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Inhibition of Weak-Base Amine-Induced Lysis of Lysosomes by Cytosol[1]

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Certain amines known to be concentrated in lysosomes, termed "lysosomotropic amines," cause the formation of lysosomal vacuoles. A cell-free system was established to examine the effects of basic substances and acidic ionophores. In this system, the drugs not only increased the internal pH, but also caused a disruption of lysosomes. The osmotic swelling of lysosomes induced by protonated bases or cations for particular ionophores, which had accumulated within lysosomes driven by the proton pump, caused the osmotic lysis of lysosomes. The lysosomal disruption was inhibited upon the addition of the cytosol fraction. This phenomenon provides an *in vitro* system for studying the osmo-regulation and intercellular dynamics of the lysosomal system, including membrane fusion. The lysosomal stabilization factor was purified from the cytosol fraction and identified as ATP-stimulated glucocorticoid receptor translocation promoter (ASTP).

Key words: ATP-stimulated glucocorticoid-receptor translocation promoter, lysis, lysosome, stability.

It has been found that the treatment of cells with a variety of chemical compounds leads to the formation in the cytoplasm of many large vacuoles. Certain amines known to be concentrated in lysosomes, termed "lysosomotropic amines," cause the formation of lysosomal vacuoles (*1*). A mechanism for the accumulation for these amines was proposed by de Duve *et al.* (*2*). The interior of lysosomes, as well as endosomes, Golgi vesicles and other intracellular organelles, is known to be acidified by an electrogenic proton pump. A concentrative uptake of amines by lysosomes may occur *via* the diffusion of neutral or less charged, more permeable, species followed by protonation and concentration in the vesicle interior of less permeable, protonated species. A degree of lysosomal swelling is possible without any accumulation of membranes in the lysosomal compartment (the fusion of many lysosomes). The osmotic swelling of vacuoles caused by weak-base amines may result in functional impairment and a retardation of membrane flow through the vacuolar compartments. The base-induced formation of vacuoles has been proposed as a model for lysosomes in cell fusion studies on lysosomal protein trafficking (*3*).

A cell-free system was established for examining of the effects of basic substances and acidic ionophores (*4*). In this system, drugs not only increased the internal pH, but also caused lysosomal disruption. The osmotic swelling of lysosomes induced by protonated bases or cations for particular ionophores, which had accumulated within lysosomes driven by the proton pump, caused the osmotic lysis of lysosomes (*4, 5*). We found that the weak bases that induce lysosomal disruption are inhibited by the addition of the cytosol fraction. This experimental system may provide a good model for studying the role of the cytosol in lysosomal membrane dynamics and/or osmo-regulation. In this study, we purified and identified the inhibitory factor of weak-base amine-induced lysosomal lysis *in vitro*.

MATERIALS AND METHODS

Materials—Bafilomycin A_1 was a gift from Dr. K. Altendorf (University of Osnabruck, Germany) and was prepared as a stock solution in dimethyl sulfoxide. Other materials were of reagent grade and obtained mostly from Sigma Chemical (St. Louis, MO).

Fluorescein Isothiocyanate Dextran (FD)-Loaded Lysosomes (FD-Lysosomes)—FD-loaded lysosomes were prepared as previously described from 8–9-week-old male Wister rats fasted overnight after intravenous injection of 20 mg/100 g body weight FD (70,000 MW) dissolved in

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Abbreviations: ASTP, ATP-stimulated glucocorticoid-receptor translocation promoter; Buffer A, 0.2 M sucrose, 20 mM Mop/TMAH (pH 7.0), 10 mM $MgCl_2$, 2 mg/ml BSA, and 0.1 M KCl; DAMP, 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine; FD, fluorescein isothiocyanate-dextran; IFL, inhibitory factor of lysosomal lysis; NEM, *N*-ethylmaleimide; TFA, trifluoroacetic acid; TGD, 20 mM Tris/Cl (pH 7.4) and 10% (v/v) glycerol in 1 mM DTT; TPBS, 0.05% Tween 20-phosphate buffered saline.

saline (*6*). Briefly, the livers were minced and homogenized with a Daunce homogenizer in 3 volumes of ice-cold 0.25 M sucrose. The postnuclear supernatant was incubated with 1 mM $CaCl_2$ for 5 min at 37°C to swell and decrease the density of mitochondria, layered on 37% Percoll, and centrifuged at 80,000 ×*g* for 15 min. The lysosome fraction was collected and used for assay.

