have heterotrimeric G-proteins been assigned a role in vesicular transport in yeast. Of course, monomeric G-proteins may also be involved in inter-vacuole fusion. Fractionation of the proteins in our in vitro reaction will be required for the molecular identification of such factors.

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