A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation

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Accepted 9 July 2001
Journal of Cell Science 114, 3619-3629 (2001) © The Company of Biologists Ltd

SUMMARY

Autophagy is a normal degradative pathway that involves the sequestration of cytoplasmic portions and intracellular organelles in a membrane vacuole called the autophagosome. These vesicles fuse with lysosomes and the sequestered material is degraded. Owing to the complexity of the autophagic pathway and to its inaccessibility to external probes, little is known about the molecular mechanisms that regulate autophagy in higher eukaryotic cells. We used the autofluorescent drug monodansylcadaverine (MDC), a specific autophagolysosome marker to analyze at the molecular level the machinery involved in the autophagic process. We have developed a morphological and biochemical assay to study authophagy in living cells based on the incorporation of MDC. With this assay we observed that the accumulation of MDC was specifically induced by amino acid deprivation and was inhibited by 3-methlyadenine, a classical inhibitor of the autophagic pathway. Additionally, wortmannin, an inhibitor of PI3-kinases that blocks autophagy at an early stage, inhibited the accumulation of MDC in autophagic vacuoles. We also found that treatment of the cells with N-ethylmaleimide (NEM), an agent known to inhibit several vesicular transport events, completely blocked the incorporation of MDC, suggesting that an NEM-sensitive protein is required for the formation of autophagic vacuoles. Conversely, vinblastine, a microtubule depolymerizing agent that induces the accumulation of autophagic vacuoles by preventing their degradation, increased the accumulation of MDC and altered the distribution and size of the autophagic vacuoles. Our results indicate that in the presence of vinblastine very large MDC-vacuoles accumulated mainly under starvation conditions, indicating that the expansion of autophagosomes is upregulated by amino acid deprivation. Furthermore, these MDC-vacuoles were labeled with LC3, one of the mammalian homologues of the yeast protein Apg8/Aut7 that plays an important role in autophagosome formation.

Key words: Autophagy, Vinblastine, Starvation, NEM-sensitive protein, PI3-kinase, LC3

INTRODUCTION

Autophagy is one of the major pathways for degradation of intracellular macromolecules in animal cells (Klionsky and Emr, 2000). It is a physiological intracellular process that plays a key role in the turnover of long-lived proteins, RNA and other cytoplasmic macromolecules (Seglen et al., 1991; Mortimore et al., 1996; Mortimore et al., 1989). Autophagocytosis can be induced by several conditions, including starvation, hormone treatment (Mortimore and Schworer, 1977; Lardeux and Mortimore, 1987; Van Sluijters et al., 2000) and stress (Schwartz et al., 1992).

Morphological and biochemical studies have shown that macroautophagy is a process that occurs in several steps. First, cytoplasm and various organelles such as endoplasmic reticulum, mitochondria and peroxisomes are sequestered by a membrane to form an autophagosome. Nascent autophagosomes are wrapped by a double smooth membrane (a phagophore) of a still unclear origin, although some evidence indicates that these structures originate from ribosome-free regions of the endoplasmic reticulum (Dunn, 1990a). Early autophagosomes lose the inner membrane, become acidic, and acquire lysosomal membrane proteins and enzymes. The sequestered material is finally degraded in vesicles called autophagolysosomes or autolysosomes (Dunn, 1990b).

Evidence has been provided suggesting that communication between the endocytic and autophagic pathways exists. Biochemical studies have indicated that autophagosomes can fuse with endosomes to form amphisomes (Berg et al., 1998). Morphologically, hepatocytic amphisomes are autophagosome-like vacuoles containing endocytic markers, such as endocytosed colloidal gold particles, but lacking lysosomal enzymes. Moreover, it has been shown that multilamellar endosomes fuse with autophagosomes in HeLa cells (Lucocq and Walker, 1997).

In contrast to other pathways of vesicular transport such as the endocytic and exocytic pathways, the autophagic route to lysosomes is still poorly characterized. Previous studies have shown that GTP-binding proteins are required in autophagic

vacuole formation (Kadowaki et al., 1996; Petiot et al., 2000). Specifically, a heterotrimeric Gi3-protein controls autophagic sequestration in HT-29 cells (Ogier-Denis et al., 1995; Ogier-Denis et al., 1996). It has also been shown that the PI3-kinase inhibitors wortmannin and LY294002 stop autophagy at the sequestration step in rat hepatocytes (Blommaart et al., 1997). Furthermore, in a recent publication evidence has been presented that the autophagic pathway is differentially controlled by class I and class III PI3-kinases (Petiot et al., 2000). Among the mechanisms controlling autophagy, protein phosphorylation plays an important role. Autophagic sequestration is reduced by tyrosine protein kinase inhibitors. It has been reported that the phosphorylation of ribosomal protein S6 and autophagy are controlled by the same signal transduction pathway (Holen et al., 1995; Blommaart et al., 1995; Van Sluijters et al., 2000).

In yeast, remarkable progress has been achieved in describing the mechanism of autophagy at the molecular level (Noda et al., 1995; Klionsky and Emr, 2000). Applying a genetic approach, a variety of autophagy defective mutants have been described such as apg 1-15 and aut 1-8 (Tsukada and Ohsumi, 1993; Thumm et al., 1994). A novel conjugation system essential for autophagy has been described in both yeast (Mizushima et al., 1998a; Shintani et al., 1999) and human (Mizushima et al., 1998b). Furthermore, it has been shown that Tor, a phosphatidylinositol kinase negatively regulates autophagy in yeast (Noda and Ohsumi, 1998) and also in mammalian cells.

Owing in part to the complexity of the autophagic pathway and the limited accessibility to external probes, little is known at the molecular level about the machinery involved in autophagy in higher eukaryotic cells. Most approaches have used proteins or radiolabeled sugars microinjected by electropermeabilization (Seglen et al., 1986) as probes. It has been reported that the autofluorescent drug monodansylcadaverine (MDC) is a selective marker for autophagic vacuoles (Biederbick et al., 1995). These authors have shown that cytoplasmic vacuoles can be labeled by MDC in vivo in different cell types. When the MDC-fluorescent fraction was separated in a sucrose gradient and analyzed by EM, typical autophagic vacuoles were observed (Biederbick et al., 1995), indicating that MDC specifically accumulates in these type of vacuoles. However, neither the regulation nor the requirements for MDC accumulation in autophagic vacuoles have been assessed. In order to analyze the molecular machinery involved in the autophagic process in mammalian cells, we have developed a morphological and biochemical assay to study authophagy in living cells based on the incorporation of MDC. With this assay autophagy can be monitored easily and accurately by measuring the fluorescence of MDC incorporated by the cells under different experimental conditions.

In order to better understand autophagy, we have used drugs that affect trafficking and/or sorting events. We tested wortmannin (WM), a PI3-kinase inhibitor, and microtubule depolymerizing agents (e.g. vinblastine) on the incorporation of MDC. We also tested the sulfhydryl-alkylating reagent N-ethylmaleimide (NEM), an agent that inhibits vesicular transport due to, at least in part, the inactivation of NSF, a general component of the transport machinery (Woodman, 1997). Our results indicate that, whereas WM and NEM abrogated the formation of autophagic vacuoles, vinblastine induced the formation of very large autophagic vacuoles in a starvation-dependent manner, indicating that the expansion of autophagosomes is upregulated by amino acid deprivation, making the autophagic process physiologically more efficient.