Preparation of Cytosol—The postnuclear supernatant of rat liver was centrifuged at 100,000 ×*g* for 1 h to obtain the supernatant fraction (cytosol). Cytosol was frozen in liquid nitrogen and stored at –80°C.

Lysosome Acidification—The acidification of vesicles was assessed from the quenching of fluorescein fluorescence (*7*). Experiments were performed at 37°C. Aliquots of lysosomes (20–30 μg) were added to curvettes containing 2 ml of isotonic buffer [0.2 M sucrose, 20 mM Mops/TMAH (pH 7.0), 10 mM $MgCl_2$, 2 mg/ml bovine serum albumin (BSA), and 0.1 M KCl] (buffer A) with other additions as indicated in the figure legends. To initiate acidification, 20 μl of 0.1 M ATP (neutralized with NaOH to pH 7.0) was added to achieve a concentration of 1 mM.

Assay of Lysosomal Lysis—FD-lysosomes were incubated in isotonic buffer A with 40 mM ammonium chloride for 10 min at 37°C. After the reaction, silicon DC-550 (d = 1.06, Fulka) (50 μl) was added and the mixture was centrifuged at 10,000 rpm for 5 min to sediment intact lysosomes. The absolute fluorescence of the resulting supernatant was measured at an excitation wavelength of 495 nm and emission wavelength of 520 nm. Lysosomal fragility was calculated from the amount of fluorescence in the supernatant as a percentage of total FD in lysosomes, where the latter was taken as the amount of fluorescence in the supernatant of Triton X-100 (final concentration 0.1%)–treated lysosomes.

Purification of the Inhibitory Factor of Lysosome Lysis—One unit of inhibitory activity is defined as the amount of inhibitor preparation required to inhibit the lysis of lysosomes (20 μg) under the conditions described for the assay of lysosomal lysis.

(Step 1) Ammonium sulfate precipitation: Cytosol was typically 100 ml in volume with a concentration of 12 mg/ml. Solid ammonium sulfate was added to give a 50% saturated solution, and the extract was stirred for 30 min at 4°C. The precipitate was collected at 21,000 ×*g* for 30 min. The pellets were resuspended in buffer [20 mM Tris/Cl (pH 7.4), 10% (v/v) glycerol, 1 mM dithiothreitol (DTT)] (TGD buffer) and then dialyzed overnight against TGD buffer. The dialysates were centrifuged at 100,000 ×*g* for 1 h to remove the precipitate.

(Step 2) Q-Sepharose chromatography: The protein solution was loaded at 0.5 ml/min onto a Q-Sepharose column (18 × 1.6 cm) equilibrated with TGD buffer. The column was washed with TGD buffer, and the factor was eluted by a 0 to 1 M NaCl gradient. The eluate was assayed for inhibitory activity; the peak of activity eluted at 0.8 M NaCl. Fractions containing significant activity were pooled.

(Step 3) Phenyl-Sepharose chromatography: The pooled fractions from the Q-Sepharose chromatography were mixed with ammonium sulfate to a concentration of 1 M, and then loaded at a flow rate of 0.25 ml/min onto a Phenyl-Sepharose column (20 × 0.5 cm) equilibrated with TGD buffer containing 1 M ammonium sulfate. The column was developed with a gradient of 1 to 0 M ammonium sulfate in TGD buffer.

(Step 4) Mono Q chromatography: The active fraction from Phenyl-Sepharose chromatography was loaded at 1 ml/min onto a Mono Q HR 5/5 column equilibrated with TGD buffer. The column was washed with 10 ml of TGD buffer, and the inhibitory factor was eluted with a 0 to 1.0 M NaCl gradient. Each fraction (1 ml) was assayed for its ability to inhibit lysosomal lysis.

