Microautophagic Vacuole Invagination Requires Calmodulin in a Ca\(^{2+}\)-independent Function*

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Microautophagy is the uptake of cytosolic compounds by direct invagination of the vacuolar/lysosomal membrane. In Saccharomyces cerevisiae microautophagic uptake of soluble cytosolic proteins occurs via an autophagic tube, a highly specialized vacuolar membrane invagination. Autophagic tubes are topologically equivalent to the invaginations at multivesicular endosomes. At the tip of an autophagic tube, vesicles (autophagic bodies) pinch off into the vacuolar lumen for degradation. In this study we have identified calmodulin (Cmd1p) as necessary for microautophagy. Temperature-sensitive mutants for Cmd1p displayed reduced frequencies of vacuolar tube formation and/or abnormal tube morphologies. Microautophagic vacuole invagination was sensitive to Cmd1p antagonists as well as to antibodies to Cmd1p. cmd1 mutants with substitutions in the Ca\(^{2+}\)-binding domains showed full invagination activity, and vacuolar membrane invagination was independent of the free Ca\(^{2+}\) concentration. Thus, rather than acting as a calcium-triggered switch, Cmd1p has a constitutive Ca\(^{2+}\)-independent role in the formation of autophagic tubes. Kinetic analysis indicates that calmodulin is required for autophagic tube formation rather than for the final scission of vesicles from the tip of the tube.

In eukaryotic cells there are many trafficking pathways between organelles. Many of these transport processes, such as the endocytic pathway, autophagocytosis, cytosome to vacuole targeting, piecemeal microautophagy of the nucleus, and biosynthetic delivery of hydrolyses, end at the lysosome, the main compartment for storage and degradation (1–6). In yeast the lysosomal organelle is called a vacuole.

Under conditions of nutrient restriction, cytosolic and membraneous material reaches the vacuole by means of autophagy (7). Macroautophagy in yeast is defined as the uptake of cytosolic elements by fusion of double membrane vesicles (autophagosomes) with vacuoles (8). These double-layered vesicles are produced from preautophagosomal structures and, during their formation, excise portions of cytosol and entrap them. Fusion of the outer autophagosomal membrane with lysosomes produces single-layered invacuolar vesicles (autophagic bodies) that are degraded (7). Macroautophagy has been studied intensively over the last decade, and many relevant components (Atg proteins) (9) have been identified, mainly by genetic screens (10–14). In contrast, only little is known about a process consisting of a direct vacuolar invagination and budding of autophagic bodies into the vacuolar lumen, which we call microautophagy of soluble compounds (15–17). Besides microautophagy of soluble cytosolic components, also larger particles can be taken up by vacuoles, e.g. during piecemeal microautophagy of the nucleus (PMN) (6), which transfers parts of the nucleus into vacuoles, and during micropexophagy (18–23), which leads to the degradation of peroxisomes. Pexophagic vacuole invagination depends on Atg proteins (24–27) and hence shares components with the macroautophagic pathway. In contrast, there is no evidence that Atg proteins are directly involved in PMN (6).

Microautophagy of soluble cytosolic components is topologically equivalent to invaginations occurring during multivesicular body formation at the endosome. It occurs through highly differentiated structures termed autophagic tubes (15). Like macroautophagy (28), autophagic tubes are induced by nitrogen starvation and rapamycin (a pharmacological agent inhibiting Tor kinase signaling). Atg proteins appear not to be directly responsible for microautophagic uptake of soluble components (15, 16), but Atg-dependent macroautophagy is a prerequisite for microautophagy of soluble components (16). Because microautophagy leads to uptake and degradation of the vacuolar boundary membrane it could compensate the enormous influx of membrane caused by macroautophagy. However, this should render microautophagic membrane invagination dependent on membrane influx via macroautophagy. Microautophagic vacuole invagination (with the exception of PMN and pexophagy) might hence be responsible for maintenance of organelar size and membrane composition rather than for cell survival under nutrient restriction. The observation that autophagic tubes show dramatically reduced transmembrane particles toward their tips (the site where autophagic bodies pinch off) is consistent with this model (15) because nascent microautophagic vesicles share this exceptional ultrastructural feature with nascent autophagosomes. Also nascent autophagosomes are virtually free of intramembraneous particles, suggesting that membrane removal by microautophagy might compensate macroautophagic membrane influx both in terms of quantity and quality.