MATERIALS AND METHODS

Materials

Minimal essential medium (α-MEM) and fetal bovine serum were obtained from Gibco Laboratories (Grand Island, NY). Vinblastine was obtained from ICN (Costa Mesa, CA). The carbocyanine DiOC6 and LysoTracker were from Molecular Probes (Eugene, OR). All other reagents were from Sigma Chemical Co. (St Louis, MO). A rabbit antibody raised against endoplasmic reticulum membrane proteins was a generous gift from Bruno Goud (Curie Institute, Paris, France). The pEGFP-LC3 plasmid was kindly provided by Noboru Mizushima and Tamotsu Yoshimori (National Institute for Basic Biology, Okazaki, Japan).

Cell culture

Chinese hamster ovary (CHO) cells were grown in minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), at 37°C in an atmosphere of 95% air and 5% CO_2, in 12-well plates to 80% confluence. HepG2 (human hepatoma) cells were grown in RPMI-1640 (Sigma) supplemented with 5% FBS.

Autophagy induction

Autophagy was induced by amino acid starvation. Cells were washed three times with PBS and incubated with 1 ml Earle's balanced salts solution (EBSS) (Sigma) at 37°C for different lengths of time in the presence or the absence of the drugs. In some experiments, cells were pre-treated with the inhibitors before induction of autophagy.

Labeling of autophagic vacuoles with monodansylcadaverine (MDC)

Following induction of autophagy by amino acid starvation, the cells were incubated with 0.05 mM MDC in PBS at 37°C for 10 minutes (Biederbick et al., 1995). After incubation, cells were washed four times with PBS and collected in 10 mM Tris-HCl, pH 8 containing 0.1% Triton X-100. Intracellular MDC was measured by fluorescence photometry (excitation wavelength 380 nm, emission filter 525 nm) in a Packard Fluorocount microplate reader. To normalize the measurements to the number of cells present in each well, a solution of ethidium bromide was added to a final concentration of 0.2 μM and the DNA fluorescence was measured (excitation wavelength 530 nm, emission filter 590 nm). The MDC incorporated was expressed as specific activity (arbitrary units).

Visualization of MDC-labeled vacuoles

Autophagic vacuoles were labeled with MDC by incubating cells grown on coverslips with 0.05 mM MDC in PBS at 37°C for 10 minutes. After incubation, cells were washed four times with PBS and immediately analyzed by fluorescence microscopy using an inverted microscope (Nikon Eclipse TE 300, Germany) equipped with a filter system (excitation filter V-2A: 380-420 nm, barrier filter: 450 nm). Images were obtained with a CCD camera (Orca I, Hamamatsu) and processed using the program Meta View, version 4.5 (Universal Images Coorporation).

Labeling of acidic compartments with Lysotracker

Acidic compartments were labeled by incubating the cells with 1 μM LysoTracker (Molecular Probes) in the culture media for 10 seconds at RT. After incubation, cells were washed with PBS and immediately analyzed by fluorescence microscopy using the

following filter system: excitation filter 510-560 nm, barrier filter 590 nm.

Indirect immunofluorescence for endoplasmic reticulum

Cells were fixed with 1 ml of 3% paraformaldehyde solution in PBS, for 15 minutes at room temperature (RT). Cells were washed with PBS and blocked twice by incubating with 0.1 M glycine in PBS for 10 minutes at RT. Cells were permeabilized with 0.01% saponin in PBS containing 0.2% BSA, and were then incubated with a rabbit antibody raised against endoplasmic reticulum membrane proteins (dilution 1:300). Bound antibodies were subsequently detected by incubation with goat anti-rabbit Texas Red-conjugated secondary antibody. Cells were mounted with 50% glycerol in PBS and analyzed by fluorescence microscopy.

Labeling of early and late endocytic compartments

Cells incubated under starvation conditions (EBSS media) for two hours were labeled with MDC as described above and subsequently incubated with 0.5 mg/ml Dextran tetramethylrhodamine (fluoro ruby) (Molecular Probes) internalized for 5 minutes to label early endocytic compartments. To label late endosomes, after the initial 5 minutes internalization of the endocytic marker, cells were washed and the probe was chased for 10 minutes. Subsequently, they were labeled with MDC. Cells were immediately analyzed by fluorescence microscopy.

Transient tranfection with GFP-LC3

CHO cells at 70% confluence were transfected with pEGFP-LC3 using the lipofectamine reagent (Gibco) according to the manufacturer's instructions. After 48 hours transfection cells were subjected to different treatments, labeled with MDC as described above and analyzed by fluorescence microscopy.

RESULTS

Monodansycadaverine (MDC)-accumulation increases during starvation

It has been reported that MDC is a specific marker for autolysosomes (Biederbick et al., 1995). However, the regulation and the molecular machinery required for MDC accumulation has not been assessed. Therefore, we studied the incorporation of MDC in cells where autophagy was stimulated by amino acid deprivation, a physiological stimulus of this process. CHO cells were incubated in α-MEM medium (control cells) or in EBSS medium (starved cells) at 37°C for two hours. Following this incubation period, both starved and control cells were incubated with MDC 0.05 mM for 10 minutes at 37°C and then washed four times with cold PBS pH 7.4. Cells were analyzed by fluorescence microscopy. As shown in Fig. 1A, starved cells showed an increase in the number of vesicles as well as in their size, indicating that starvation induced the formation of the MDC-labeled vacuoles. MDC was concentrated in spherical structures distributed in the cytoplasm and also localized in the perinuclear region, probably corresponding to the microtubule organizing center.

Intracellular MDC was measured by fluorometry (see Materials and Methods). Two hours starvation increased MDC uptake more than twice compared with control cells (Fig. 1B). Similar results were obtained with HepG2, a hepatocytic cell line (data not shown). To analyze the kinetics of MDC uptake, CHO cells were incubated in EBSS medium for different periods of time and subsequently labeled with MDC. Thirty minutes of amino acid deprivation was enough to double the amount of MDC that accumulated in the starved cells (Fig. 1C). This is consistent with previous results indicating that autophagy is rapidly induced following amino acid deprivation (Dunn, 1990a). After 90 minutes of starvation a steady state of MDC incorporation was reached. The accumulation of MDC was almost completely prevented by adding to the starvation media a 4× solution of a mixture of amino acids (data not shown) confirming that the incorporation of MDC was