(Step 5) Phenyl-Superose chromatography: The active fraction from the Mono Q column was mixed with ammonium sulfate to a concentration of 1 M, and loaded onto a Phenyl-Superose column equilibrated with TGD buffer containing 1 M ammonium sulfate. The column was developed with a 1 to 0 M ammonium sulfate gradient. Fractions were assayed for inhibitory activity and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Purification steps 4 and 5 were repeated to obtain a single band on SDS-PAGE.

Lysylendopeptidase Digestion—Purified protein (16 μg) dissolved in 50 mM Tris/HCl (pH 9.5) was digested with lysylendopeptidase (0.5 mg) at 37°C overnight. The reaction was stopped by freeze-drying and the residue was dissolved in water containing 0.1% trifluoroacetic acid (TFA) and applied to a column of μ-Bondasphere (2.1 × 15 cm, C18, Millipore) equilibrated with water containing 0.1% TFA. The column was eluted with a gradient of 0–80% acetonitrile containing 0.1% TFA. A peptide was detected by its absorbance at 210 nm. The peptide peak was collected and freeze-dried for peptide sequence analysis. Sequencing was performed on an ABI model 476A gas-phase sequencer with on-line PTH identification.

Electrophoresis and Immunoblotting—SDS-PAGE was routinely performed as described by Laemmli. The proteins separated by SDS-PAGE were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon, 0.45 μm, Millipore). The membrane was incubated overnight at 4°C in 0.05% Tween 20-phosphate–buffered saline (TPBS) containing 5% non-fat dry milk powder. This was followed by incubation with immune or preimmune sera in the same solution overnight at 4°C. The membranes were washed twice with TPBS and incubated with alkaline phosphatase-conjugated anti-rabbit IgG goat antibody (ZYMED Laboratories Inc.) in TPBS containing 3% BSA for 2 h at room temperature. The membranes were washed three times with TPBS, and the color was developed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as a substrate for alkaline phosphatase.

Immunoprecipitation—The purified inhibitory factor (5 μg) was incubated with anti-ATP-stimulated glucocorticoid-receptor translocation promoter (ASTP) IgG (*8*) or control IgG for 2.5 h at 4°C in 50 mM Tris/HCl (pH 7.4), 2.5 mM $MgCl_2$, and 1 mM DTT. After incubation, the mixture was centrifuged at 14,000 ×*g* at 4°C to remove insoluble materials, and the supernatant was collected and used for the assay of lysosome lysis.

Protein—For most assays, protein levels were quantified using the Amido black/solid-phase method according to Schaffner and Weissmann, with bovine serum albumin as the standard (*9*).

RESULTS

Cytosol Stabilizes Lysosomes against Osmotic Disruption Due to the Accumulation of Bases—NH_4Cl induced the vacuolation of lysosomes within cells (*1*). We tried to establish a cell-free system for the formation of large vacuoles. When ATP was added to isotonic medium [0.1 M KCl, 0.2 M sucrose, 10 mM $MgCl_2$, 2 mg/ml BSA, and 20 mM Mops/TMAH (pH 7.0)] containing FD-labeled lysosomes, fluorescein fluorescence decreased due to lysosomal acidification driven by proton pump activity. Afterwards, NH_4Cl was added to the medium, and the fluorescence intensity of the lysosomes increased instantaneously, followed by a further gradual increase. FD, which was quenched under the acidic atmosphere of lysosomes, was released into the medium and dequenched during lysosomal lysis (Fig. 1). Bafilomycin A_1, a vacuolar ATPase inhibitor, suppressed the increase in fluorescein intensity. This indicates that the phenomenon is dependent on the activity of a proton pump. Why are lysosomes within cells stable in the presence of weak bases unlike the case *in vitro*? One important difference between the conditions surrounding lysosomes in culture and *in vitro* is the presence of cytosolic components within cells. Therefore, we added cytosol fraction to the assay medium. As shown in Fig. 1, the rise in the fluorescence intensity after the addition of NH_4Cl was stopped and the release of FD was also suppressed. This shows that the cytosol stabilizes lysosomes against NH_4Cl-induced lysis. The amount of cytosol to cause the complete inhibition of lysosomal lysis was around 25–50 μg protein/ml (Fig. 2). The lysosomal stabilization by cytosol was sensitive to *N*-ethylmaleimide (NEM) (Fig. 3).