In contrast to macroautophagic or peroxisomal degradation pathways, microautophagic uptake of soluble proteins into the yeast vacuole is poorly understood. Microautophagic activity can be reconstituted in a cell-free system composed of purified vacuoles and cytosolic extracts (16). Vacuoles internalize a reporter enzyme (firefly luciferase) in an ATP-dependent fashion. After the uptake reaction, internalized luciferase reporter can be reisolated with the vacuoles; it is protected against proteolytic removal by microautophagy might compensate macroautophagic membrane influx in both terms of quantity and quality.

The abbreviations used are: PMN, piecemeal microautophagy of the nucleus; GTP\(_{S}\), guanosine 5’-O-(3-thiotriphosphate); PIPES, 1,4-piperazinediethanesulfonic acid; BAPTA, 1,2-bis(aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; SNARE, soluble NSF attachment protein receptors.

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miniscient assay. Using a pharmacological approach including low molecular weight inhibitors, the *in vitro* uptake reaction could be dissected into different kinetic stages (17).

In the present study we have used a combination of the three approaches described above to analyze the role of calmodulin in microautophagy of soluble compounds. Calmodulin is a 16-kDa protein that in yeast binds three calcium ions via EF hands (29–32). Calmodulin is essential for cell viability, but its ability to bind calcium ions is not essential, at least in yeast (33). It is involved in a large variety of cellular processes, such as organization of the actin cytoskeleton (34), chromosome segregation during mitosis (35), membrane fusion (36), endocytosis (37), and nuclear division (38). In the yeast *Saccharomyces cerevisiae* Cmd1p performs at least two essential functions (39). First, it plays an important role in polarized cell growth via an interaction with an unconventional type V myosin, Myo2p (40). Second, its interaction with a 110-kDa protein component of the spindle pole body is required during mitosis (41, 42). That calmodulin might have at least two further essential functions is indicated by the existence of a total of four intragenic complementation groups (34). This illustrates the potential of calmodulin to participate in different biochemical events by different mechanisms. Although many calmodulin activities are calcium-regulated, there are also examples for functions of apicalcalmodulin.

Calmodulin is induced 2–3-fold by the drug rapamycin (43), which triggers a starvation response and induces autophagy *in vivo* (28). We have analyzed the requirement for calmodulin for microautophagic membrane invagination, its kinetics, and the role of free calcium and of calcium binding by calmodulin.

**MATERIALS AND METHODS**

Sources of Chemicals—W-5, rapamycin (Alexis, Gruenberg, Germany), W-7, BAPTA (Calbiochem, Darmstadt, Germany), EGTA (Roth, Karlsruhe, Germany), Ophiobolin A (Sigma), and GTPyS (Roche Applied Science) were suspended as 10–100× stock solution in PS buffer (200 mM sorbitol, 10 mM PIPES/KOH, pH 6.8) (for W-5, W-7, BAPTA, EGTA, and GTPyS) or Me₂SO (for Ophiobolin A and rapamycin) and stored at −20 °C. Yeast lytic enzyme was purchased from ICN Biochemicals (Eschege, Germany).

Yeast Strains—Strains Y10000 (BY4742, wild type), Y13470 (ΔVac17), Y12889 (ΔNvj1), and Y10253 (ΔVac8) were purchased from Euroscarf (Frankfurt, Germany). Strain K91-1A was kindly provided by Y. Kaneko. Strains DBY5734 (CMD1 wild type), DBY5706 (cmd1–226), DBY5708 (cmd1–228), DBY5713 (cmd1–233), DBY5719 (cmd1–239) (34), CRY1 (CMD1 wild type), IGY149 (cmd1–6), IGY041 (cmd1–3), and IGY148 (cmd1–5) (33) were provided by David Botstein and Trisha Davis, respectively.

Yeast Cells—Yeast cells were cultured, and cytosol from strains K91-1A, BY4742, and ΔVac8 was prepared as described previously (16).

Vacuole Preparation—Vacuole preparation was performed as described previously (16), but by using yeast lytic enzyme (from Arthrobacter Luteus, ICN catalog number 360944; final concentration, 3.27 mg/ml) instead of oxalycatase. For storage of vacuoles, glycerol (10% v/v from a 50% stock) was added to a fresh vacuole suspension. The suspension was frozen as little nuggets in liquid nitrogen and stored at −80 °C (17).

Purification of Calmodulin—Wild type calmodulin (pG028) was expressed in *Escherichia coli* GM1 (33) and purified by phenylsepharose chromatography as described before (44).

