BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 204–208 (1998)
ARTICLE NO. RC989416

Overexpression of Cation-Dependent Mannose 6-Phosphate Receptor Prevents Cell Death Induced by Serum Deprivation in PC12 Cells

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Received August 22, 1998

PC12 cells express well cation-independent mannose 6-phosphate receptors (CI-MPR), but not cation-dependent (CD)-MPR as much. To examine CD-MPR dependency of transport of cathepsins B and D to lysosomes in PC12 cells, we prepared the cells overexpressing CD-MPR. Immunoreactivity for cathepsin B became more distinct and larger in size in the transfected cells than in wild-type cells. No difference in the distribution of cathepsin D was seen between these two cells. The viability of the cells following serum deprivation was significantly higher in the transfected cells than in wild-type cells. This increased viability of the transfected cells was blocked by CA074, a specific inhibitor of cathepsin B, while pepstatin A suppressed the action of CA074. The results suggest that CD-MPR preferentially transport cathepsin B in PC12 cells, and cathepsins B and D participate in the regulation of PC12 cell apoptosis.

Soluble lysosomal enzymes in higher eukaryotic cells require mannose 6-phosphate residues as the recognition signal to be transported from the trans Golgi network to lysosomal compartments by specific receptors (5, 11). This signal-mediated transport of soluble lysosomal enzymes is carried out by large and small mannose 6-phosphate binding proteins; the larger protein is the 270 kDa transmembrane protein, cation-independent mannose 6-phosphate receptor/insulin-like growth factor II receptor (CI-MPR) and the smaller one is the 46 kDa transmembrane protein, cation-dependent (CD)-MPR (10, 13). These two MPRs are involved in intracellular transport of lysosomal enzymes to lysosomes, but they are not considered to be redundant in function (14, 18, 19).

Lysosomes are an important organelle having an acidic milieu to maintain cellular metabolism by degrading extra- and intracellular unneeded substances (30). Cathepsins B and D are representative cysteine and aspartic proteinases and ubiquitously present in lysosomes of mammalian cells. We have previously shown that autophagic vacuoles/autolysosomes appear in the apoptotic processes of nerve cells in the CA1 region of the gerbil hippocampus after brief ischemic insult (23). The autophagy occurring in such dying cells may play an important role in the protection of the cells from death (3).

Apoptosis is characterized by morphological alterations consisting of shrinkage of cytoplasm, nuclear chromatin condensation, fragmentation of cells into apoptotic bodies and heterophagocytosis by neighboring cells (7, 29). It has been well established that the caspase family of proteinases play a central role in the execution of apoptosis (22). Besides caspases, it is also interesting that lysosomal cysteine and aspartic proteinases are involved in apoptosis of cultured cells (4, 16). Using primary culture, Maeda et al. have shown that leupeptin induces apoptosis of hepatocytes without any additional death stimuli, but aspartic, serine and calpine proteinase inhibitors do not (16), suggesting that lysosomal cysteine proteinases are involved in the apoptotic cascade. Moreover, human cathepsin D is found to be one of positive mediators of apoptosis, and further, the enzyme is confirmed to induce apoptosis of HeLa cells when overexpressed (4). It is therefore important to understand the regulatory mechanism of lysosomal cysteine and aspartic proteinases in the cell

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0006-291X/98 $25.00

death cascade or their relationship with the cascade using the caspase family of proteinases.

In the course of the study on the relationship between cathepsins B and D, and MPRs, we prepared PC12 cells overexpressing CD-MPR, which showed more distinct and larger immunodeposits for cathepsin B in the cytoplasm than in the wild-type (wt) cells. We therefore analyzed the CD-MPR gene-transfected PC12 cells by immunochemical as well as immunocytochemical methods. In this, we noticed that the viability of the transfected cells was significantly increased than that of wt cells. Here we show that this alteration in the viability of PC12 cells overexpressing CD-MPR is regulated by cathepsins B and D.

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rat     MFPLSGCWRTELLLLLLLAVAVRESWQIEEKSCDLVGEKDKESKNEVALLERLRPLFNKS  60
mouse   ---F----------------------------------------------------------  60
human   ---FYS----G------.---------T---T-------G---EK-L--VK--K------  59

rat     FESTVGQGSDTYSYIFRVCREAGNHSSGAGLVQINKSNEKETVVGRINETHIFNGSNWIM  120
mouse   ----------------------S---------------G---------------------  120
human   ------------I------------T-----------G-------L-------------  119

rat     LIYKGGDEYDNECGKEQRRAVVMISCNRHTLAGNFNPVSEERGKIQDCFYLFEMDSSLAC  180
mouse   --------------------------------A----------V----------------  180
human   --------------------------------D----------V----------------  179

