Four stages during microautophagy in vitro

Determination of four sequential stages during microautophagy in vitro

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Summary

Microautophagy is the transfer of cytosolic components into the lysosome by direct invagination of the lysosomal membrane and subsequent budding of vesicles into the lysosomal lumen. This process is topologically equivalent to membrane invagination during multivesicular body formation, and to the budding of enveloped viruses. Vacuoles are lysosomal compartments of yeasts. Vacuolar membrane invagination can be reconstituted in vitro with purified yeast vacuoles, serving as a model system for budding of vesicles into the lumen of an organelle. Using this in vitro system, we defined different reaction states. We identified inhibitors of microautophagy in vitro and used them as tools for kinetic analysis. This allowed us to characterize four biochemically distinguishable steps of the reaction. We propose that these correspond to sequential stages of vacuole invagination and vesicle scission. Formation of vacuolar invaginations was slow and temperature dependent whereas the final scission of the vesicle from a preformed invagination was fast and proceeded even on ice. Our observations suggest that the formation of invaginations rather than the scission of vesicles is the rate limiting step of the overall reaction.
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Introduction

The lysosome is a major lytic compartment in eukaryotic cells (1). Substrates are delivered to its lumen by several pathways: via endocytosis, via direct transport across the lysosomal membrane and via autophagy (2-4). Autophagy, defined as non-selective uptake and degradation of cytoplasm in the lysosome, can occur either as macro- or as microautophagy. In macroautophagy, nascent autophagosomes engulf parts of the cytoplasm and subsequently fuse with the lysosome. During microautophagy, vesicles bud into the lysosomal lumen by direct invagination of the boundary membrane, resulting in degradation of both cytoplasmic components and lysosomal membrane. Microautophagy can lead to the degradation of soluble components, or to the selective uptake of entire organelles, as exemplified by peroxisome degradation (micropexophagy). In order to distinguish these two forms, which we presume to follow different mechanisms, we refer to the microautophagic uptake of soluble components as type I and to that of organelles as type II.

Vacuoles are the lysosomal compartment of the yeast Saccharomyces cerevisiae. Tubular invaginations leading to type I microautophagy have been observed both in living yeast cells and in isolated vacuoles (5). These membrane tubules are induced upon starvation. They are dynamic and often branched structures showing a sharp kink of the vacuolar membrane at the site of invagination. As the lumen of the tubules is continuous with the cytoplasm, budding of vesicles from the tip of the tubules results in unilamellar autophagic bodies containing cytosol, destined for degradation by vacuolar hydrolases. Because of a lateral heterogeneity along the autophagic tubes, with a high density of transmembrane particles at the base and a smooth zone at the tip where budding occurs, the autophagic bodies reaching the vacuolar lumen are largely devoid of transmembrane particles. The process of type I vacuole invagination and vesicle formation can be reconstituted in vitro using purified...
yeast vacuoles (6). Solute uptake can be quantified using luciferase as a reporter enzyme. Luciferase uptake depends on ATP, salt concentration, temperature and cytosol. Cytosolic extracts prepared from starved cells stimulate luciferase uptake by isolated vacuoles with an activity twofold higher than that of extracts from nonstarved cells. Cytosolic extracts from starved atg-mutants support the in vitro-reaction to similar extent as extracts from nonstarved wild type cells. This observation suggests that Atg proteins may be involved in regulation of type I microautophagy, but not in the uptake reaction itself. Microautophagy does not depend on known factors for vacuole fusion and vesicle trafficking, such as Sec17p/[-SNAP, Sec18p/NSF, or the SNAREs Vam3p, Vam7p or Nyv1p. Scission of the invaginated membrane hence occurs via a mechanism distinct from homotypic vacuole fusion.

Pioneering genetic screens have identified many genes required for macroautophagy (3,4,7-31) and for autophagic peroxisome degradation (30-33). They have shed light on the mechanisms underlying these important protein degradation pathways. However, mutants selectively defective in microautophagy of soluble cytosolic components have not been identified so far. No method for the selective quantitation of type I microautophagy in living cells is available other than morphological observation of vacuolar membrane invaginations (5,6). In order to further characterize microautophagic vesicle formation, we used a cell free system that reconstitutes type I vacuole invagination and we performed time course experiments to dissect the reaction into four steps.
Experimental procedures

*Chemicals* - Nystatin, amphotericin, FCCP and valinomycin were purchased from Sigma, Deisenhofen, Germany. Nocodazol, colchicine, rapamycin, K252a and aristolochic acid were purchased from Alexis, Gruenberg, Germany. GTP\[S\] was purchased from Roche Molecular Biochemicals, Mannheim, Germany. All other reagents were analytical grade. Drugs were suspended as 50x or 100x stock solution in DMSO and stored at -20°C. Nystatin stock solutions were renewed after 1 week of storage.

*Yeast culture, cytosol preparation and vacuole preparation* were performed as described previously (6). For storage of vacuoles, protease inhibitors (0.2 mM pefabloc SC, 0.2 µg/ml leupeptin, 1 mM α-phenanthroline, 1 µg/ml pepstatin A) and glycerol (10 % w/v from a 50% stock) were added to a fresh vacuole suspension. The suspension was frozen as little nuggets in liquid nitrogen and stored at -80°C for a maximum of 3 weeks.