Preparation of Cytosol Depleted for Calmodulin—8.5 mg of cytosol (strain K91-1A; stock concentration, 45 mg/ml) were incubated with 1 mM phenylmercuri sulfonfyl fluoride, 0.1 mM Pefabloc SC, 0.5 mM o-phenanthroline, 0.5 μg/ml pepstatin A, and 1 μM affinity purified rabbit antibodies to calmodulin (or control antibody, respectively) for 30 min on ice. 50 μl of protein G-agarose beads (washed in PS buffer, 150 mM KCl) were added and incubated for another 30 min while shaking gently at 4 °C. The beads were separated by centrifugation (20,800 × g, 1 min, 2 °C, fixed angle table top centrifuge), and the cytosol was recovered. The efficiency of immunodepletion was checked on a Western blot.

In Vitro Microautophagy Assay—A standard reaction had a volume of 45 μl and was composed of: vacuoles (0.2 mg/ml, either freshly prepared or thawed from a −80 °C stock), 6.8 mg/ml (K91-1A cells), or 3.2 g/ml (BY4742 cells and ΔVac8 cells) cytosol from starved cells, 105 mM KCl, 7 mM MgCl₂, 2.2 mM ATP, 88 mM disodium creatine phosphate, 175 units/ml creatine kinase, 17 μg/ml luciferase, 100 μM dithiothreitol, 0.1 mM Pefabloc SC, 0.5 mM o-phenanthroline, 0.5 μg/ml pepstatin A, 200 mM sorbitol, 10 mM PIPES/KOH, pH 6.8. This mixture was incubated for 1 h at 27 °C. For measuring luciferase uptake, the samples were chilled on ice, diluted with 300 μl of 150 mM KCl in PS buffer, and centrifuged (6500 × g, 3 min, 2 °C, fixed angle table top centrifuge), and the pellet was washed once more with 150 mM KCl in PS buffer and resuspended in 55 μl of 150 mM KCl in PS buffer. Proteinase K was added (0.3 mg/ml from 18× stock) and incubated on ice for 2 min. Digestion was stopped by adding 55 μl of 1 mM phenylmethylsulfonyl fluoride/150 mM KCl in PS buffer. Luciferase activity was determined using an assay kit according to the manufacturer’s instructions (Berthold detection systems, Pforzheim, Germany). 25 μl of sample were mixed with 25 μl of lysis buffer, and 50 μl of substrate mix (45) were added directly before counting light emission in a microplate luminometer (LB 96 V; Berthold Technologies, Bad Wildbad, Germany). Alkaline phosphatase activity was determined in a 25-μl aliquot as described previously (16) to serve as an internal reference for the quantity of pelleted vacuoles. Uptake activity was calculated as the quotient of luciferase activity over alkaline phosphatase activity (counts/s/μg protein).

Thin Section Electron Microscopy—Yeast cells were cryomobilized by high pressure freezing as described previously (46). In short, living specimens were sucked into cellulose microcapillaries of 200-μm diameter, and 2-mm-long capillary tube segments were transferred to aluminum platelets of 200-μm depth containing 1-hexadecane. The platelets were sandwiched with platelets without any cavity and then frozen with a high pressure freezer (HPM 010; Bal-Tec, Balzers, Liechtenstein). Extranuclear hexadecane was removed from the frozen capillary tubes under liquid nitrogen and transferred to 2-ml microtubes with screw caps (Sarstedt number 72.694) containing substitution medium prewarmed up to −90 °C. The samples for ultrastructural studies were kept in a freeze substitution unit (Balzers FSU 010; Bal-Tec) in 2% osmium tetroxide in anhydrous acetone at −90 °C for 32 h, warmed up to −60 °C within 3 h, kept at −60 °C for 4 h, warmed up to −40 °C within 2 h, and kept there for 4 h. After washing with acetone, the samples were transferred into an acetone–Epon mixture at −40 °C, infiltrated at room temperature in Epon, and polymerized at 60 °C for 48 h. Ultrathin sections, stained with uranyl acetate and, if required with lead citrate, were viewed in a Philips CM10 electron microscope at 60 kV.

**RESULTS**

A search for pharmacological inhibitors of microautophagic vacuole invagination *in vitro* (17) had hinted at a role of calmodulin in this...
process. Therefore, we analyzed the role of calmodulin for this reaction in detail. Because knock-out of the sole calmodulin gene in yeast (Cmd1) causes cell death, we used yeast strains containing temperature-sensitive cmd1 alleles to investigate autophagic tube formation under nitrogen starvation conditions. Ohya and Botstein (34) defined four intragenic complementation groups for Cmd1 that affect four different essential functions of calmodulin. We have chosen one representative conditional allele from each complementation group for initial analysis. Concomitantly with the induction of autophagy, the cells were shifted to the restrictive temperature to inactivate calmodulin. At permissive temperature all of the mutants showed little effect on the frequencies of autophagic tube formation (80–95% of the wild type control), except for cmd1–233, which showed a 50% reduction already under these conditions (Fig. 1A). At restrictive temperature cmd1–226 and cmd1–228 showed stronger reductions of autophagic tubes in comparison with the wild type, whereas tube frequency did not significantly decrease in cmd1–233 and in cmd1–239. cmd1–239 and cmd1–226 had severely aberrant tube morphology at restrictive temperature (see below). Reduced frequency of tubes is consistent with a deficiency in membrane invagination.