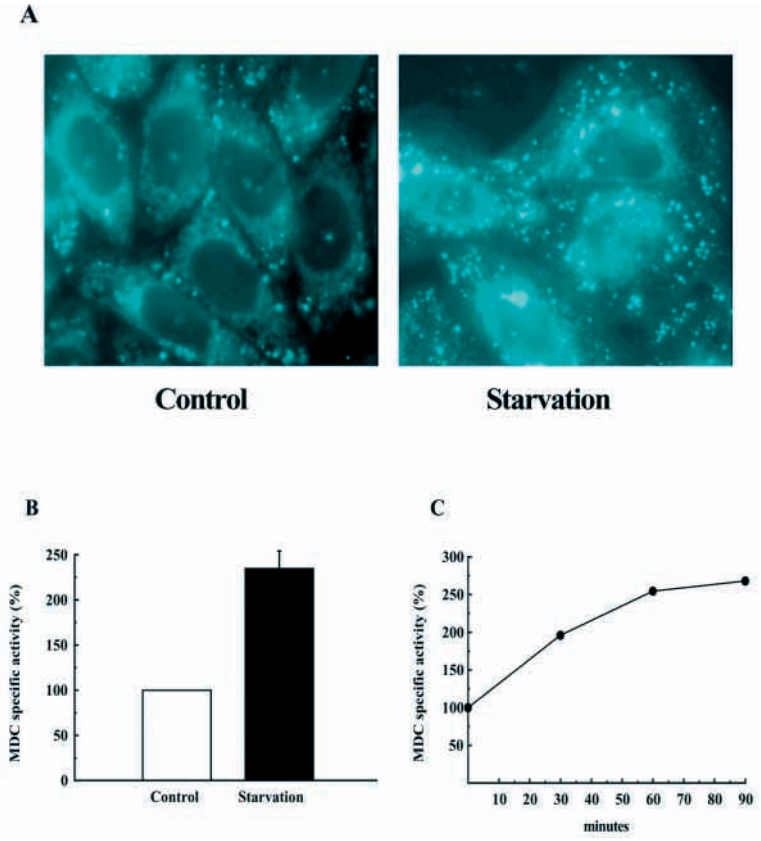

Fig. 1. Monodansycadaverine (MDC)-labeled vesicles are induced by starvation. (A) CHO cells were incubated in α-MEM medium (control cells) or in EBSS medium (starved cells) at 37°C for two hours. Following this incubation period, both starved and control cells were incubated with MDC at 0.05 mM for 10 minutes at 37°C and then washed four times with PBS pH 7.4. Cells were immediately analyzed by fluorescence microscopy using an inverted microscope as described in Materials and Methods. Images were obtained with a CCD camera and processed using the Meta View program. (B) Cells were incubated in α-MEM medium (control cells) or in EBSS medium (starved cells) at 37°C for two hours. Following this incubation period, both starved and control cells were incubated with MDC 0.05 mM for 10 minutes at 37°C, washed four times with PBS pH 7.4, and then lysed in 10 mM Tris-HCl, pH 8 containing 0.1% Triton X-100. Intracellular MDC was measured by fluorescence photometry as indicated in Experimental Procedures. The data represent the mean±s.e.m. of at least three independent experiments. (C) CHO cells were incubated in starvation medium for different periods of time. MDC labeling and measurement was performed as in B. Data are representative of at least three independent experiments.

specifically induced by amino acid deprivation.

To characterize the MDC-labeled structures we carried out co-localization studies with various cellular markers in cells incubated under starvation condition. A marked co-localization was observed with Lysotracker, which accumulates in acidic compartments and lysosomes (Fig. 2A-C), indicating that several of the MDC-labeled vacuoles have acquired an acidic pH. To investigate whether endosomes overlap with MDC-labeled autophagic vacuoles we have internalized rhodamine-conjugated dextran for 5 minutes to label early endosomes. Fluorescence microscopy showed rodhamine-dextran in punctated spherical structures that did not co-localize with MDC (Fig. 2D-F). This result indicates that MDC-vacuoles are distinct from the early endosome compartment. To label late endocytic compartments the endocytic marker was internalized for 5 minutes. Cells were then washed and the probe was chased during 10 minutes. As shown in Fig. 2G-I the fluid phase marker chased to late endocytic compartments partially co-localized with the MDC-containing vacuoles. This is consistent with previous observations that the autophagic and endocytic pathways converge after the early endosome level (Gordon and Seglen, 1988; Tooze et al., 1990).

Since it has been postulated that autophagosomes originate from ribosome-free endoplasmic reticulum membranes (i.e. isolation membrane, phagophore) we used an antibody against ER proteins. It is important to mention that for immunofluorescence studies cells are fixed and fixation decreases the MDC staining. Therefore, MDC-structures are not visualized as clearly as in non-fixed cells. As shown in Fig. 2K, a typical ER reticular pattern was observed; however, the marker was not detected on the limiting membrane of the MDC-labeled vesicles, as in the case of LC3 (see below). In addition, we have used the carbocyanine DiOC6 to identify ER membranes in living cells. A similar reticular distribution was observed and no overlapping with MDC-vesicles was detected (data not shown).