*In vitro microautophagy assay* - A standard reaction had a volume of 50 µl and was composed of: vacuoles (0.2 mg/ml, strain DBY5734 (6), either freshly prepared or thawed from a -80°C stock), 3 mg/ml cytosol from starved K91-1A-cells (6), 105 mM KCl, 7 mM MgCl\(_2\), 2 mM ATP, 80 mM disodium creatine phosphate, 175 U/ml creatine kinase, 17 µg/ml luciferase, 100 µM DTT, 0.1 mM pefabloc SC, 0.1 µg/ml leupeptin, 0.5 mM α-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 150 mM KCl in PS buffer (200 mM sorbitol, 10 mM PIPES/KOH pH 6.8), centrifuged (6800 g, 4 min, fixed angle table top centrifuge), the pellet washed once more with 150 mM KCl in PS buffer and resuspended in 50 µl 150 mM KCl in PS buffer. Proteinase K was added (0.3 mg/ml from 10x stock) and incubated on ice for 10 min.
Digestion was stopped by adding 50 µl 1 mM PMSF/150 mM KCl in PS buffer. Luciferase activity was determined using an assay kit according to the manufacturer’s instruction (Berthold detection systems, Pforzheim, Germany): 25 µl sample were mixed with 25 µl lysis buffer and 25 µl substrate A (containing ATP). 25 µl Substrate B (1 mM luciferin) 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 (6). Uptake activity was calculated as the quotient of luciferase activity over alkaline phosphatase activity (counts per second/ \( \text{OD}_{405} \) per min) and referred to an uninhibited standard reaction (60 min, 27°C), which was set to 100%. In some experiments, horseradish peroxidase (40 µg/ml) was used instead of luciferase. For activity determination, a 25 µl-sample (vacuoles after washing and proteinase K-digestion, in PS buffer/ 1 % triton X 100) was mixed in an opaque 96-well microtiter plate with 25 µl substrate (5 mM luminol, 10 mM H\(_2\)O\(_2\), 100 mM Tris/HCl pH 8.5, freshly prepared) and 25 µl cosubstrate (200 µM luciferin, 100 mM Tris/HCl pH 8.5). Luminescence was measured in a microplate luminometer (LB 96 V, Berthold Technologies, Bad Wildbad, Germany) and background activity (vacuoles without horseradish peroxidase) was subtracted. Uptake activity was calculated as described above.

**Thin section electron microscopy** - Yeast cells were cryofixed using a propane-jet freezing device (JFD 030, Bal-Tec, Balzers AG) and freeze-substituted in 0.5% uranyl acetate in ethanol at -90°C for 35 h, at -60°C for 4 h, and -50°C for 2 h in a freeze-substitution unit (FSU 010, Bal-Tec, Balzers AG). After washing with ethanol at -35°C, the samples were infiltrated with Lowicryl HM20 and UV-polymerized at -35°C for 48 h. Ultrathin sections stained with uranyl acetate and lead citrate were viewed in a Philips CM 10 electron microscope.
Results

One of the most important approaches to explore complex biochemical processes is kinetic analysis. A dissection into distinguishable reaction phases can serve to ascribe the interactions of relevant components and their dynamic changes to defined steps of the overall process and greatly facilitates the formulation of hypotheses on the molecular mechanism. Therefore, the identification of intermediates has always been an important and fruitful part in the analysis of complex biochemical reactions. In order to better understand vacuolar membrane invagination we sought to identify distinct reaction stages for this process. Since proteins and lipids involved in vacuolar membrane invagination are not known yet we attempted to generate tools for kinetic analysis by identifying low molecular weight inhibitors. Such pharmacological approaches ("chemical genetics") have proven useful to dissect complex physiological phenomena (34). If, as for vacuoles, sufficient quantities can be prepared, relevant target proteins for the inhibitors can even be identified by direct fractionation of the membrane on affinity matrices, or by crosslinking approaches (35-40). In a pilot study we screened 46 commercially available low molecular weight substances for inhibitory activity in the previously described (6) in vitro microautophagy reaction. These compounds were selected because they target processes known to be involved in membrane transport, such as protein phosphorylation, lipid metabolism, membrane fluidity and cytoskeleton rearrangements. The majority of the agents (26 out of 46) did not influence microautophagy in vitro at all (Tab. 1).

13 of the 46 agents showed effects on the in vitro uptake assay, but they were not pursued further because they affected luciferase uptake only at unreasonably high concentrations, inhibited luciferase activity or lysed vacuoles (Tab. 2).
Seven of the substances (Tab. 3) showed nearly complete inhibition of luciferase uptake without affecting vacuolar integrity, as judged by light microscopy and by reversibility of inhibitor action (see below; Fig. 4). None of these inhibitors had any effect if added after the uptake reaction, confirming that vacuoles were not lysed by the inhibitors.