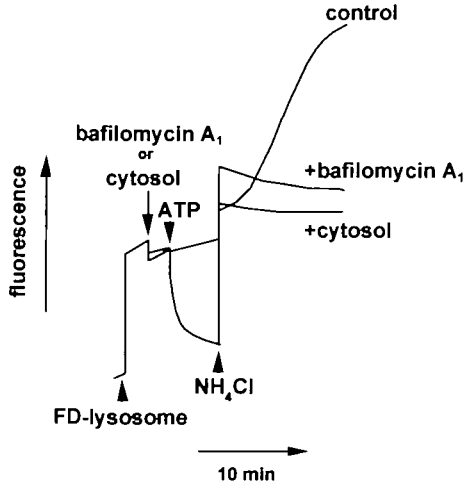

Fig. 1. **Lysosomal lysis induced by weak bases and its inhibition by bafilomycin A_1 and cytosol.** Aliquots of FD-lysosomes were incubated in isotonic buffer containing 0.2 M sucrose, 0.1 M KCl, 2 mg/ml BSA, 10 mM $MgCl_2$, 20 mM Mops/TMAH (pH 7.0), and ATP (1 mM). NH_4Cl (40 mM), bafilomycin A_1 (10 nM), and cytosol (50 μg/ml) were added at the indicated time points.

Inhibition by Cytosol of Lysosomal Lysis Induced by Other Bases and Acidic Ionophores—Figure 4 shows lysosomal lysis by various bases. Methylamine, atropine and chloroquine, all vacuoligenic in cell culture, caused lysis. But tetramethylamine, tributylamine, and aniline, which do not induce the vacuolization of lysosomes, did not cause lysis. The same phenomena were observed with acidic ionophores, such as nigericine, monensin, but not valinomycin and gramicidine, amphotercin B and nystatin, used in place of the bases. Suppression by cytosol was observed in all cases of lysis induced by weak-base amines or acidic ionophores (Fig. 4).

Purification of Inhibitory Factors of Lysosomal Lysis—Cytosolic inhibitory factors of the lysis of lysosomes from rat liver were purified in eight steps. The purification consisted of sequential chromatography on Q-Sepharose, Phenyl-Sepharose, Mono Q, and Phenyl-Superose columns. Table I summarizes the procedures. The inhibitory activity in rat liver cytosol was eluted from Q-Sepharose, Phenyl-Sepharose, and Mono Q columns as single peaks. The activity of the inhibitor was separated into two peaks by Phenyl-Superose chromatography. The first active peak (Inhibitory Factor of lysosome Lysis-1; IFL-1) was purified about 500-fold and the second active peak (IFL-2) about 600-fold. On SDS-PAGE, IFL-1 gave a single band at 48 kDa, while IFL-2 gave mainly two bands at 48 and 50 kDa. Thus, IFL-1

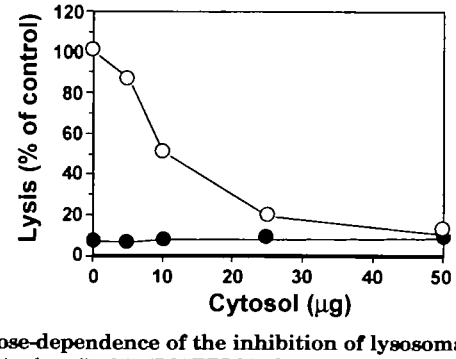

Fig. 2. **Dose-dependence of the inhibition of lysosomal lysis by cytosol.** As described in "MATERIALS AND METHODS," FD-lysosomes were incubated with 40 mM ammonium chloride for 10 min in the presence of the indicated amount of cytosol (○). 10 nM bafilomycin A_1 was added to each assay to assess vacuolar ATPase-dependent lysis (●). Values are expressed relative to the control without cysotol.