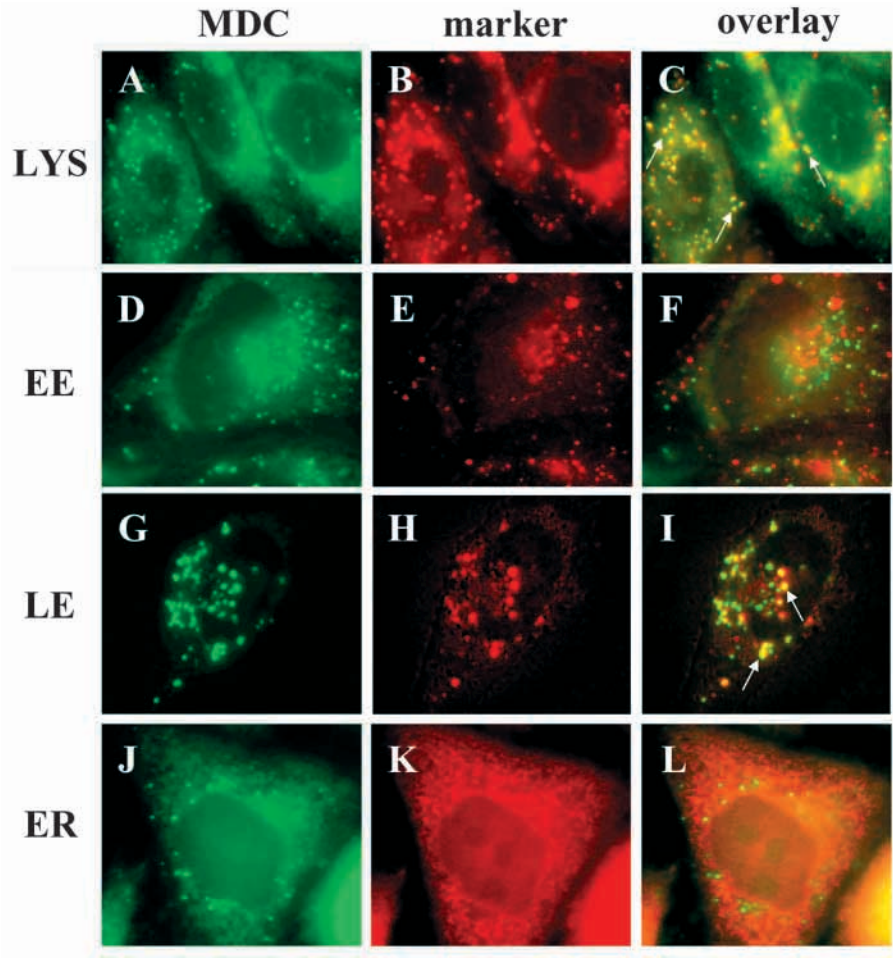

Fig. 2. Characterization of the MDC-labeled vacuoles. CHO cells were incubated in EBSS medium (starvation) at 37°C for two hours. Following this incubation period, cells were labeled with MDC as described in Fig.1 and then incubated with 1 μM LysoTracker (LYS) for 10 seconds at RT. Cells were washed with PBS and immediately analyzed by fluorescence microscopy using the following filter system: excitation filter 510-560 nm, barrier filter 590 nm. MDC-labeled vacuoles are displayed in green and LYS in red. Arrows indicate some of the structures showing overlapping in the merged images (A-C). To label early endocytic compartments (EE), cells incubated under starvation conditions for two hours, were labeled with MDC and subsequently incubated with 0.5 mg/ml dextran tetramethylrhodamine for 5 minutes (D-F). To label late endosomes (LE), after the initial 5 minutes internalization of the endocytic marker, cells were washed and the probe was chased for 10 minutes before labeling with MDC. Cells were immediately analyzed by fluorescence microscopy (G-I). Endoplasmic reticulum (ER) was detected by indirect immunofluorescence (J-L). Cells were fixed, permeabilized and incubated with a rabbit antibody raised against ER membrane proteins (dilution 1:300) and with goat anti-rabbit Texas Red-conjugated secondary antibody. Cells were mounted with 50% glycerol in PBS and analyzed by fluorescence microscopy.

These results indicate that MDC accumulates in vacuoles of the autophagic pathway that are acidic and that partially overlap with late endocytic structures, but that are not labeled by ER markers, therefore they probably represent more mature compartments.

MDC incorporation is blocked by inhibitors of the autophagic pathway

It is known that 3-methyladenine (3-MA) is a specific inhibitor of early stages of the autophagic process (Seglen and Gordon, 1982). To test if the incorporation of MDC was indeed via autophagy, CHO cells were pre-incubated for 3 hours with 10 mM 3-MA and autophagy was induced by amino acid deprivation for 90 minutes. As expected, MDC incorporation was inhibited by 3-MA (Fig. 3B). An inhibitory effect on the incorporation of MDC was also observed by fluorescence microscopy (Fig. 3A).

It has previously been shown that phosphatidylinositol 3-kinases participate in the regulation of autophagy (Blommaart et al., 1997). To determine if the incorporation of MDC was

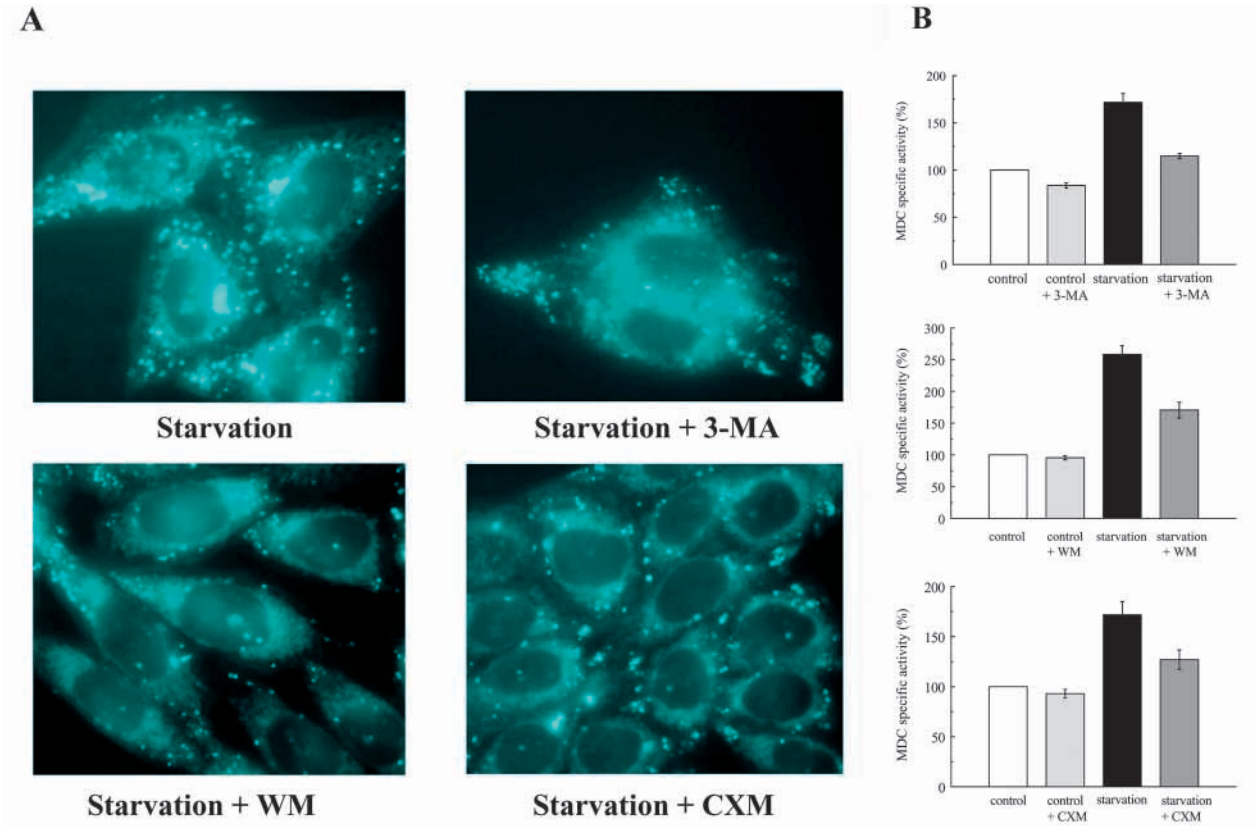

Fig. 3. The accumulation of MDC is inhibited by inhibitors of the autophagic pathway. CHO cells were incubated in α-MEM medium (control cells) or in EBSS medium (starved cells) for two hours in the absence or the presence of 10 mM 3-methyladenine (3-MA), 200 nM wortmannin (WM) or 2 μg/ml cycloheximide (CXM). In the case of 3-MA and CXM, cells were pre-incubated for 3 hours (3-MA) or 5 hours (CXM) with the corresponding inhibitor before the induction of autophagy. (A) Following the incubation period indicated above, cells were incubated with 0.05 mM MDC for 10 minutes at 37°C and then washed four times with PBS pH 7.4. Cells were immediately analyzed by fluorescence microscopy as indicated in Fig. 1. (B) Cells treated as indicated above were incubated with 0.05 mM MDC for 10 minutes at 37°C, washed four times with PBS pH 7.4, and then lysed in 10 mM Tris-HCl, pH 8 containing 0.1% Triton X-100. Intracellular MDC was measured by fluorescence photometry as indicated in Experimental Procedures. The data represent the mean±s.e.m. of two to four independent experiments.

also regulated by PI3-kinases we tested the effect of wortmannin (WM), a potent PI3-kinase inhibitor, on the formation of MDC-labeled vacuoles. Cells were incubated in α-MEM medium (control cells) and in EBSS (starvation medium) at 37°C in the presence or the absence of 200 nM WM. As shown in Fig. 4A, WM inhibited the accumulation of MDC in starved cells. Furthermore, we analyzed the effect of WM by fluorescence microscopy. Fig. 4B shows that the accumulation of MDC in autophagic vacuoles was substantially inhibited by WM.