Inhibition of luciferase uptake by these drugs depended on their concentration (Fig. 1 A) but also on the presence of cytosol. Type I microautophagy in vitro is strongly stimulated by the addition of cytosol (6). In reactions lacking cytosol, uptake persisted, albeit only with 20% of the activity of the control samples. Under these conditions none of the agents listed in table 1 impaired uptake any more (Fig. 1 B). Uptake in the absence of cytosol was still ATP-dependent, indicating that the signal measured had been generated by an energy-dependent process. Since the action of all inhibitors tested required cytosol, these substances might prevent binding of cytosolic factors to the vacuolar membrane.

The absence of effects of microtubule-directed drugs such as nocodazole and colchicine is notable since vacuoles are very large organelles and occupy up to a third of the total cell volume. Our favoured hypothesis on vacuolar membrane invagination had been that long filaments, such as microtubules, might push towards the vacuolar lumen, deforming the vacuolar boundary membrane. This hypothesis appeared particularly attractive since microtubules are very dynamic structures in yeast, growing and shrinking vigorously in the course of the cell cycle. Furthermore, they can be close to the vacuolar membrane (41). Microtubule destabilizing agents did not inhibit vacuole invagination in vitro (Fig. 2 A), even at concentrations high enough to suppress growth of intact cells by disassembly of the spindle (42; A. Mayer, unpublished observation). This suggested that vacuolar membrane invagination can occur independently of microtubules. Electron microscopic analysis of
starved yeast cells (Fig. 2 B) supported this conclusion. Cytoplasmic microtubules in yeast are organised exclusively by the spindle pole body. They emanate directly from this structure and are generally very short (43). If cytoplasmic microtubules initiated vacuolar invaginations the spindle pole bodies should be close to the site of invagination. However, microautophagic membrane invaginations could be observed on parts of the vacuolar membrane not facing the nucleus and the spindle pole body. Hence, vacuolar invaginations can obviously develop without the aid of microtubules.

Using the pharmacological inhibitors identified (Tab. 1) we could now attempt a kinetic analysis of type I microautophagy. In order to test whether the inhibitors affected different stages of vacuolar invagination, a standard uptake reaction was started. Inhibitors were added at different time points (Fig. 3) and the reaction was continued until the end of a standard incubation period. Agents influencing very early steps of type I microautophagy would be ineffective if added late during the reaction, whereas agents acting on late events of microautophagy would be active throughout the reaction. As a control, samples were transferred to ice, a treatment which blocks many processes depending on membrane or protein dynamics (44,45). Inhibitors could be grouped into two categories according to their kinetic properties. Inhibition curves of FCCP/valinomycin, rapamycin and K252a developed almost in parallel to each other, crossing the ice curve at 35 min and keeping their inhibitory potential throughout the incubation (Figs. 3 A,C,E). We designate these as late acting (class B) inhibitors. In contrast, the reaction had become largely resistant to class A inhibitors (GTP\textsuperscript{S}, nystatin and aristolochic acid) after 30 min, resulting in a curve convex towards the top (Fig. 3 B,D,F). The ice curve showed a very shallow slope during the first 30 min but a steep rise between 30 and 45 min, indicating that most of the luciferase uptake had taken place during this relatively short period of time. Inhibition by transfer on ice became ineffective late during the reaction because, after 45 min, class B inhibitors
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(FCCP/valinomycin, rapamycin, K252a) still blocked further luciferase uptake efficiently but transfer on ice did not. The kinetics shown thus define three reaction states: 1. GTP$^\text{S}$, nystatin and aristolochic acid sensitive; 2. cold sensitive; 3. FCCP/valinomycin, rapamycin and K252a sensitive.

In order to exclude the possibility that the inhibitors had damaged the vacuoles nonspecifically we tested whether an inhibitory block could be reversed by removal of the inhibitor. Two-step reactions were performed: During a first 30 min incubation, inhibitors were present in a complete microautophagy reaction. The agents were then removed by sedimenting the vacuoles, discarding the supernatant and resuspending the membranes in fresh medium. Aliquots of these samples were either set on ice, or they were incubated at 27°C in the presence or absence of an inhibitor. This second incubation was stopped after 60 min and luciferase uptake was determined in all samples (Fig. 4). During the first incubation without inhibitor at 27°C, only about 20% of the total signal was generated, consistent with the very shallow slope of the ice curve during this period of time (Fig. 3). All inhibitors were reversible by reisolating and washing the membranes, except nystatin (Fig. 4 G). The yellow nystatin partitions into membranes (46) and is retained by vacuoles even after reisolation, as evident from the intense staining of the organelles after nystatin treatment (not shown).