rat     SPEVSHLSVGSILLVIFASLVAVYIIGGFLYQRLVVGAKGMEQFPHLAFWQDLGNLVADG  240
mouse   ------------------------------------------------------------  240
human   ---I----------T--------VV---------------------------------  239

rat     CDFVCRSKPRSVPAAYRGVGDDQLGEESEERDDHLLPM*  278
mouse   ----------N--------------------------*  278
human   ----------N--------------------------*  277
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FIG. 1. Comparison of rat CD-MPR protein sequence with those of mouse and human. These sequences were aligned using 38 Genetyx-CD software package. − indicates the same residue; . indicates lack of a residue.

MATERIALS AND METHODS

Cells and culture. For the experiments, PC12 cells were used and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing a high level of glucose (4.5 g/L) supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). The PC12 cells transfected with or without cDNA of rat CD-MPR, which were harvested from the culture dishes, were washed several times with DMEM and plated at a density of $2.5 \times 10^3/cm^2$.

cDNA cloning and transfection. For the transfection study, we isolated the cDNA for rat CD-MPR from a PC12 cell cDNA library, screened using oligonucleotide probes synthesized to the regions conserved in human and mouse cDNA sequences (12, 15, 26). The transfection of CD-MPR was carried out by using the calcium phosphate precipitation method (1) after the open reading frame of CD-MPR linked with FLAG tag nucleotide sequences at the 3′ end before the stop codon was constructed into pcDNA3. Cells which expressed neomycin phosphotransferase were isolated by selection with G418 (450 μg/ml).

Antisera. Rabbit polyclonal antibodies to rat cathepsins B and D, and CI-MPR were purified by affinity chromatography, as previously reported (8,9,20,24). An antibody against a synthetic peptide of CD-MPR corresponding to its cytoplasmic domain was also prepared and affinity-purified using the peptide. Monoclonal antibody against FLAG was purchased (Eastman Kodak).

Cell death assay. In each experiment, cells, cultured in the presence of serum, or in the absence of serum but in the presence of NGF (100 ng/ml) were used as controls. As experimental conditions, cells were cultured in the absence of serum, or in the absence of serum but in the presence of 200 μM CA074 (N-(L-3-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline), a specific inhibitor of cathepsin B with or without 100 μM pepstatin A. The survival assay of the cells were performed 24 hr after the onset of culture by trypan blue exclusion test (17, 25). We also examined the survival rate of the cells by the TUNEL method (6, 23). These experiments were carried out in triplicate, and the data were statistically analyzed by the student's t-test.

Immunocytochemistry. PC12 cells, cultured under various experimental conditions were obtained 24 hr after the onset of culture and fixed with 4% paraformaldehyde buffered in 0.1 M phosphate buffer, pH 7.2 at 4°C for 30 min. Immunostaining for CI- and CD-MPRs, cathepsins B and D was performed, as previously described (24, 31).

Immunoblot analysis. Extracts from wt and CD-MPR-gene-transfected PC12 cells before or after serum-deprived culture were obtained by the treatment with cell lysis buffer containing 150 mM NaCl, 50 mM Tris, 1 % Triton X-100 including the protease inhibitor cocktail containing 0.06 mg/ml antipain-HCl, 0.01 mg/ml bestatin, 0.02 mg/ml chymostatin, 0.06 mg/ml E-64, 0.01 mg/ml leupeptin, 0.01 mg/ml pepstain, 0.06 mg/ml phosphoramidon, 0.4 mg/ml Pefabloc SC, 0.2 mg/ml EGTA and 0.01 mg/ml aprotinin (Boehringer Mannheim). Ten μg of proteins from each sample was subjected to 12.5 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to PVDF membrane (Immobilon, Millipore). The blots were incubated with anti-cathepsin B or D and visualized with the ECL detection system (Amersham). Quantitative analysis of the immunosignals was carried out using Scanning Imager (Molecular Dynamics).

Cell fractionation. Wt PC12 cells were homogenized in 0.25M sucrose by passing 10 times through 27 gauge needle at 4°C, and centrifuged at 1200 g for 5 min at 4°C. The supernatant was used as postnuclear supernatant (PNS). The PNS was treated with the cell lysis buffer and 10 μg of proteins from the sample was subjected to 12.5 % SDS-PAGE, followed by immunoblotting with anti-cathepsins B and D.

Northern blotting analysis. Total RNA was prepared from both wt cells and transfectant by the AGPC method (2). Each sample was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes (27). Hybridization was performed with a ^{32}P-labeled cDNA probe for rat CD-MPR. The membranes were subjected to autoradiography.