Taken together, these results indicate that the accumulation of MDC-labeled vesicles correlates with the induction of autophagy by amino acid deprivation, and is abrogated by proven inhibitors of the autophagic pathway such as 3-MA and WM. Therefore, autophagocytosis can be followed both biochemically and morphologically in living cells using this system based on the incorporation of MDC.

To test if protein synthesis was required for the autophagy-dependent accumulation of MDC, CHO cells were pre-incubated with cycloheximide for 5 hours prior to induction of autophagy by amino acid deprivation. The results indicate that the starvation-induced accumulation of MDC was inhibited by pre-incubation with cycloheximide (Fig. 3A,B). By contrast, simultaneous induction of autophagy and treatment with cycloheximide for 1 hour did not affect MDC incorporation (data not shown). This result indicates that cycloheximide does not directly inhibit the accumulation of MDC but that the synthesis of a protein (or proteins) with a rapid turnover is required for the autophagic process.

The formation of autophagosomes requires an N-ethylmaleimide sensitive fusion protein

It is known that NSF, the N-ethylmaleimide (NEM)-sensitive fusion protein, is required for multiple transport events (Woodman, 1997; Colombo, 1996). ATP hydrolysis by NSF results in the disassembly of cis-SNARE complexes allowing the formation of trans-SNARE complexes and subsequent membrane fusion (Gerst, 1999; Woodman, 1997). The ATPase activity of NSF is abrogated by NEM, thereby inactivating NSF.

It has previously been shown that, similar to in vitro observations, treatment of intact CHO cells with NEM caused the accumulation of uncoated Golgi-transport vesicles (Orci et al., 1989), probably due to inactivation of endogenous NSF. To test if an NEM-sensitive protein was required in the autophagic pathway we treated the cells with NEM. Fig. 4 shows that pre-

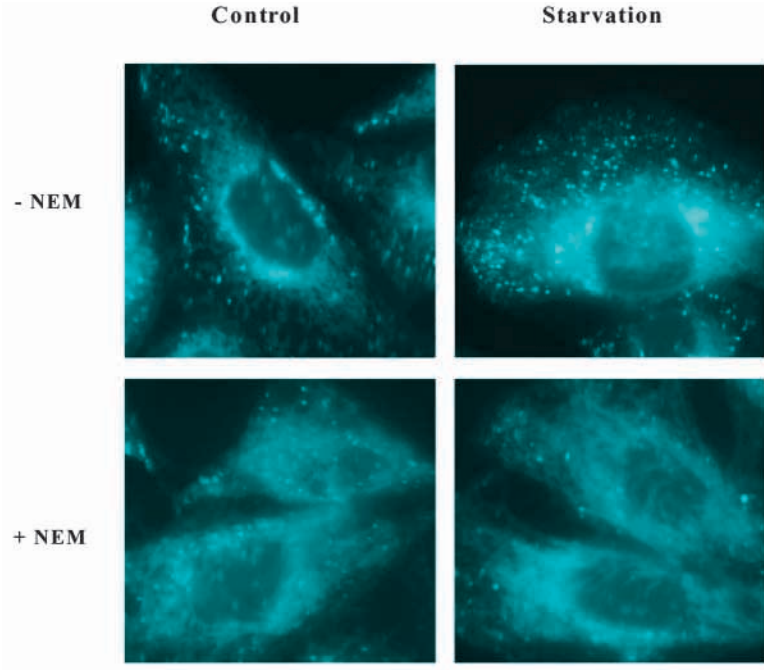

Fig. 4. N-ethylmaleimide (NEM) completely blocks the formation of MDC-labeled vacuoles. Cells were pre-incubated for 15 minutes in the presence or the absence of 50 μM NEM. Autophagy was subsequently induced by incubating the cells in starvation medium for 60 minutes. Cells were labeled with 0.05 mM MDC for 10 minutes at 37°C and then washed four times with cold PBS pH 7.4. Cells were immediately analyzed by fluorescence microscopy as indicated in Fig. 1.

incubation of the cells with 50 μM NEM completely eliminated the starvation-dependent accumulation and labeling of the vesicles with MDC. This result suggests that an NEM-sensitive protein (e.g. NSF) is required for the initial steps in the autophagic pathway, such as the sequestration step, although it is likely that it might be required for other events such as fusion with late endocytic/lysosomal compartments. Since NEM may affect several proteins, we cannot discard the possibility that another NEM-sensitive protein(s) might be required.

Vinblastine alters the distribution and size of MDC-labeled vesicles

It has been shown that the microtubule disrupting agent vinblastine blocks the autophagic-lysosomal pathway at some point subsequent to autophagosome formation (Gordon and Seglen, 1988; Hoyvik et al., 1986). Vinblastine depolymerizes microtubules involved in the intracellular transport of endosomes and lysosomes, thus preventing fusion between autophagosomes and the lysosomal compartment. To examine the effect of microtubule depolymerizing agents on the accumulation of MDC, CHO cells were grown in full nutrient conditions in the presence or absence of 50 μM vinblastine. Vinblastine-treatment moderately increased the accumulation of MDC measured by fluorometry (data not shown). We also studied the accumulation of MDC by fluorescence microscopy in vinblastine-treated cells. As shown in Fig. 5C, vinblastine treatment increased the number of MDC-labeled vacuoles. Furthermore, the labeled vesicles presented an altered distribution, probably as a consequence of the disruption of the microtubule architecture. Cells were more rounded and the MDC vacuoles were uniformly distributed around the nucleus.

We further examined the effect of vinblastine on the formation of MDC-labeled autophagic vesicles in cells incubated under starvation. Under these conditions autophagy will be stimulated but the maturation and degradation of the autophagic vesicles will be prevented because vinblastine hampers fusion with the lysosome. To our surprise, as shown in Fig. 5D, there was a marked increase in the size of the labeled vacuoles, an effect that was inhibited by pre-incubation with 3-MA (Fig. 5F). We have used Metamorph software to quantify the number and size of the MDC-labeled vacuoles The data in Fig. 5G show the quantification of the images displayed in Fig. 5A-D, although similar results were obtained by quantifying more than 50 cells subjected to the same treatments. A marked increase in the number of vesicles was induced under starvation conditions; however, in the presence of vinblastine the number of vacuoles was only moderately increased, even in cells incubated in an amino acid free media. By contrast, the area media of the vesicles generated in the presence of vinblastine was increased. It is interesting to note that the increase in size of these autophagic vacuoles was mainly observed under starvation conditions (compare insets in Fig. 5C and D), suggesting that the expansion of autophagosomes is upregulated by a signal transduction mechanism that depends on amino acid deprivation. Since vinblastine blocks fusion between autophagosomes and lysosomes, the maturation and degradation of autophagic vacuoles is hampered, causing the accumulation of autophagosomes. The size of the vacuoles accumulated is increased, mainly under starvation conditions, because vinblastine does not block the formation and fusion of early stages of this pathway.

We have characterized the vacuoles generated in the presence of vinblastine under starvation condition, by co-localization with several subcellular markers. Similar to the results described above, the MDC-labeled vacuoles co-localized with Lysotracker (Fig. 6A-C). Endocytic markers were not detected in autophagic vacuoles after a short 5 minute uptake (Fig. 6D-F); however, few MDC-labeled vacuoles seem to be reached by rhodamine-dextran when the endocytic marker was chased for 15 minutes (Fig. 6G-I). Membranes of the endoplasmic reticulum were identified by indirect immunofluorescence. In Fig. 6K a typical reticular structure is observed with spaces that are occupied by the MDC-labeled vesicles, however the ER marker was not detected on the autophagosomal delimiting membrane (Fig. 6L).