Uptake was almost completely abolished if the same inhibitors as in the first incubation were added to the second stage (bar 2). This indicates that the membranes had not passed the initial block during reisolation and resuspension. Vacuoles lost about half of their uptake potential if the first incubation was performed either on ice or in the presence of inhibitor (compare Fig. 4 B and Figs. 4 A, C-H), i.e. under all conditions that prevented uptake. The reason for this effect is unclear. It is possible that addition of ATP and cytosol renders accessory vacuolar components more labile and prone to inactivation if the reaction cannot proceed.
Next, we tested whether there would be stable reaction intermediates. If such intermediates existed the reaction step leading to these intermediates should be essentially irreversible. Then, it should be possible to accumulate these intermediates in the presence of late acting (class B) inhibitors. These intermediates should have passed early reaction steps and therefore be resistant to early acting (class A) inhibitors. After release from a class B block, the vacuoles should thus complete the reaction in the presence of class A inhibitors. If stable intermediates did not exist, however, a reaction blocked by class B inhibitors should remain sensitive to subsequent class A inhibition. Since most inhibitors were reversible we could test whether stable intermediates might accumulate. Vacuoles were incubated with different inhibitors for 30 minutes, reisolated and then incubated for 60 min with class A or class B inhibitors (Fig. 5 C-F). There was no resistance to any inhibitor if the vacuoles were reisolated after 30 minutes, even if the preincubation had been performed in the absence of any inhibitors. Thus, a stable reaction intermediate did not exist. This was unexpected because an undisturbed reaction – performed without reisolating and resuspending the vacuoles – became resistant to class A inhibitors within this period of time (Fig. 3). Since vacuole invagination generates large and complex membrane deformations, we assume that the mechanical stress coinciding with reisolation and resuspension may destabilize such a structure and destroy an intermediate resistant to class A inhibitors.

Microautophagic uptake into the vacuole requires membrane invagination followed by scission of a vesicle (5). In the in vitro reaction most luciferase uptake occurred in a 15 minutes period following an initial lag of 30 min (Fig. 3), suggesting that biochemical changes were necessary to prepare the vacuolar membrane for subsequent rapid microautophagic uptake. Notably, the formation of tubular invaginations showed a lag period of about 30 minutes that corresponds well to the lag in luciferase uptake (6). We asked whether, once an invagination had formed during the lag period, rapid formation of a vesicle
and luciferase uptake could be assayed as a separate event. We examined the potential for such rapid uptake by incubating vacuoles in complete reaction mixture, but without luciferase. During this initial incubation, all biochemical steps required to generate a membrane invagination could proceed, but an uptake signal would not be generated due to lack of the reporter. After 60 min at 27°C, samples were supplemented with luciferase. Vesicle formation and luciferase uptake would only be scored from this time onwards. After incubation for 2 min, vacuoles were diluted, reisolated, washed and digested as usual.

Luciferase uptake was quantified (Fig. 6 A). Uptake activity of a standard reaction containing luciferase throughout a 60 min incubation was determined in parallel to serve as a reference. Approximately 40% of the uptake activity in this reference sample could also be observed in the sample incubated with luciferase for only 2 min. Although this proportion varied between different batches of vacuoles, it was consistent within one experiment. Rapid uptake after preincubation of the vacuoles was not only observed using luciferase as a reporter enzyme, but was equally efficient with horseradish peroxidase (Fig. 6 B). This excludes an effect relying on special properties of luciferase.

Luciferase that had been taken up rapidly could not be distinguished from luciferase that had been taken up in the course of 60 minutes. Both were protected from digestion by proteinase K, which was added at the end of every uptake reaction to degrade luciferase on the surface of vacuoles or in solution. Furthermore, both were rapidly degraded by intravacuolar proteases. To measure this degradation, we reisolated the vacuoles at the end of an uptake reaction in order to remove luciferase that had not been taken up. We incubated the vacuoles further in the absence of luciferase and monitored luciferase activity after different periods of chase (Fig. 6 C,D). Chase of the vacuoles in isotonic medium led to a rapid decline of luciferase activity. This decline could be prevented by lysing the vacuoles by a brief osmotic shock, followed by readjustment to isotonic conditions prior to the chase incubation.
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Osmotic lysis results in a strong dilution of the vacuolar hydrolases and of luciferase taken up into these vacuoles, slowing down its degradation. In contrast, degradation of luciferase that had not been taken up into the vacuolar lumen, which might occur via contaminating or accidentally released proteases, should at best be stimulated by osmotic release of additional vacuolar proteases. Sensitivity of luciferase degradation to osmotic vacuole lysis thus demonstrates complete transfer of this reporter into the vacuolar lumen, even by the rapid uptake reaction. Rapid uptake was also sensitive to the same inhibitors as the standard reaction occurring over one hour (Fig. 7). Therefore, both modes of uptake result in full transfer of proteins from the medium into the vacuolar lumen by the same mechanism. This mechanism includes the generation of a state of competence which requires a large fraction of the total reaction time. Since microautophagic uptake into vacuoles comprises both the formation of vacuolar membrane invaginations and the scission of vesicles from their tips (5,6), we propose that this state of competence reflects the formation of a vacuolar invagination and that rapid uptake reflects the scission of vesicles from a preformed invagination.