RESULTS AND DISCUSSION

We cloned the cDNA of rat CD-MPR containing an open reading frame which encoded for a polypeptide of 279 amino acids (Figure 1). The predicted amino acid sequence showed 91.0% and 97.8% amino acid identity with human (26) and mouse (12, 15) CD-MPR.

To examine the relationship of cathepsins B and D with MPRs, we examined immunoreactivity for CD- and CI-MPRs in PC12 cells. As shown in Figure 2 A,B, immunoreactivity for CI-MPR was widely distributed in the cytoplasm of the cells, whereas that for CD-MPR was weakly or faintly detected in a part of the perinuclear region. We then prepared PC12 cells overexpressing CD-MPR and the expression level of the CD-MPR mRNA was confirmed to be highly expressed in the cells (Figure 2C); in addition to the endogenous CD-MPR mRNA in both wt and transfected cells, a band corresponding to the transcript of a cDNA fragment containing the open reading frame region of the CD-

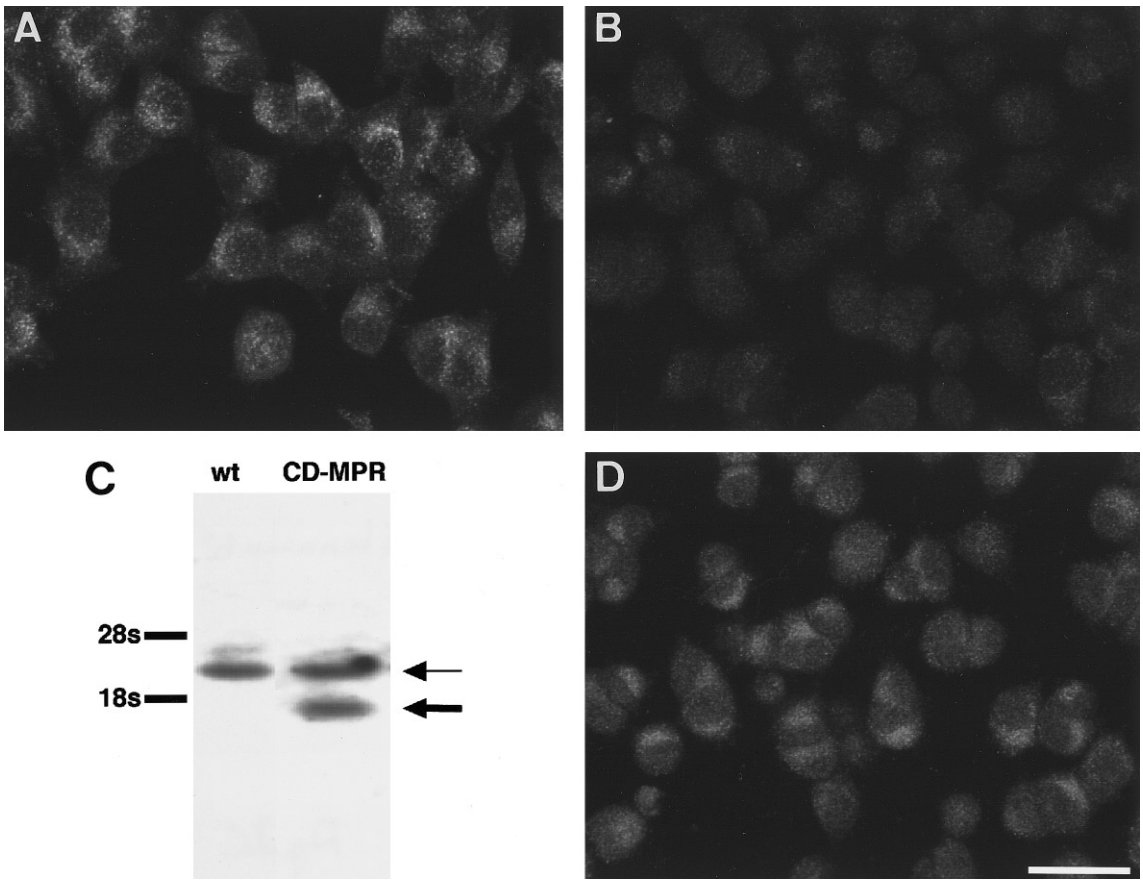

FIG. 2. Immunostaining of CI- and CD-MPRs in wild-type (wt) and CD-MPR-gene-transfected PC12 cells. (A,B, and D) Immunoreactivity for CI-MPR (A) and CD-MPR (B) in wt cells, and that for CD-MPR (D) in transfected cells. Bar indicates 20 μm. (C) Northern blot analysis of the CD-MPR mRNA in wt and CD-MPR-gene-transfected (CD-MPR) cells. Thin arrow indicates bands of the endogenous mRNA; thick arrow indicates the mRNA of the transfected CD-MPR gene.