It is known that the maturation of the autophagic vacuoles occurs in various steps. First, an early autophagosome is formed by closure of cup-shaped isolation membranes (Seglen et al., 1996; Mizushima et al., 2001). Second, a late autophagosome is formed upon acquisition of the acidifying H^+-ATPase and, finally, autophagolysosomes containing acid hydrolases are generated by fusion with pre-exisiting lysosomes (Dunn, 1990b). Since it is known that vinblastine blocks fusion between autophagosomes and lysosomes, our results indicate that MDC is able to label not only

autophagolysosomes but also more immature vesicles (e.g. late autophagosomes) that have an acidic pH. In earlier reports it has been shown that ER membrane markers could be observed only in membranes of early autophagosomes but disappeared in late autophagic vacuoles. Since we have not detected ER markers in the MDC-labeled vacuoles generated in the presence of vinblastine, it is likely that the earliest structures of the autophagosomal pathway such us the phagophore or isolation membranes are not labeled with MDC.

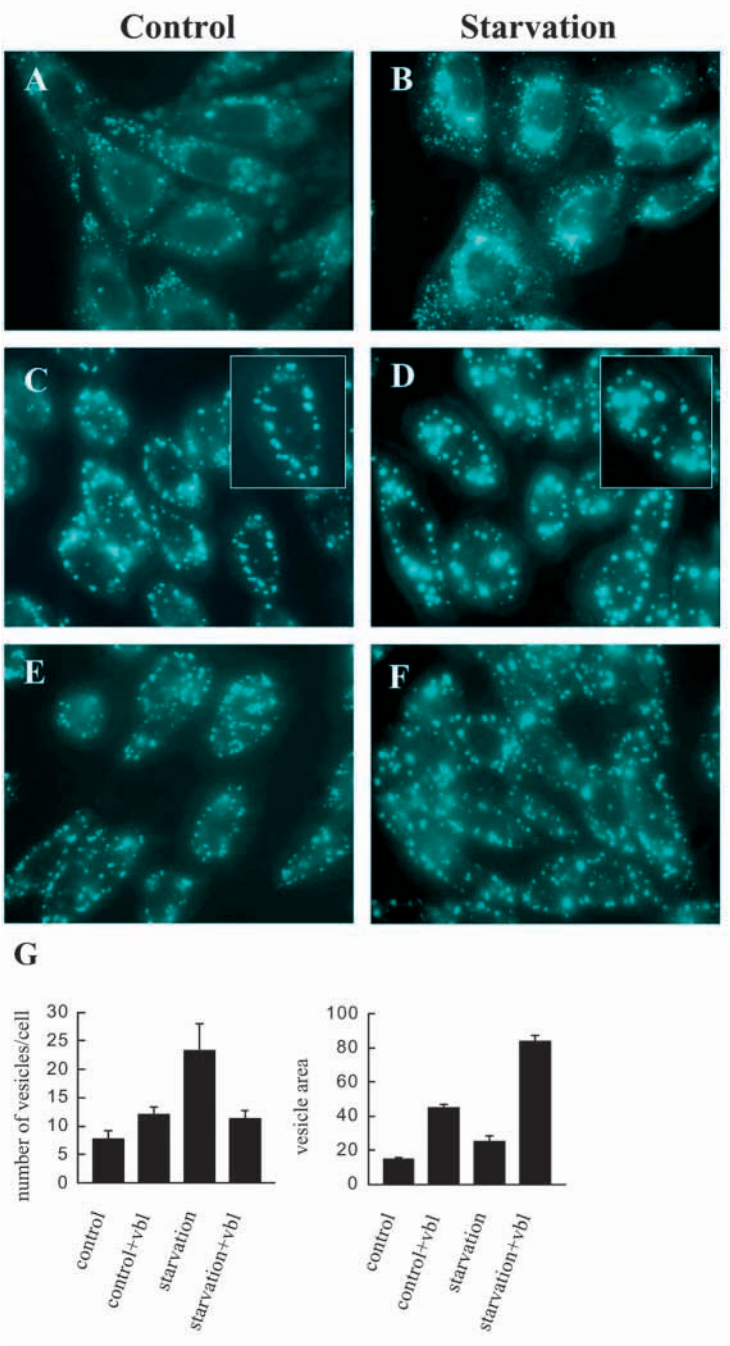

Fig. 5. Vinblastine-treatment causes a marked increase in the size of autophagic vacuoles. (A) CHO cells were incubated in full nutrient conditions (A,C,E) or in starvation medium (B,D,F) for 3 hours, in the presence (C,D,E,F) or absence (A,B) of 50 μM vinblastine. In E and F cells were pre-incubated for 3 hours with 10 mM 3-MA. Both starved and control cells were subsequently incubated with 0.05 mM MDC for 10 minutes at 37°C and then washed four times with cold PBS pH 7.4. Cells were immediately analyzed by fluorescence microscopy as indicated in Fig. 1. (G) The number and size of MDC-labeled vacuoles displayed in Fig. 5A-D were quantified by Methamorph software, using the integrated morphometry analysis. Data represent the mean±s.e.m.

MDC-vacuoles are labeled with LC3 a protein that associates with autophagosome membranes

Little is known about the molecules present in autophagosomal membranes in mammalian cells. Apg8/Aut7 is an essential protein for autophagy in yeast (Lang et al., 1998; Kirisako et al., 1999) that is upregulated by amino acid starvation (Kirisako et al., 1999). Three mammalian homologs have been identified: GABARAP (GABA receptor-associated protein), GATE-16 (Golgi associated ATPase enhancer of 16 kDa), and LC3 (microtubule-associated protein light chain 3) (Mann and Hammarback, 1994; Wang et al., 1999; Legesse-Miller et al., 2000). It has been shown that LC3 is a cytoplasmic protein that is processed into another form, LC3II, which associates with the autophagosome membranes (Kabeya et al., 2000; Mizushima et al., 2001). LC3II is present both inside and outside autophagosomes and the amount of this form increases by amino acid deprivation. To assess if the MDC-labeled vacuoles recruit LC3, we transiently overexpressed GFP-LC3 in CHO cells. As shown in Fig. 7, in cells incubated in complete medium (control) GFP-LC3 presented a diffuse distribution. By contrast, after incubation in an amino acid-free media for two hours, GFP-LC3 presented a large punctate pattern with several ring-shaped structures. Some of the brightest spots do not seem to overlap with MDC. However, several MDC-labeled vacuoles are clearly decorated by GFP-LC3 (inset) indicating that this protein binds peripherally to the delimiting membrane of these vacuoles. Furthermore, in cells incubated under starvation condition but in the presence of vinblastine, the large MDC-labeled vacuoles are also marked with GFP-LC3, suggesting an association of this protein with the delimiting membrane. These results confirm the autophagic nature of the MDC-labeled structures.