For detailed morphological analysis we chose the mutants showing the most severe temperature-dependent reduction of tube frequency (cmd1–226) or the most severely aberrant tube morphology (cmd1–233). We analyzed a representative subset of autophagic tubes by electron microscopy of quick frozen and freeze-substituted cells. This procedure minimizes fixation artifacts on vacuole morphology and enabled us to evaluate tube morphology in detail using static snap shots of a large number of autophagic tubes (Fig. 1B). We could classify tubes into different morphological categories and quantify their relative abundance in wild type versus mutant strains (single random sections have been analyzed). The mutants formed many enlarged invaginations, enlarged tube tips, or even completely disorganized structures, indicating not only a deficiency in invagination per se as supposed before for cmd1–226 (Fig. 1A). These phenotypes are consistent with a deficiency in completion of the invagination/budding process of autophagic bodies at the tubular tips (cmd1–239) leading to a kind of “frustrated” autophagic tubes incapable of fulfilling their final function.

We further investigated the role of Cmd1p using the cell-free system reconstituting microautophagy. We ran in vitro luciferase uptake assays with vacuoles derived from the temperature-sensitive strains shown above. Even when cultured at permissive temperature, all of the mutants showed a clear reduction of uptake activity, indicating that the effect of the mutations in vitro is more pronounced than in vivo (Fig. 3). This is not unusual for temperature-sensitive mutants. After the shift to restrictive temperature, the relative uptake activities of mutant vacuoles dropped even further when compared with wild type vacuoles. The temperature shock effect was most severe for cmd1–226 and cmd1–233, confirming the in vivo effects (Fig. 1). Strains cmd1–228 and cmd1–239 also showed decreased activities, but these changed only a little after temperature shock. The lack of further temperature-dependent reduction of activity for cmd1–228 is in contrast to the in vivo behavior of this allele. Potential reasons could be a secondary effect in vivo, a general process.
instability of the mutant protein in vitro, rescue of mutant calmodulin by wild type calmodulin from cytosol, or refolding of the inactivated Cmd1p into its active conformation during vacuole preparation. If the cytosol used in the in vitro assays was immunodepleted for Cmd1p, the results were comparable as for nondepleted cytosol (data not shown), indicating that the addition of Cmd1p via cytosol could not mask the effect of temperature-sensitive mutants under standard assay conditions. Therefore, all further experiments were performed with nondepleted wild type cytosol.

The reconstituted microautophagic uptake reaction provides the possibility of performing experiments in the presence of low molecular weight inhibitors (16, 17). Therefore, we ran in vitro uptake reactions in the presence of the calmodulin antagonists W-5, W-7, and Ophiobolin A (Fig. 4A). All three Cmd1p antagonists inhibited luciferase uptake at micromolar concentrations. In agreement with published data demonstrating that the potency of W-5 is significantly lower than that of its structural analog W-7, W-5 and W-7 showed clearly different IC50 values in our experiments (W-7, 110 μM; W-5, 200 μM). The inhibitor concentrations used in the uptake reactions were higher than the concentrations required to block vacuolar fusion in vitro (49). This may be due to the fact that microautophagy in vitro requires the addition of high concentrations of cytosol, whereas vacuole fusion could be assayed without the addition of cytosol. Cmd1p might also function in a different protein complex resulting in altered sensitivity to the inhibitors.

To exclude unspecific inhibitory effects caused by vacuole lysis or inhibition of the luciferase reporter enzyme, we also added inhibitors at the end of the luciferase uptake reaction. Because uptake has already been completed at this point, such treatment would only allow unspecific lysis or inactivation of the reporter enzyme to reduce the signal. Inhibition was only observed if inhibitors had been added at the beginning but not if added at the end of the reaction, confirming that the inhibitors did not compromise vacuolar integrity or luciferase activity (Fig. 4B).