MPR gene appeared in the transfected cells. Immunoreactivity for CD-MPR became intensely distributed in the perinuclear region of the transfected cells (Figure 2D), while no difference was detected in the distribution of CI-MPR between the CD-MPR-transfected and wt cells (data not shown).

The transfected PC12 cells were then immunostained with anti-cathepsins B and D. Immunodeposits for cathepsin B in the transfected cells became more distinct and larger in size than those in wt cells (Figure 3A,B), whereas immunoreactivity for cathepsin D showed no clear-cut difference in the distribution between the transfected and wt cells (Figure 3C,D). This difference will be discussed later.

We performed cell fractionation by a 15% Percoll gradient using PNS from the transfected and wt PC12 cells. In wt cells, cathepsin B-positive fractions were spread from the heavier fractions to the light membrane fractions, whereas those in the transfected cells appeared mostly in the heaviest fraction (Figure 3E). No clear-cut alteration was detected in the distribution of cathepsin D between the transfected and wt cells (Figure 3E). We then compared the protein amounts of the active forms of cathepsins B and D in wt and transfected PC12 cells before and 24 hr after serum-deprived cultureby Western blotting. In wt cells, the amount of the 29 kDa cathepsin B protein significantly decreased to approximately 30 % of its initial amount 24 hr after the onset of culture, whereas that of the 42 kDa cathepsin D protein increased to 250% (Fig. 3F). In the CD-MPR-gene transfected cells, however, no alteration was detected in the protein amount of cathepsin B, although the amount of the cathepsin D protein increased also to 250% of the initial amount (Figure 3F). We further examined the viability of the transfected cells following serum deprivation. The survival rate of the cells was significantly increased than that of wt cells (Figure 3G). This increased survival rate of the transfected cells was blocked, when CA074, a specific inhibitor of cathepsin B (21) was added to the culture media, while the inhibition by CA074 was restored by the further addition of pepstatin A (Figure G).

It has been suggested that interactions of MPRs with lysosomal enzymes differ depending on the phosphor-