DISCUSSION

Autophagy is the major inducible pathway for the general turnover of cytoplasmic components (Dunn, 1990a; Dunn, 1990b; Klionsky and Emr, 2000). The initial event is the sequestration of cytoplasmic components and organelles in double-membrane structures (e.g. nascent autophagosomes) that probably originate from specialized regions of the endoplasmic reticulum. The maturation of autophagosomes appears to occur in a stepwise manner. The first step is the acquisition by the nascent autophagosome of lysosomal membrane-associated proteins but not lysosomal enzymes. Next, a proton-ATPase is incorporated and the compartment becomes acidified. Finally, lysosomal hydrolases are delivered to this maturing compartment and the intravacuolar materials are degraded (Dunn, 1990b). The

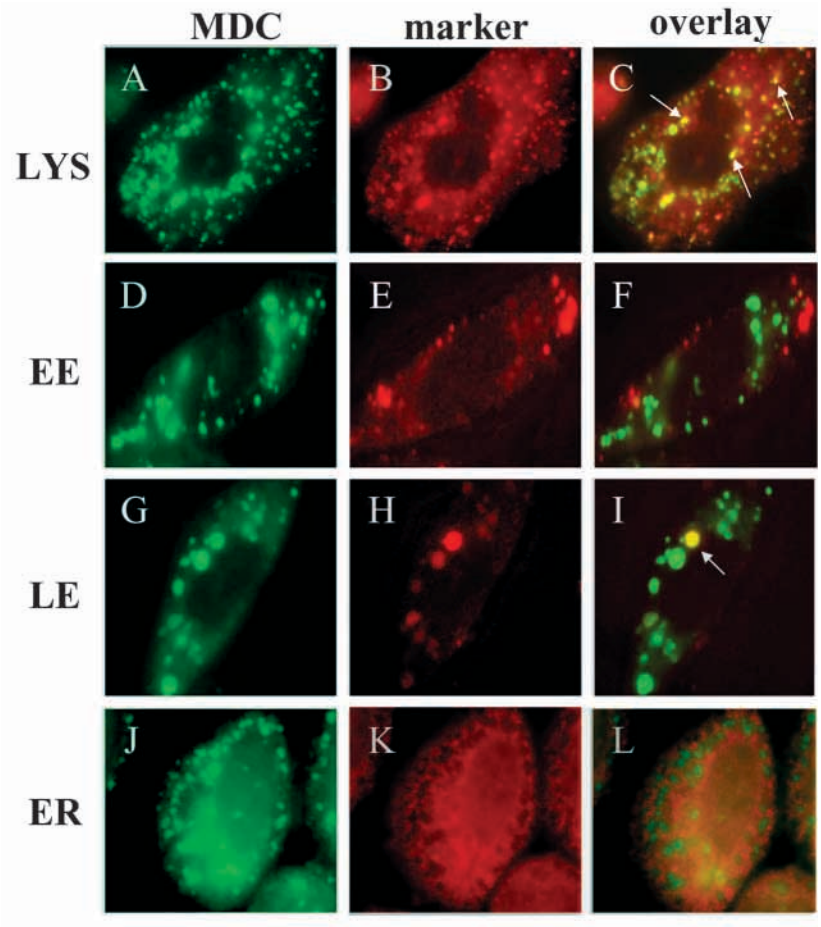

Fig. 6. Characterization of the MDC-labeled vacuoles generated in the presence of vinblastine. CHO cells were incubated in starvation medium for two hours at 37°C in the presence of 50 μM vinblastine. Following this incubation period, cells were labeled with MDC and then incubated with 1 μM LysoTracker (LYS) for 10 seconds at RT. Cells were washed with PBS and immediately analyzed by fluorescence microscopy using the following filter system: excitation filter 510-560 nm, barrier filter 590 nm. MDC-labeled vacuoles are displayed in green and LYS in red. Arrows indicate some of the structures showing overlapping (A-C). To label early endocytic compartments (EE), cells incubated under starvation conditions for two hours were labeled with MDC and subsequently incubated with 0.5 mg/ml dextran tetramethylrhodamine for 5 minutes (D-F). To label late endosomes (LE), after the initial 5 minutes internalization of the endocytic marker, cells were washed and the probe was chased for 10 minutes before labeling with MDC. Cells were immediately analyzed by fluorescence microscopy (G-I). Endoplasmic reticulum (ER) was detected by indirect immunofluorescence. Cells were fixed, permeabilized and incubated with a rabbit antibody raised against endoplasmic reticulum membrane proteins (dilution 1:300) and a goat anti-rabbit Texas Red-conjugated secondary antibody. Cells were mounted with 50% glycerol in PBS and analyzed by fluorescence microscopy (J-L).

crucial event in the transformation of an autophagosome into an autophagolysosome is the fusion with lysosomes. Mature autophagic vacuoles are defined mainly by the acquisition of lysosomal enzymes and by their appearance at the electron microscope. The ultrastructural hallmarks of fully developed autophagic vacuoles are myelin-like membrane whirls in the lumen of these organelles (Seglen et al., 1996; Papadopoulos and Pfeifer, 1987).

In this study we have developed a novel system for monitoring the autophagic pathway based on the incorporation of the autofluorescent marker MDC. This reagent has been described as a selective marker for autophagic vacuoles (Biederbick et al., 1995). However, it was not previously determined whether the accumulation of MDC was under the control of the known regulators of the autophagic pathway. In the present report we have shown that the accumulation of MDC-labeled vesicles correlates with the induction of autophagy by amino acid deprivation. The accumulation of MDC-labeled vacuoles was also visualized by fluorescence microscopy, allowing the morphological study of different stages in the development of autophagic vacuoles. Furthermore, the incorporation of MDC was abrogated by incubation with inhibitors known to block autophagy (e.g. 3-MA, WM), indicating that the accumulation of MDC responds to the classical regulators of this pathway. Interestingly, we have observed that treatment of the cells with the alkylating agent N-ethylmaleimide completely blocked the accumulation of MDC, even under starvation conditions, suggesting that an NEM-sensitive protein is required for the initial steps in the formation of autophagic vacuoles.

Due to the complexity of this multi-step process it is not clear whether autophagy requires protein synthesis or not, and the literature on this issue is contradictory. For example, cycloheximide has been found not to inhibit autophagy stimulated by glucagon (Wong and Woo, 1987), while others have observed that cycloheximide pretreatment decreased autophagic degradation or inhibited the formation of autophagic vacuoles (Marzella and Glaumann, 1980; Oliva et al., 1992). In our study we have observed that pre-incubation with cycloheximide for more than three hours before induction of autophagy by amino acid deprivation partly inhibited the accumulation of MDC induced by starvation. This result indicates that the synthesis of a protein (or proteins) with a rapid turnover is required for this process. This observation is consistent with a previous report showing that pre-treatment with cycloheximide for 3 hours caused inhibition of autophagy-induced degradation. Apparently, a protein with a very fast turnover is necessary for lysosomal fusion (Lawrence and Brown, 1993).