We exploited this fast mode of uptake to further investigate the sequence of events. If any of the inhibitors identified above was present during the 60 min preincubation without luciferase, the rapid uptake reaction was blocked (Fig. 7, light bars). The same was true for lack of ATP or cytosol during the preincubation. If the 60 minutes preincubation was performed in the absence of inhibitors, but inhibitors were added at the end of the preincubation directly before addition of luciferase, rapid uptake was hardly influenced (Fig. 7, dark bars). Only rapamycin and K252a, two late-acting class B inhibitors (Fig. 3), were still able to reduce luciferase uptake by 50%. All other inhibitors, including the class B inhibitor FCCP/valinomycin that showed the same kinetic characteristics as rapamycin and K252a in an undisturbed standard reaction (Fig. 3), could influence uptake only during preincubation.
Therefore, the rapid uptake reaction can be used to dissect the late phase of type I microautophagy in vitro. It allows the preceding, FCCP/valinomycin sensitive step to be distinguished from the last step that is influenced by rapamycin and K252a.
Discussion

We used the inhibitors of in vitro microautophagy to identify four stages in this model reaction (Fig. 8). Stage I is defined by sensitivity to GTP\(\gamma\)S, aristolochic acid and nystatin, stage II is sensitive to cooling, stage III to FCCP/valinomycin, and stage IV to K252a and rapamycin. We utilized these drugs primarily as convenient tools for the kinetic dissection of in vitro microautophagy. Identification of their real targets would require numerous alternative approaches. However, several hints about these targets can be extracted from the inhibitor effects. Targets for the active inhibitors might be GTPases (GTP\(\gamma\)S), sterols and membrane fluidity (nystatin), and, via phospholipases, the composition of membrane phospholipids (aristolochic acid). An obvious candidate for a GTPase, Ypt7p, a Rab-GTPase involved in homotypic vacuole fusion, is not required for type I microautophagy (6). Nystatin exerts its fungicidal activity at least partially by forming pores in the plasma membrane and disrupting the membrane potential (47). It inhibits homotypic vacuole fusion in vitro by interfering with priming, the ATP-dependent activation of vacuolar SNAREs by Sec17p/Sec18p. Inhibition was related to the binding of ergosterol by nystatin, but was not due to disruption of the vacuolar membrane potential (38). As nystatin concentrations used in our study were about ten times lower than the concentrations used for inhibiting vacuole fusion (38), inhibition of type I microautophagy by nystatin cannot be explained by disruption of the vacuolar membrane potential. This notion is confirmed by the observation that disrupting the vacuolar membrane potential by FCCP/valinomycin affects a much later stage in luciferase uptake than does nystatin (Fig. 3). Unlike in vacuolar fusion, where nystatin inhibits SNARE priming (38), type I microautophagy cannot be influenced by this effect because type I microautophagy is independent of SNAREs (6). We propose that nystatin most likely interferes with type I microautophagy by sequestering ergosterol that may have a direct role in membrane invagination and/or membrane scission. This interpretation is consistent
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with morphological studies on type I microautophagic membrane invagination in yeast which demonstrated that the vesicles pinching off into the lumen of the vacuole are drastically depleted of integral membrane proteins (5) and show freeze fracture structures typical for pure lipid bilayers, suggesting that vacuolar membrane invagination might be a lipid driven process (5).

Progression of type I microautophagy can be stopped by chilling on ice up to stage II. At later stages, luciferase uptake is still influenced by K252a, FCCP/valinomycin and rapamycin, but can no longer be stopped by cooling. A significant proportion of the actual uptake reaction can even occur on ice (Fig. 6). Based on known properties of membrane bilayers we can speculate on possible explanations. Cooling of phospholipid membranes can result in a phase transition (48). Such phase transitions can increase the rigidity of membrane bilayers and make them less fusogenic (48, 49). However, there are also examples, particularly of highly curved bilayers, such as small liposomes, which become more fusogenic below the phase transition temperature (50, 51). The phase transition may render the bilayer structure incompatible with the imposed curvature and favour fusion processes that relieve these conformational restraints. If this were the case for the highly curved tubular membrane invaginations associated with type I microautophagy, low temperatures would allow and possibly favour vesicle formation.

Stage III depends on electrochemical gradients, as indicated by its sensitivity to the protonophor FCCP and the ionophor valinomycin. While in an undisturbed reaction the activities of FCCP/valinomycin kinetically overlapped with those of K252a and rapamycin (Fig. 3), they could be dissected in an assay that selectively measured rapid luciferase uptake following a preincubation period. This rapid uptake, which occurs only after a state of competence has been created, was insensitive to FCCP/valinomycin, but still sensitive to
K252a and rapamycin (Fig. 7 B). These properties thus distinguish an additional step of the reaction that we term stage IV. The precise role of electrochemical gradients in type I microautophagy remains to be elucidated. Possibly, some cytoplasmic factors have to associate with the vacuolar membrane in a membrane potential dependent manner in order to allow vesicle budding. This could explain why FCCP/valinomycin, but also other inhibitors, work only in the presence of cytosol (Fig. 1 B). Some cytoplasmic proteins, such as actin, can associate with the vacuolar membrane only if the membrane potential is undisturbed (J. Kunz, unpublished observation). The functional significance of this effect is under investigation.