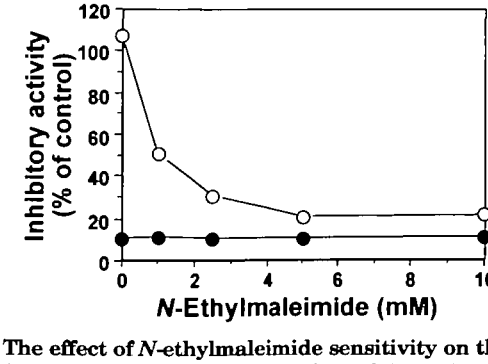

Fig. 3. **The effect of *N*-ethylmaleimide sensitivity on the inhibition of lysosomal lysis by cytosol.** Cytosol (50 μg) was treated with various concentrations of *N*-ethylmaleimide (NEM) at 37°C for 10 min and then quenched of excess NEM by DTT (○). NEM-treated cytosol was added to the medium and assayed as described in "MATERIALS AND METHODS." 10 nM bafilomycin A_1 was added to each assay to assess vacuolar ATPase-dependent lysosomal lysis (●). Values are expressed relative to the control without NEM.

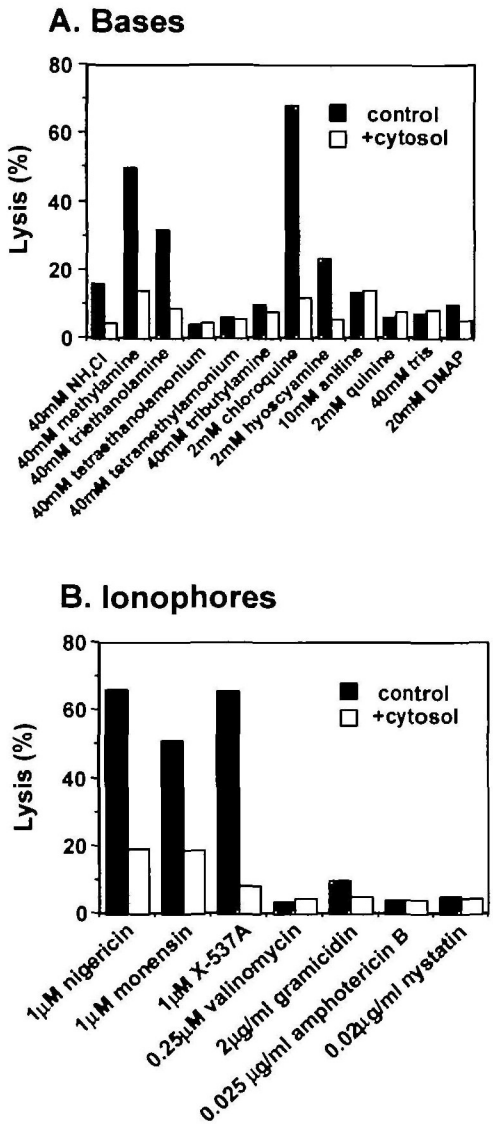

Fig. 4. **Effect of cytosol on the ATP-dependent lysis of lysosomes induced by various bases and ionophores.** FD-lysosomes were incubated with various bases and ionophores as indicated as described in "MATERIALS AND METHODS." The lysosomes were separated from the medium by centrifugation. Values were calculated as percentages of lysis caused by the supernatant in equivalent assays containing 0.1% Trition X-100. The inhibition of lysosomal lysis by cytosol (50 μg/ml) was evaluated.

was purified to apparent homogeneity, as judged by SDS-PAGE.

Micro-Sequencing of 48 kDa—Edman degradation of 48 kDa IFL-1 was not successful because the protein had a blocked N-terminus. To obtain the internal amino-acid sequence, IFL-1 was digested with lysylendopeptidase. The resulting peptides (a–c) were separated by HPLC and then analyzed. The amino acid sequences of three peptides were determined (Table II). The partial amino acid sequences of IFL-1 are identified as ATP-stimulated glucocorticoid-receptor translocation promoter (ASTP) (*10–12*).