An alternative strategy to selectively inactivate components on the vacuolar surface in vitro is to use affinity purified antibodies that can be added to an in vitro uptake reaction. The in vitro reaction was sensitive to affinity purified antibodies to Cmd1p (Fig. 4C). This effect was specific because it was not observed with purified nonimmune antibodies (total IgGs derived from a nonimmunized animal) at the same concentration and could be rescued by the addition of purified Cmd1p (Fig. 4D). The IC50 values of anti-Cmd1p in the microautophagic uptake reactions are ~5-fold higher than the IC50 values required for inhibition in vacuolar fusion (49), again suggesting that Cmd1p might function in a different protein complex in the two reactions.

Microautophagic vacuolar uptake can be resolved into sequential kinetic stages by adding inhibitors at different time points (17). According to their ability to block the reaction at different kinetic stages, inhibitors have been classified. Early acting class A inhibitors (nystatin, GTPγS, and aristolochic acid) inhibit formation of vacuolar membrane invaginations (see Fig. 9), whereas late acting class B inhibitors (valinomycin/FCCP, K252a, and rapamycin) affect subsequent steps leading to formation of vesicles from the tubes. To determine the time course of Cmd1p action during microautophagy, we performed kinetic analyses with the calmodulin inhibitor W-7. W-7 was added at different time points of the reaction (Fig. 5). Then the incubation was continued until the end of a standard 60-min reaction period. Class A inhibitors lose their activity if added late during the reaction, whereas class B inhibitors remain active throughout the reaction. As a control, the samples were transferred to ice, a treatment interfering with many aspects of membrane dynamics. The addition of W-7 inhibits the reaction at all time points, even crossing the ice curve. This suggests that W-7 inhibits the late stage of microautophagic invasion that cannot be blocked by chilling. It behaves as a class B inhibitor, and its action differs from that of GTPγS, a class A inhibitor used as a control for early action (17).

Late acting class B inhibitors can be further classified depending on their ability to inhibit "rapid uptake" (17). Rapid uptake of luciferase occurs after preincubation of vacuoles under standard conditions supporting microautophagic membrane invagination, but in the absence of the reporter enzyme. This allows the vacuoles to complete all preparatory reactions for uptake, e.g. to form an invagination, without producing a luciferase signal. The formation of vesicles can then be scored by adding luciferase for a short period of time. This allows only rapid uptake from preformed invaginations but is too short for the formation of new invaginations. Thus, this criterion can be used to distinguish the preparation for uptake (tube formation) from its completion (vesicle scission). After 60 min of incubation without luciferase, the reporter enzyme is added to an uptake reaction. The samples are incubated for another 5 min to permit reporter enzyme uptake. Uptake is terminated by chilling, diluting, and centrifuging the reactions. Pelleted vacuoles are analyzed for luciferase uptake. To dissect the late activity of Cmd1p antagonists, both W-5 and W-7 were used in rapid uptake assays (Fig. 6). W-5 and W-7 inhibited only when added at the start of the overall reactions, i.e. already during the 60-min preincubation without luciferase, but not when added between the 60-min preincubation and luciferase addition. The early acting class A inhibitor GTPγS and the late acting class B inhibitor rapamycin were used as controls (17). GTPγS did not affect rapid uptake at all, whereas rapamycin strongly impairs rapid uptake. These results thus suggest that calmodulin is required for the invagination of the vacuolar membrane but not for the scission of vesicles from it.

Cmd1p is involved in a large variety of cellular processes, and most of them involve Cmd1p as a calcium sensor (50, 51). To test whether this is also the case for microautophagy, we used yeast strains expressing mutant forms of Cmd1p with reduced Ca2+ affinities. These cmd1–3 (D20A, E31V, D56A, E67V, D93A, and E104V), cmd1–5 (E31V, E67V, and E104V), and cmd1–6 (D20A, D56A, and D93A) mutants contain amino acid substitutions in each of the three calcium-binding domains of Cmd1p that remove groups coordinating Ca2+ ions (33). Vacuoles
derived from such mutants showed wild type uptake activity in vitro, suggesting that Cmd1p can fulfill its function without the need to bind Ca\(^{2+}\) (Fig. 7A). In line with this, tube morphologies and frequencies, assayed by fluorescence microscopy in vivo, were also like wild type for the three mutants (data not shown). If cytosol immunodepleted for Cmd1p was used in the in vitro uptake assays with cmd1–3 vacuoles,
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FIGURE 7. Calmodulin mutants deficient in calcium binding. A, in vitro microautophagic reactions were run that contained cytosol (K91-1A) and vacuoles from strains expressing calmodulins with mutations in the calcium-binding domains (cmd1–6, cmd1–3, and cmd1–5). The data from three independent experiments were averaged with S.D. Titration of calcium chelators BAPTA and EGTA. In vitro microautophagic reactions were run in the presence of different chelator concentrations as indicated. After 60 min, uptake was assayed. The data from three to five independent experiments were averaged. The error bars indicate the standard deviation. B, uptake activity was the same as for nondepleted cytosol (data not shown), confirming that a potential defect caused by the mutant calmodulins on the membranes was not masked by redundancy with wild type Cmd1p added via the cytosolic extracts. This matched our previous observations for the temperature-sensitive cmd1 alleles (Fig. 3).