Stage IV is the only stage in type I microautophagy that can experimentally be separated from the preceding steps by reisolating the vacuoles (Fig. 7 A). It depends on completion of these preceding stages and can be prevented by all inhibitors blocking stages I-III. Once stages I to III have been completed, stage IV, the actual uptake reaction, can be influenced only by rapamycin and K252a (Fig. 7 B). This final uptake reaction no longer depends on cytosol or ATP (Fig. 7 A). In the course of this last step of in vitro microautophagy the reporter enzyme becomes inaccessible to external proteases and susceptible to vacuolar lumenal proteases. Thus, stage IV corresponds to the scission of vesicles into the vacuolar lumen. In our in vitro system such a process might be favoured by cooling and mechanical stress during reisolation or vortexing. Since stages I-III are necessary to establish competence for the final uptake reaction we propose that molecular events in these stages involve the formation of vacuolar invaginations and/or the formation of the vesicular expansion that can be observed at the tip of the tubes prior to vesicle scission (5). This hypothesis is consistent with the observation that the formation of vacuolar invaginations occurs with a lag period of 20-30 min (6), a time frame that correlates well with the lag period of luciferase uptake (20-30 min) and with the development of resistance to inhibitors of stages I and II (20-30 min). Resistance to FCCP/valinomycin (stage III) is attained significantly
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later. Therefore, we cannot attribute this event to tube formation or vesicle scission with certainty at the moment. The electrochemical gradient across the vacuolar membrane may favour vesicle scission in an undisturbed reaction at 27°C but become dispensable upon cooling, perhaps due to membrane rearrangements discussed above. Alternatively, stage III may represent a late stage of tube formation or vesicular expansion at the tip of the tube.

In the present study we have identified several inhibitors of vacuolar membrane invagination and vesicle scission that could be assigned to four kinetic stages. It is obvious that, following an approach of "chemical genetics", this pilot screen can be expanded to larger compound libraries. Identified inhibitors may serve as tools to isolate components necessary for microautphagic membrane invagination, e.g. by means of affinity chromatography or chemical crosslinking. When combined with the kinetic resolution of different reaction stages, this approach may pave the way to a more detailed understanding of this complex reaction.
Acknowledgements

We thank Christa Baradoy for assistance, members of the Mayer lab for critical discussions and Alexandra Moreno-Borchardt for critically reading the manuscript. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 446, A.M.) and Studienstiftung des Deutschen Volkes (J.K.)
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Footnotes

1 The abbreviations used are: apg, autophagy; aut, autophagocytosis; DMSO, dimethysulfoxide; FCCP, carbonylcyanide-p-trifluormethoxyphenylhydrazone; HRP horseradish peroxidase; IC_{50/90} inhibitor concentration yielding 50/90% inhibition; OD_{405}, optical density at 405 nm; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethysulfonyl fluoride; PS, PIPES/sorbitol; UV, ultraviolet;
Fig. 1. Titration of inhibitors of \textit{in vitro}-microautophagy and cytosol dependence.

\textbf{A} Titration: Inhibitors were added (from 50x- to 100x stocks) to standard \textit{in vitro}-microautophagy reactions at the indicated concentrations. For FCCP+valinomycin, the concentration of valinomycin was kept constant at 1 µM. After a 60 min reaction at 27°C, luciferase uptake was determined. Uptake after a 60 min incubation on ice (<5% of uninhibited uptake) was subtracted from all values; uptake activity in the uninhibited reaction was set to 100%.

\textbf{B} Cytosol dependence: \textit{In vitro}-microautophagy reactions were performed with (3 mg/ml, upper panel) or without (lower panel) cytosol. Inhibitors were added as follows: 5 µM FCCP+1 µM valinomycin, 1 mM GTP\textsubscript{$\gamma$S}, 40 µM rapamycin, 100 µM K252a, 500 µM aristolochic acid, 3 µM nystatin. After a 60 min incubation, cytosol was added in order to render all samples chemically equal, vacuoles were washed and luciferase uptake was determined. Uninhibited uptake was set to 100 %. Note that the absolute uptake activity without cytosol was only about 20% of that with cytosol. Data shown are the mean of 4 experiments with standard deviation.

Fig. 2. Invaginations of the vacuolar membrane form independently of microtubules.

\textbf{A} \textit{In vitro}-microautophagy reactions were performed in the presence of nocodazole or colchicine and assayed for activity. \textbf{B} Thin section of a BJ3505 cell (\textit{pep4}; E. Jones, Carnegie Mellon University, Pittsburgh, PA) that had been starved for 3 h in SD-N, quick-frozen in liquid propane, freeze substituted in 0.5% uranyl acetate and embedded in Lowicryl HM20. The section shows an invagination of the vacuolar membrane (>) originating far away from the spindle pole body.

Fig. 3. Kinetics of inhibition of \textit{in vitro}-microautophagy.