We have observed that, in cells incubated in the presence of vinblastine (which allows the accumulation of autophagosomes by blocking degradation), very large MDC labeled vesicles are formed. Interestingly, the increase in size was mainly observed in cells incubated under starvation conditions, suggesting that a signal transduction mechanism regulates the size of autophagosomes. Several autophagy-

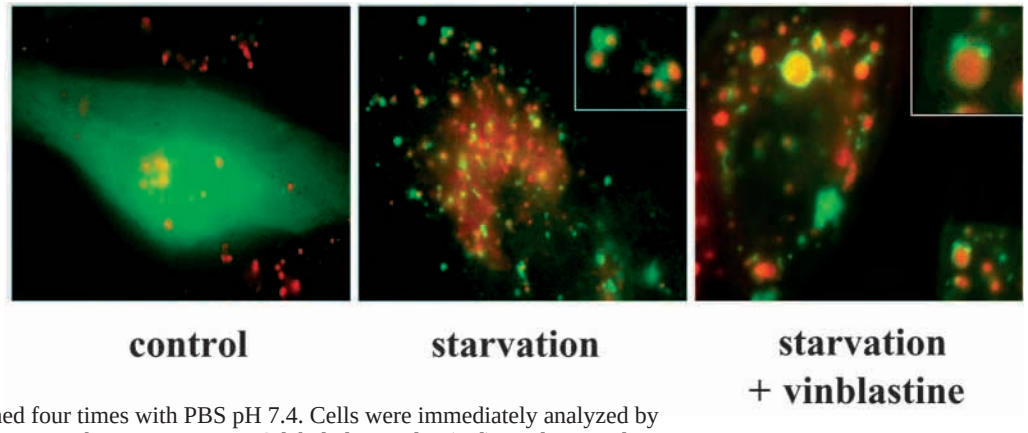

Fig. 7. MDC-vacuoles are labeled with LC3 a protein that associates with autophagosome membranes. CHO cells were transfected with pEGFP-LC3 as described in Materials and Methods. After 48 hours transfection cells were incubated for two hours at 37°C in α-MEM medium (control), EBSS medium (starvation), or EBSS medium containing 50 μM vinblastine. Following this incubation period, cells were incubated with MDC at 0.05 mM for 10 minutes at 37°C and then washed four times with PBS pH 7.4. Cells were immediately analyzed by fluorescence microscopy. Typical images are shown. Insets: MDC-labeled vacuoles (red) are decorated with GFP-LC3 (green).

defective mutants (apg and aut mutants) have been isolated in yeast (Tsukada and Ohsumi, 1993; Thumm et al., 1994). Among them, Apg8/Aut7, an essential protein for autophagy, is transcriptionally upregulated in response to starvation (Kirisako et al., 1999). In apg8Δ/Aut7 cells autophagosomes were hardly detected in the cytoplasm by EM, suggesting that this protein plays a critical role in the formation of autophagosomes (Kirisako et al., 1999). However, aberrant autophagosome-like structures were observed only under starvation conditions. By contrast, Lang et al., reported the presence of autophagosomes in rapamycin-induced Aut7Δ cells (Lang et al., 1998). Interestingly, these autophagosomes were much smaller in size, implying that Aut7 function seems to be required for proper autophagosome size. Therefore, the results that we have obtained in the presence of vinblastine are consistent with the idea that, under the stimulus of nitrogen starvation, a factor (or factors) promotes the expansion of the autophagic vacuole, making the autophagic process physiologically more efficient. In a recent publication, Klionsky and collaborators have dissected autophagosome biogenesis in yeast into distinct nucleation and expansion steps (Abeliovich et al., 2000). The authors have shown that autophagy is induced in the absence of de novo protein synthesis, but the autophagosomes formed are significantly smaller than normal, suggesting that the synthesis of a protein (or proteins) is required for the regulation of autophagosome expansion.

Besides the putative origin of the nascent autophagic vacuoles from membranes of the endoplasmic reticulum (ER), we have observed by immunofluorescence that the MDC-labeled vacuoles were not decorated with an antibody that recognizes ER membranes. This is in agreement with a previous observation that the ER marker sec61b was not present in MDC-labeled vacuoles (Biederbick et al., 1995). The authors have suggested that perhaps the ER-marker proteins rapidly disappear after the formation of autophagic vacuoles. Alternatively, MDC accumulates only in mature autophagic vacuoles, such as autophagolysosomes. Our results indicate that MDC accumulates in an acidic compartment (labeled with LysoTracker) that is distinct from the early endosome compartment but that partially overlaps with late endosomes. This is consistent with previous observations that the autophagic and endocytic pathways converge after the early endosome level (Gordon and Seglen, 1988; Tooze et al., 1990). Interestingly, in the presence of vinblastine, a drug that blocks fusion between autophagosomes and lysosomes (Kovacs et al., 1982; Gordon and Seglen, 1988), there was an increase in the formation of MDC-labeled vacuoles. The increased accumulation of MDC-labeled vacuoles correlates with previous reports showing that the microtubule inhibitor vinblastine causes an accumulation of autophagosomes (Fengsrud et al., 1995) by preventing their degradation. This result suggests that MDC can accumulate not only in autophagolysosomes but also in earlier compartments of the autophagic pathway (e.g. autophagosomes). The autophagic nature of the MDC-vacuoles generated in the presence of vinblastine was confirmed by the localization of LC3, a mammalian homolog (Kabeya et al., 2000) of yeast Apg8p/Aut7, which plays a critical role in the formation of autophagosomes as mentioned above. It has been shown that in amino acid-starved cells, LC3 is detected mainly on the membranes of autophagosomes (Kabeya et al., 2000; Mizushima, et al., 2001). We have observed that several MDC-labeled structures, generated in starved cells and in the presence of vinblastine, were clearly marked with GFP-LC3 (Fig. 7), suggesting that these MDC-labeled vacuoles are autophagosomes. The vacuoles were also labeled with Lysotracker, a marker of acidic compartments, indicating that they have already acquired a vacuolar H^+-ATPase. By contrast, we have not detected ER markers in the MDC-labeled vesicles, therefore, it is likely that autophagosome precursors such us the phagophore or isolation membranes are not labeled with MDC, even in vinblastine-treated cells.

In summary, in this report we have described a new biochemical and morphological assay that allows a fast and simple measure of autophagy. Several assays have been used to monitor autophagy. However, most of them involve multiple steps and the use of more complicated methodologies such as electroporation, application of radioactive compounds, and electron microscopy. By contrast, the system presented here allows not only a quantitative measurement of autophagy but also a morphological analysis of the autophagic pathway by fluorescence microscopy. We have labeled autophagic vacuoles with MDC in different stages of their development by combining autophagy induction and inhibitors. We have observed a marked increased in the size of autophagic vacuoles

in cells incubated with vinblastine under nitrogen-starvation conditions, indicating that the expansion step is regulated by a signal transduction mechanism initiated by the depletion of amino acids (Van Sluijters et al., 2000). Further work will be necessary to identify signaling molecules that may control this expansion step. Lastly, we have found that an NEM-sensitive protein seems to be required for the initial step of autophagosome formation. Although the molecular cell biology of autophagy remains poorly understood, some of the regulatory elements of this process have been elucidated. We believe that this new assay will allow us to gain further insight into the molecular machinery that regulates the autophagic pathway using transfected cells that overexpress proteins involved in intracellular trafficking.

We thank L.S. Mayorga and S. I. Patterson for critical reading of this manuscript. This work was partly supported by grants from CONICET (PIP 0695/98), Agencia Nacional de Promoción Científica y Tecnológica (PICT97 # 01-00111-02072 and PICT99 # 1-6058), CIUNC (Universidad Nacional de Cuyo) and Fundación Antorchas.

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