If Cmd1p could indeed act in a Ca\(^2+\)-independent fashion during microautophagy, the overall in vitro reaction might be independent of Ca\(^2+\) altogether. We addressed this question by titrating the Ca\(^2+\) chelators BAPTA and EGTA into the uptake reaction (Fig. 7B). Neither chelator inhibited the reaction up to a concentration of 5 mM. BAPTA even stimulated the reaction below 5 mM. This phenomenon could be due to an inhibition of proteases or lipases or to the chelation of disturbing metal ions. The concentration of free Ca\(^2+\) can be calculated with good precision if the pH, buffers, and ionic strength are taken into account (52). We based this estimation on the conservative assumption that all vacuoles contain 2–4 mM Ca\(^2+\) in their lumen (53), that they would completely release all Ca\(^2+\) into the reaction mixture (which does not happen), and that the vacuolar lumen comprises approximately 5% of the total volume of the reaction (determined by microscopy and by sedimentation experiments). Even in this worst case, for which we completely neglect compensatory Ca\(^2+\) uptake via the vacuolar Ca\(^2+\) pumps Pmc1p (54) and Vcx1p (55), a chelator concentration of 5 mM would reduce the concentration of free Ca\(^2+\) to less than 60 nM under the salt and buffer conditions of the in vitro assay. This is below the resting concentration in yeast cytosol, which has been measured to be between 100 and 350 nM (56–59). 60 nM is also well below the K\(_d\) values for calmodulin (11 \(\mu\)M) (60) or other typical Ca\(^2+\)-regulated proteins. Therefore, a regulatory function of calcium in microautophagic membrane invagination is quite unlikely.

Taken together our data indicate that microautophagy requires calmodulin in a Ca\(^2+\)-independent fashion. Proteins interact with calmodulin in a Ca\(^2+\)-independent fashion via IQ motifs. By sequence analysis of known vacuole-associated proteins, we have identified three vacuolar proteins containing putative IQ motifs: Myo2, Vtc2p, and Vtc3p. Myo2p acts as a molecular motor to transport vacuolar vesicles along actin cables into the daughter cell. A Myo2p-Vac17p-Vac8p complex is responsible for this reaction (61). Therefore, we tested whether the Myo2p-Vac17p-Vac8p complex could be involved in the vacuolar invagination reaction using knock-out strains lacking the vacuolar Myo2p receptors Vac17p and Vac8p. Vacuoles derived from these strains showed the same luciferase uptake activity as wild type organelles (Fig. 8). Vac8p can also act as the receptor protein for Nvj1p during PMN (6), a process topologically related to microautophagy of soluble components. To investigate whether microautophagy of soluble components and PMN share common components, we also tested vacuoles from an nvj1 deletion strain for vacuolar invagination in vitro. This strain showed wild type activity (Fig. 8), suggesting that microautophagy of soluble compounds and PMN follow different mechanisms.

Vtc2p and Vtc3p also contain putative IQ and IQ-like motifs in their central domains. Vtc proteins exist in a high molecular weight complex comprising Vtc1 through 4 (62–65). This Vtc complex functions in vacuolar lipid turnover. This function is of particular interest for vacuolar membrane invagination because ultrastructural analysis had indicated that the vesicles pinching off from the tips of the invaginations are largely devoid of integral membrane proteins and appear like pure lipid bilayers (15). Accordingly, we have recently identified specific lipids as essential for invagination. In a separate detailed study, we have ana-

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lyzed the role of Vtc proteins for vacuolar uptake activity in vitro. There was a strong dependence of the reaction on the Vtc complex. Mutation of the Vtc genes or inactivation of the Vtc complex by antibodies strongly reduced vacuolar uptake activity. The central domains of Vtc2p and Vtc3p, which carry the IQ motifs, bound calmodulin in a Ca\(^{2+}\)-independent fashion. Mutation of the predicted IQ motifs greatly affected sorting and function of the Vtc complex. This suggests that Vtc2p and Vtc3p represent targets for calmodulin in microautophagic vacuole invagination.