For each curve, a 6x standard reaction mixture was incubated. At the indicated timepoint, a 50
µl sample was withdrawn. The indicated inhibitors were added from a 50x stock solution and the 60 min incubation was completed (resulting in X min without inhibitor followed by 60-X min with inhibitor). For the “ice”-curve, samples were put on ice for the rest of the incubation instead of adding inhibitor; for the control curve, DMSO without inhibitor was added at the indicated timepoints. After completion of the 60 min incubation, vacuoles were washed and luciferase uptake was determined. Data shown are the mean of four independent experiments with standard deviation. To facilitate comparison the ice curve and control curve are shown in all diagrams.

Fig. 4. Reversibility of inhibition

A-H 3x standard reactions were preincubated with the indicated inhibitors (as in Fig. 3) for 30 min. Vacuoles were reisolated (3 min, 8000 g, 4°C, fixed angle table top centrifuge) and the supernatant containing the inhibitor was discarded. Vacuoles were resuspended in complete reaction medium and split into three aliquots. One 50 µl aliquot was diluted immediately (300 µl 150 mM KCl in PS buffer) and put on ice to measure the uptake that had taken place during the first incubation (1). The second aliquot received the same inhibitor as in the preincubation. The sample was incubated with the inhibitor for another 60 min at 27°C (2). The third aliquot was incubated for 60 min at 27°C without inhibitor being added, measuring the luciferase uptake after reversion of a block induced by the inhibitor during the first incubation (3). After a total incubation time of 90 min, all samples were processed for measuring uptake activity. Uptake activity without any inhibitor being present during both incubations (b, 2+3) was set to 100%. Data are the mean of four independent experiments with standard deviation.

Fig. 5. Test for stable intermediates

A-F. Two-step reactions were performed as in Fig. 4. Different inhibitors were present in the
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first and the second incubation. Inhibitor concentrations used were: 7.5 µM FCCP, 45 µM rapamycin, 100 µM K252a, 750 µM aristolochic acid, 3 µM nystatin, 1 mM GTP[S]. The inhibitors present in the second incubation are indicated under the corresponding bars. In each diagram, uptake activity in the absence of inhibitor in the second incubation was set to 100%. Data were averaged from two independent experiments with standard deviation.

**Fig. 6. Rapid uptake of luciferase.**

Standard in vitro-microautophagy reactions were performed with either (A) luciferase or (B) horseradish peroxidase (HRP) as reporter enzymes. In parallel, reactions were started without either enzyme. After 60 min incubation on ice or at 27°C, these samples were put on ice. Luciferase or horseradish peroxidase were added and vortexed. After 2 min incubation, the vacuoles were washed and digested by proteinase K as usual. Data shown are the mean from a double determination.

**C,D** Exposure to vacuolar hydrolases. Standard uptake reactions (C) or rapid uptake reactions (D) were performed as described in A without protease inhibitors. Vacuoles were reisolated and either suspended in isotonic buffer A (90 mM KCl, 10 mM PIPES/KOH pH 6.8, 200 mM sorbitol, 0.5 mM Mg-ATP, 20 mM creatine phosphate, 125 µg/ml creatine kinase, 1 mM DTT, 1 mg/ml BSA) or lysed osmotically in water (5 min, on ice). After lysis, buffer A was added from a concentrated stock solution in order to make all samples chemically equal. Reactions were incubated at 30°C. At the time points indicated, samples were taken, supplemented with protease inhibitors (1 mM PMSF, 0.2 mM pefabloc, 1 mM o-phenanthroline, 1 µg/ml pepstatin A) and transferred on ice. Luciferase activity was measured as described in Experimental Procedures and referred to the initial value, which was set to 100%.

**Fig. 7. Inhibitor sensitivity of rapid uptake.**
In vitro-microautophagy reactions without luciferase were incubated for 60 min. Inhibitors (as in Fig. 3) had been added to these samples either before (light bars) or after (dark bars) this 60 min preincubation period. Then, luciferase was added to all samples (2 min), vacuoles were washed and digested and luciferase uptake was determined. Two experiments with double determination were averaged.

**Fig. 8.** Schematic overview of the kinetic steps of microautophagic vacuole invagination. The assignment of the biochemically defined steps to the morphological changes represents our current working hypothesis.
Tab. 1 Low molecular weight inhibitors without effect on *in vitro* microautophagy.