Immunoprecipitation of Inhibitory Factor by Anti-ASTP Antibody—We performed immunoblot analysis using an

TABLE II. **Sequences of peptides derived from IFI-1.**

Sequence
a) LGQLNIDISNI
b) LTGEPLYNAVVWLDLRYT
c) SIGWVTTQSPESG

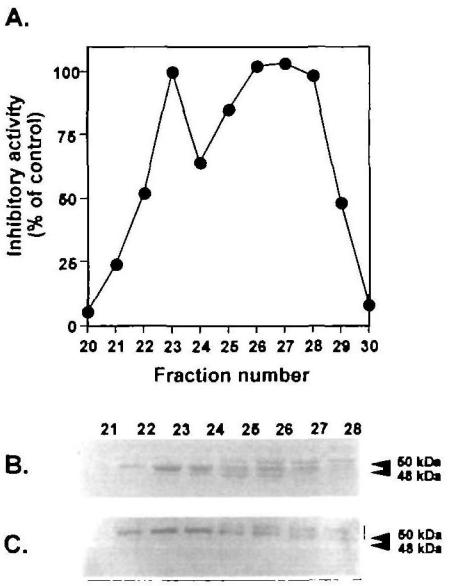

Fig. 5. **Chromatography of ILF on Phenyl-Superose.** (A) Elution profile of inhibitory activity. (B) SDS-PAGE of the protein present in the fractions containing the inhibitory activity. (C) Immunoblot analysis of purified inhibitors using anti-ASTP antibody. Values are expressed relative to the control, incubating with cytosol (50 μg/ml) instead of a test fraction.

TABLE I. **Purification of inhibitors (IFL-1 and IFL-2).**

Fraction	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification (fold)
Cytosol 50% saturated	8,250	12,600	1.53	100	1
Ammonium sulfate precipitation	583	9,210	15.8	73	10
Q-Sepharose	111	6,420	56.3	50	37
Phenyl-Sepharose	54.2	3,050	56.2	24	37
Mono Q (#1)	29.8	2,930	98.3	23	64
Phenyl-Superose (#1)	3.68	1,770	481	14	314
Mono Q (#2) Phenyl-Superose (#2)	0.880	1,460	1,660	12	1,085
Peak 1 (IFL-1)	0.0919	72	784	0.6	512
Peak 2 (IFL-2)	0.144	132	920	1.0	601

One unit of inhibitory activity is defined as the amount of inhibitor preparation required to inhibit the lysis of lysosomes (20 μg) under the conditions described for the assay of lysosomal lysis.

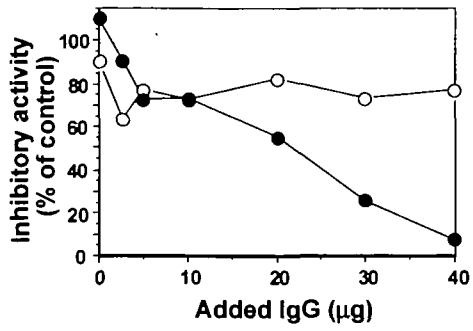

Fig. 6. **Immunoprecipitation of inhibitor (IFL) by anti-ASTP antibody.** The purified inhibitor (5 μg) was incubated in the presence of various amounts of anti-ASTP IgG (●) or control IgG (○), and the resulting supernatant was assayed for lysosomal lysis as described in "MATERIALS AND METHODS." Values are expressed relative to the control without anti-ASTP antibody.

antibody against ASTP to confirm that IFL-1 is identical to ASTP. Anti-ASTP antibody (*8, 12*) recognized 48 kDa IFL-1 and 48 and 50 kDa IFL-2 on SDS-PAGE (Fig. 5). This shows that 50 kDa IFL-2 may be an isomer of the 48 kDa IFL-1, and IFL-2 consists of a heteroisomer of the 48 and 50 kDa species. The purified IFL-1 was immunoprecipitated with anti-ASTP antibody as shown in Fig. 6. These results confirm that the inhibitory factor of lysosomal lysis (IFL-1) is ASTP.

DISCUSSION

The basis for the lysosomotropic effect of weak-base amines appears to be their ability to become protonated within the acidic interior of the lysosomes. This results in the intralysosomal accumulation of protonated amine, leading to osmotic swelling (vacuolation) of the lysosomes and giving rise to neutralization of the lysosome pH. In cultured cells, the bases induce the formation of a large vacuole. In contrast to the phenomenon *in vivo*, lysosomal lysis occurs in a cell-free system in the presence of ATP (dependent on the activity of an ATP-driven proton pump). This is due to osmotic swelling induced by protonated bases that accumulate within lysosomes driven by the proton pump, accompanied by the osmotic influx of water. We found that the lysosomal disruption is inhibited by the addition of cytosol to the cell-free system, and purified the inhibitory factor (IFL) and identified it as ASTP. As the inhibitory activity of lysosomal lysis in rat liver cytosol was eluated from Q-Sepharose, Phenyl-Sepharose, Mono Q columns as single peaks, IFL/ASTP is a major factor for the stabilization of lysosomes in the cytosol.