**DISCUSSION**

The specific calmodulin content of purified vacuolar membranes was comparable with that of bulk cytosol. The fact that neither depletion nor rescue experiments with cytosol showed any effect could indicate that the fraction of Cmd1p that is necessary for microautophagy is tightly bound to the vacuolar membrane and cannot be easily substituted by the simple addition of new Cmd1p. It is also possible that the population of Cmd1p, which is necessary for microautophagy, has to be preactivated in a membraneous protein complex and that this putative structure cannot be reconstituted in vitro. Unspecific side effects appear unlikely because we have inactivated calmodulin by three different strategies, i.e. conditional alleles, antibodies, or low molecular weight inhibitors. All three approaches yielded independent evidence for a calmodulin requirement.

Cmd1p is important for organization of the actin framework in yeast, and cmd1Δ--226 mutants show abnormal actin organization at restrictive temperature (34). This could be relevant to microautophagy because vacuoles contain actin bound to their surface (47, 66). Defects in the organization of autophagic tubes could be due to a disorganized actin structure on vacuoles. Up to now it was not known what drives the membrane invagination itself. Although an involvement of tubulin appears unlikely (17), one cannot exclude the possibility that actin could support membrane invagination by stabilizing preformed invaginations, by applying mechanical force directed toward the lumen of the vacuole, or by driving the budding process of autophagic vesicles. Although our results did not support a role for Myo2p associated with vacuoles via Vac17p and Vac8p, it is tempting to speculate that the actin cytoskeleton might be involved in vacuolar membrane invagination and support this reaction by modulation of the polymerization state. We have initiated studies to test this possibility.

Apocalmodulin (the Ca\(^{2+}\)-free form of Cmd1p) binds to various actin-binding proteins (brush border myosin I, Myr4, P190), cytoskeletal, and membrane proteins (neuromodulin, neurogranin, PEP-19, Igloo, syntrophin, and IQGAP), enzymes (glycogen phosphorylase b kinase, adenylyl cyclase, inducible NO synthase, glutamate decarboxylase, and cGMP-dependent protein kinase), channels (sarcoplasmatic reticulum Ca\(^{2+}\)-release channel), and receptors (inositol-1,4,5-trisphosphate receptor) (39). Endocytosis in yeast requires Cmd1p but is apparently Ca\(^{2+}\)-independent (37). In contrast to its function during vacuole fusion, where Cmd1p functions in its Ca\(^{2+}\)-bound forms (49), apocalmodulin suffices to support microautophagic membrane invagination. The \(K_d\) of Ca\(^{2+}\)-calmodulin has been reported to be approximately 11 \(\mu\)M under physiological conditions (57). Yet this is not necessarily representative of the Ca\(^{2+}\)-binding of calmodulin in a protein complex. Binding studies suggested that interaction of calmodulin with peptides could increase its affinity for Ca\(^{2+}\) up to 1,000-fold, yielding a \(K_d\) of 11 nm Ca\(^{2+}\) (39). Ca\(^{2+}\)-binding by calmodulin containing complexes might then occur at extremely low Ca\(^{2+}\) concentrations and even in the presence of chelators. Because the relevant concentrations would be well below the Ca\(^{2+}\) concentration in yeast cytosol (100–350 nM) (56–59), however, such an interaction would probably not mediate Ca\(^{2+}\)-dependent regulation but only a constitutive function of Ca\(^{2+}\). Thus, although we cannot fully exclude that Cmd1p might still bind some Ca\(^{2+}\) also in the microautophagic reaction, it appears that such potential Ca\(^{2+}\)-binding would probably not contribute to a regulatory function in vacuolar invagination. This conclusion is consistent with previous reports suggesting that cmd1Δ, mutants defective for Ca\(^{2+}\)-binding behave normally under conditions inducing macro- and microautophagy (such as starvation) or heat shock (33).

We propose that Cmd1p acts in different associations in microautophagy and in vacuole fusion. Several observations support this hypothesis. First, it can be expected for structural reasons because apocalmodulin and Ca\(^{2+}\)-calmodulin bind target proteins differently. They utilize different binding motifs, the IQ motif and noncontiguous binding sites, respectively (reviewed in Ref. 39). Second, Ca\(^{2+}\)-binding to calmodulin reduces its \(\alpha\)-helical content from 69 to 10% (67). This dramatic conformational change is likely to favor interaction with different proteineous partners. Third, the inhibitor and antibody concentration required to block microautophagy are higher than the concentrations required to inhibit vacuole fusion (49). Finally, vacuole-associated mutant calmodulin could not be readily exchanged for soluble wild type calmodulin in microautophagic uptake, whereas this was possible for vacuole fusion (49). Thus, the fraction of Cmd1p active in microautophagy is not equivalent to that required for vacuolar fusion, i.e. Cmd1p has different targets in these reactions, or it has more than one target in at least one of these processes.