| inhibitor                                                                 | concentration tested |
|--------------------------------------------------------------------------|----------------------|
| nocodazole                                                               | 200 µM               |
| colchicine                                                               | 1 mM                 |
| inositol-1,4,5-trisphosphate                                             | 500 µM               |
| adenosphostin                                                            | 50 µM                |
| R59949 (diacylglycerolkinase inhibitor)                                   | 200 µM               |
| methylarachidonoylf fluorophosphonate                                    | 500 µM               |
| farnesyl transferase inhibitor (Alexis Nr. 290-005)                      | 2 mM                 |
| perillic acid                                                            | 1 mM                 |
| oleic acid                                                               | 300 µM               |
| myristoyllysophatidylcholine                                             | 100 µM               |
| cholesterol                                                              | 2 mM                 |
| caffeine                                                                 | 5 mM                 |
| 3-methyladenine                                                          | 5 mM                 |
| phorbol-12-myristate-13-acetate                                          | 1 mM                 |
| KN62 (protein kinase inhibitor)                                          | 1 mM                 |
| KT5833 (protein kinase inhibitor)                                        | 500 µM               |
| H9 (protein kinase inhibitor)                                            | 1 mM                 |
| GDP[S]                                                                   | 1 mM                 |
| NaF                                                                      | 2 mM                 |
| Na3VO4                                                                   | 200 µM               |
| sodiumpyrophosphate                                                     | 200 µM               |
| deltamethrin                                                            | 1 mM                 |
| cantharidine                                                            | 1 mM                 |
| microcystin LR                                                           | 100 µM               |
| calyculine A                                                             | 20 µM                |
| ocadaic acid                                                            | 20 µM                |
Tab. 2 Non-specific inhibitors of \textit{in vitro} microautophagy. IC$_{50}$ (inhibitor concentration yielding 50% inhibition) and IC$_{90}$ (inhibitor concentration yielding 90% inhibition) and their side effects in the \textit{in vitro} reaction.

| inhibitor          | side effect and/or IC$_{50}$ |
|--------------------|-------------------------------|
| edelfosine         | IC$_{50}$=100 µM; vacuole lysis |
| cyclosporin        | IC$_{50}$=100 µM; published IC$_{50}$=10 nM (52) |
| neomycin           | IC$_{50}$=250 µM; vacuoles difficult to resuspend |
| U73122             | IC$_{50}$=150 µM; maleimido group inactivates luciferase |
| manoalid           | IC$_{50}$=5 µM; vacuoles cannot be sedimented |
| podophyllotoxin    | IC$_{50}$=250 µM; published IC$_{50}$=12 nM (53) |
| paclitaxel         | IC$_{90}$>500 µM; published IC$_{50}$=12 nM (53) |
| polymyxin B        | IC$_{50}$=500 µM; detergent-like properties at this concentration |
| staurosporin       | IC$_{50}$=200 µM; published IC$_{50}$=2.7 nM (54) |
| mellitin           | vacuole lysis above 10 µM |
| F48/80             | inhibition also if added after uptake reaction |
| phospholipase A2   | vacuole lysis |
| phenylarsinoxide   | inhibits luciferase by inactivating SH-groups |

Tab. 3 Inhibitors of \textit{in vitro} microautophagy. IC$_{50}$ (inhibitor concentration yielding 50% inhibition) and IC$_{90}$ (inhibitor concentration yielding 90% inhibition) were determined by titration in the \textit{in vitro} invagination reaction (Fig. 1).

| inhibitors and their effect in other model systems | effect on \textit{in vitro} microautophagy |
|---------------------------------------------------|------------------------------------------|
|                                                   | IC$_{50}$ | IC$_{90}$ |
| GTP[S ; inhibits \textit{in vitro} vacuole fusion at 1 mM (35) | 200 µM | 1 mM |
| FCCP; dissipates vacuolar membrane potential at 10 µM (35) | 2 µM | 10 µM |
| nystatin; inhibits \textit{S. cervisiae} growth at 7 µM (55) | 1 µM | 3 µM |
| amphotericin B; same action and potency as nystatin (47) | 2 µM | 5 µM |
| rapamycin; induces macroautophagy at 0.2 µM (56) | 20 µM | 40 µM |
| K252a; inhibits hepatic autophagy with IC$_{50}$=30 µM (57) | 50 µM | 200 µM |
| aristolochic acid; inhibits phospholipase A2, IC$_{50}$=400 µM (58) | 250 µM | 750 µM |
Titration of inhibitors of in vitro microautophagy

- FCCP
- FCCP+1 µM
- Valinomycin
- K252a
- aristolochic acid
- nystatin
- amphotericin B
- rapamycin
- GTPγS

Action of inhibitors in presence and absence of cytosol

+ cytosol

- cytosol

Kunz et al., Fig. 1
Kunz et al., Fig. 3
Reversibility of inhibitors

| Step | Description |
|------|-------------|
| 1    | Dilute, incubate on ice (60') |
| 2    | Add inhibitor, incubate at 27°C (60') |
| 3    | Incubate without inhibitor at 27°C (60') |

| 1 | 2 | 3 |
|---|---|---|
| a | b | c | d | e | f | g | h |
| ice | none | GTPγS | aristolochic acid | rapamycin | FCCP | nystatin | K252a |

Uptake activity (%)

| 0 | 20 | 40 | 60 | 80 | 100 |
|---|----|----|----|----|-----|
| 1 | 2 | 3 |

Kunz et al., Fig. 4
Kunz et al., Fig. 5
a) Luciferase

b) Horseradish peroxidase

c) Degradation of luciferase after a 60 min uptake reaction

d) Degradation of luciferase after rapid uptake

Kunz et al., Fig. 6
Kunz et al., Fig. 7
Stage

0  I  II  III  IV

Nystatin
GTPγS
Aristolochic acid

0°C
Valinomycin/
FCCP

K252a
Rapamycin

Kunz et al., Fig. 8