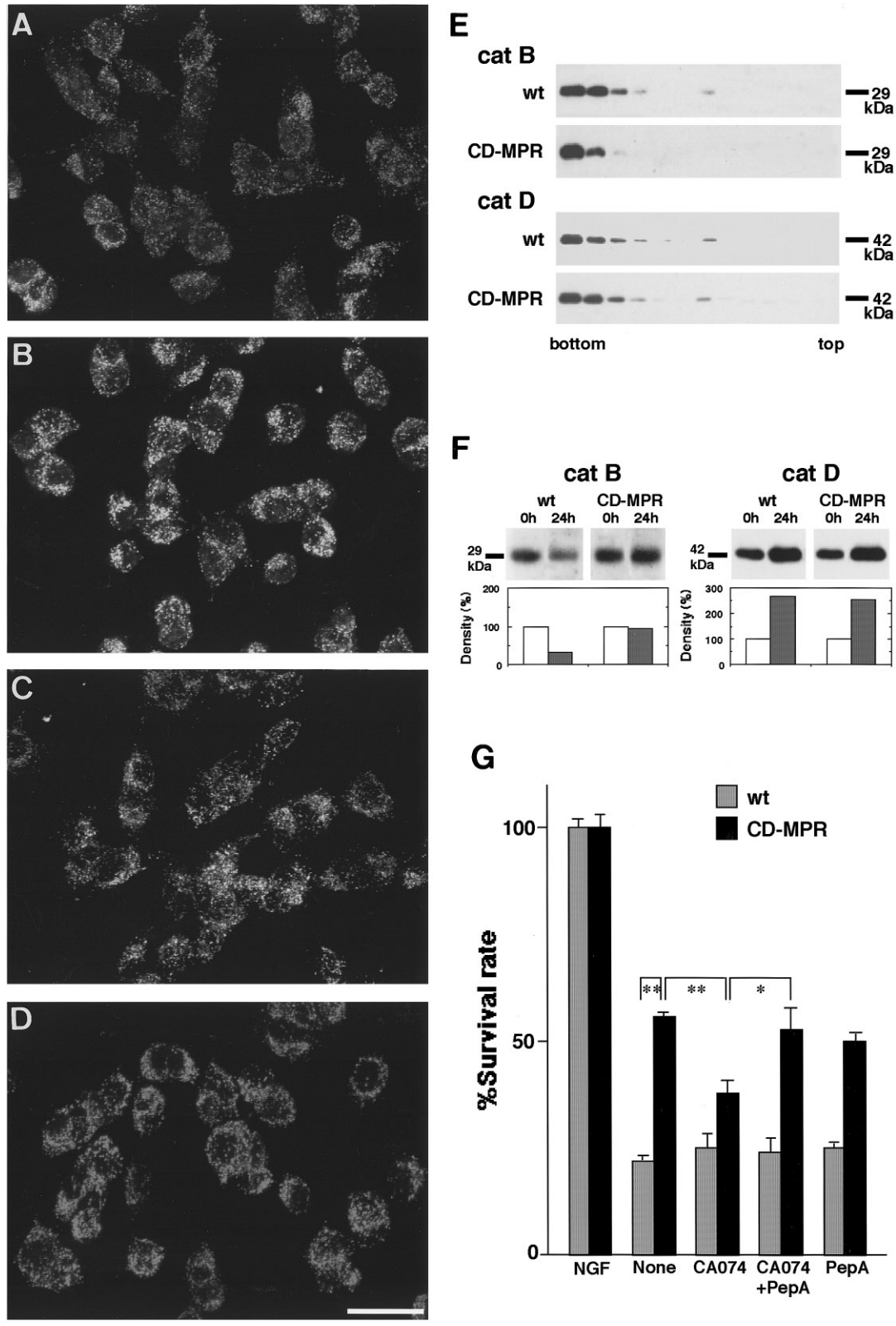

FIG. 3. Immunohistochemical and immunochemical analyses of cathepsins B and D in wild-type (wt) and CD-MPR-gene-transfected PC12 cells and their viability. (A-D) Immunoreactivity for cathepsin B in wt (A) and transfected cells (B), and that for cathepsin D in wt (C) and transfected (D) cells. Bar indicates 20 μm. (E) Subcellular fractionation of cathepsins B (cat B) and D (cat D) in wt and transfected (CD-MPR) cells. (F) Changes in the protein amounts of cathepsins B (cat B) and D (cat D) in wt and transfected (CD-MPR) cells before (0h) and 24 hr (24h) after the onset of serum-deprived culture. (G) The survival rate of wt and transfected (CD-MPR) cells 24 hr after the onset of culture under serum deprivation with 100 ng/ml NGF, 200 μM CA074, 200 μM CA074 and 100 μM pepstatin A (PepA), or 100 μM pepstatin A. The numbers of surviving cells cultured in the absence of serum but in the presence of NGF were estimated as 100%. Vertical bars indicate $\pm$ standard deviation. * indicates $P<0.05$ and ** $P<0.02$; none indicates serum-free medium alone.

ylation of their oligosaccharides; CI-MPR preferentially transports lysosomal enzymes possessing oligosaccharides with two mannose 6-phosphate residues, while CD-MPR preferentially transports lysosomal enzymes having multiple oligosaccharides with one mannose 6-phosphate residue (14, 17, 18). However, phosphorylation state is believed to differ from one cell type to another. In PC12 cells overexpression of CD-MPR altered the intracellular distribution of cathepsin B, but did not that of cathepsin D, suggesting that transport of cathepsin B to lysosomes may be more dependent on CD-MPR than CI-MPR in the cells. From the study on fibroblasts lacking CI-MPR and/or CD-MPR transport of cathepsin D has been shown to depend on CI-MPR, which is consistent with the present data exhibiting that no alteration was induced in the intracellular distribution of the enzyme by overexpression of CD-MPR in PC12 cells (14). It is interesting that the change in the intracellular distribution of cathepsin B in PC12 cells overexpressing CD-MPR occurred concomitantly with that in the viability of the cells which was significantly augmented in serum-deprived culture. It has been shown that overexpression of cathepsin D induce apoptosis of HeLa cells (4), while a lysosomal cysteine proteinase inhibitor induces cell death of primary cultured hepatocytes (16). These results may be incompatible with the present data showing that CA074, a specific inhibitor of cathepsin B prevented the increased viability of PC12 cells overexpressing CD-MPR following serum deprivation and this blocked survival rate by CA074 was restored by the further addition of pepstatin A. From these lines of evidence exhibiting that overexpression of CD-MPR efficiently transports cathepsin B to lysosomes in PC12 cells, resulting in the increase in the viability of the cells, it may be inferred that there is a cell death pathway regulated by cathepsins B and D, in which cathepsin D may act as a death factor and this death-inducing ability is prevented by cathepsin B, as reported separtely (28).

ACKNOWLEDGMENT

This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture of Japan.

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