Possible explanations for the inhibition of osmotic lysosomal lysis by cytosol are (i) inhibition of proton pump activity, (ii) inhibition of base accumulation, (iii) inhibition of water permeability, (iv) increase in the permeability of solute inside the lysosome, (v) increase in the mechanical intensity of the lysosome membrane, and (vi) induction of lysosomal fusion. The acidification of lysosomes is not inhibited by cytosol as shown in Fig. 1. This suggests that the proton pump activity is not inhibited by the addition of cytosol. The increase in the fluorescence intensity caused by bases (neutralization of intralysosomal pH) was not inhibited by cytosol. The effect of weak-base amines is not the result of some unique chemical characteristic, but is due to weak base activity. Bases and acidic ionophores induce lysosomal lysis through osmotic swelling, which is suppressed by cytosol. These results show that the permeability of the bases is not decreased by cytosol. These data suggest that the effect of cytosol is not due to (i) or (ii). It is well established that the disruption of lysosomes occurs early in a number of kidney diseases as well as after drug administration. It might be that the osmotic water permeability of lysosomes is low, reducing the likelihood of swelling and rupture. But rat renal lysosomes exhibit very high water permeability (*13*). Rat liver lysosome water eflux was measured by the self-quenching of entrapped FD, and cytosol did not increase the permeability of water (unpublished observation). Rat liver lysosomes are permeable to some inorganic and aliphatic organic anions and inorganic cations (*14*). As anion or cation transport across a lysosomal membrane may affect the intralysosomal pH, a protective mechanism assuming (iv), an increase in the permeability of solute inside the lysosome, is also not satisfactory. It is possible that IFL/ASTP may protect against lysis through (v), a direct effect on membrane configuration or fluidity. It has been reported that membrane fluidity does indeed affect lysosomal sensitivity (*15, 16*), and this needs further investigation.

Alternatively, (vi), lysosomal fusion, may be behind the effect of cytosol on the inhibition of lysis. It has been shown that lysosomes, themselves, rapidly exchange membrane and contents (*17–19*). In the beige mouse (an animal model of human Chediak-Higashi syndrome), large lysosomes originate from fusion events among the lysosomes themselves (*19*). These findings provide evidence that lysosomes themselves fuse within cells. Studies of the mechanisms of membrane fusion have achieved great progress in recent years, and an NEM-sensitive factor is reported to function in membrane fusion between vesicles of the vacuolar system (*20*). Vacuoles also form upon incubation with indigestible substances such as sucrose (*21, 22*) or a tripeptide of the D-isomer of alanine (*23*). This shows that the indigestible material within lysosomes may affect membrane trafficking. The protein toxin VacA, produced by cytotoxic strains of *Helicobacter pylori*, causes vacuolar degeneration of cells. It is presumed that VacA alters membrane trafficking events taking place at the late endosomal stage (*24, 25*). These findings suggest that the vacuolation of endocytic organelles is caused by a disruption in membrane trafficking. IFL/ASTP may act as a fusogen for lysosomes similar to glycerolaldehyde-3-phosphate dehydrogenase, a protein with fusogenic characteristic similar to viral proteins, where exposure of the hydrophobic regions of protein (*26*) and fusion of lysosomes prevents osmotic lysis by increasing lysosomal volume.

We have found previously that ARF ruptures lysosomes (*27, 28*). Many reports have been published suggesting that lysosomal rupture with the release of proteases such as cathepsin may induce apoptosis (*29*). The stability of the lysosomal membrane may be important in the initiation of apoptosis. The present results show that there is a regulatory factor of lysosome stability in cytosol. We speculate that IFL/ASTP works as a modulator of lysosomal membrane dynamics. The mechanism of the effect of IFL/ASTP on lysosomal stabilization remains to be determined.

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