Potential Cmd1p-interacting proteins in vacuolar preparations have been identified in previous publications. These included the V-ATPase components Vph2p, Vph1p, Vma6p, Vma2p, and Vma3p and a V-ATPase associated component, Vtc4p (68, 69). The V-ATPase acidifies the vacuolar lumen and is a source of the electrochemical gradient across the vacuolar membrane. The maintenance of a proton gradient across the vacuolar membrane is necessary for microautophagy (16, 17). However, an interaction of calmodulin with the V-ATPase is probably unrelated to its role in microautophagic vacuole invagination because this interaction depends on Ca\(^{2+}\) and is sensitive to BAPTA (68). In contrast, the vacuolar Vtc complex, which consists of Vtc1p, Vtc2p, Vtc3p, and Vtc4p, contains putative IQ and IQ-like motifs (on Vtc2p and Vtc3p). IQ motifs can bind calmodulin in a Ca\(^{2+}\)-independent fashion. In a separate study, we have investigated the role of the Vtc complex in microautophagic uptake. Inactivation of the complex by deletion and by antibodies led to severe reductions of uptake activity. This makes the Vtc complex a bona fide target for Cmd1p in microautophagic membrane invagination.

We could determine the kinetic stage of Cmd1p action. Cmd1p acts rather late in microautophagy. It is important for the invagination process itself, both for building autophagic tubes and for their correct structural organization. However, once autophagic tubes could form in a preincubation period, final scission of vesicles into the vacuolar lumen is insensitive to calmodulin inhibition. Based on these results we have integrated the requirement of calmodulin into our current model of
microautophagy (Fig. 9). Our data places the calmodulin requirement between stages II and III of the reaction sequence, i.e. at the end of the phase ascribed to membrane invagination. This is consistent with a role as a binding partner of the Vtc complex for several reasons. Previous studies have indicated that a phase separation takes place on the autophagic tube, leading to a depletion of membrane integral proteins and an enrichment of lipids on the invagination (15). On the other hand, recent studies in our group provided evidence for a role of the Vtc complex in vacuolar lipid metabolism3 and for a requirement of specific lipids on the invagination (15). Therefore, we propose that the Vtc complex is one site of a Ca2+-independent action of calmodulin for vacuolar membrane invagination. It will be interesting to investigate the precise effects of calmodulin on the Vtc complex in future studies of its molecular structure in combination with binding and mutagenesis studies.

It remains an enigma what finally drives the scission of autophagic bodies into the vacuolar lumen. Although topologically equivalent to homotypic vacuole fusion scission of microautophagic vesicles is independent of proteins with a central role in homotypic vacuole fusion, such as SNAREs and Sec18p/NSF (16). There are also kinetic differences. GTPγS inhibits the final step of fusion pore opening in vacuole fusion, whereas calmodulin is required for the preceding step inducing hemifusion (70–72). In contrast, calmodulin acts after the GTPγS-sensitive step in microautophagic vacuole invagination.

Scission of clathrin-coated vesicles, mitochondria, or chloroplasts depends on dynamin-like GTPases. These can form oligomeric collars that surround the neck of nascent vesicles (73–77). However, these processes are topologically different from budding during microautophagy. The curvature that needs to be induced for scission of an endocytic vesicle is opposite to that required for scission of a microautophagic vesicle into the lumen of the vacuole. Therefore, it seems unlikely that the scission mechanisms are identical. If dynamin-like GTPases worked as active “pinches,” surrounding and squeezing the neck of the nascent vesicle, they would have to do that from the luminal side of the vesicles in microautophagic vacuole invagination, which contradicts the pinchase concept. The vacuolar surface carries the dynamin-like GTPase Vps1p, which is required for vacuole fragmentation and fusion (78). The polymeric state of dynamin is required for its role in endocytotic membrane scission (73–77). GTPγS leads to depolymerization of Vps1p in vitro (78) but affects microautophagic uptake only at a very early stage rather than at the late scission of vesicles (Figs. 5, 6, and 9). For all of these reasons, Vps1p is unlikely to act in the scission of microautophagic vesicles by mechanically promoting scission. It leaves open the possibility, however, that Vps1p could regulate other components involved in microautophagic membrane invagination. Such regulatory roles have also been discussed for dynamin-mediated scission of endocytic vesicles (79).

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Microautophagic Vacuole Invagination Requires Calmodulin in a Ca$^{2+}$-independent Function
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