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Cathepsin X cleaves the C-terminal dipeptide of alpha- and gamma-enolase and impairs survival and neuritogenesis of neuronal cells

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ABSTRACT

The cysteine carboxypeptidase cathepsin X has been recognized as an important player in degenerative processes during normal aging and in pathological conditions. In this study we identify isozymes alpha- and gamma-enolases as targets for cathepsin X. Cathepsin X sequentially cleaves C-terminal amino acids of both isozymes, abolishing their neurotrophic activity. Neuronal cell survival and neuritogenesis are, in this way, regulated, as shown on pheochromocytoma cell line PC12. Inhibition of cathepsin X activity increases generation of plasmin, essential for neuronal differentiation and changes the length distribution of neurites, especially in the early phase of neurite outgrowth. Moreover, cathepsin X inhibition increases neuronal survival and reduces serum deprivation induced apoptosis, particularly in the absence of nerve growth factor. On the other hand, the proliferation of cells is decreased, indicating induction of differentiation. Our study reveals enolase isozymes as crucial neurotrophic factors that are regulated by the proteolytic activity of cathepsin X.

1. Introduction

Increased neuroinflammatory reaction is recognized as playing a central role during normal brain aging, however the molecular mechanisms linking neuroinflammation with neurodegeneration have not been established. Identification of factors involved in these complex interactions is needed to understand the contribution of inflammatory components to age-related cognitive decline. Recent studies have suggested a lysosomal cysteine protease, cathepsin X, to be an important player in degenerative processes during normal aging and in pathological conditions (Wendt et al., 2007). While a number of other abundantly expressed cathepsins have been described in different neuronal death mechanisms, the pattern of cathepsin X localization implies its involvement in inflammatory processes and indicates a role for cathepsin X in inflammation induced neurodegeneration (Stichel and Luebbert, 2007). Cathepsin X is abundantly expressed in various immune cells such as monocytes, macrophages and dendritic cells (Kos et al., 2005). It is expressed in almost all cells in the mouse brain, with a preference for glial cells and aged neurons (Wendt et al., 2007).

In our recent studies we showed that cathepsin X stimulates macrophage/dendritic cell adhesion (Obermajer et al., 2006, 2008b) and lymphocyte migration (Jevnikar et al., 2008), and modulates inflammatory responses (Obermajer et al., 2008a) by activating β_2 integrin receptors Mac-1 and LFA-1 by proteolytic cleavage of the C-terminal part of the β-subunit. However, the natural substrates and precise mechanism of action for this protease in other cell types, particularly neuronal cells, are not known.

Enolase is a multifunctional glycolytic enzyme with diverse location in cells and tissues. The αα isozyme (alpha enolase, non-neuronal enolase) is present in various cells, including macrophages, neurons and glial cells (Ueta et al., 2004). The αγ and γγ isozymes (gamma enolase, neuron-specific enolase) are found only in neurons and neuroendocrine cells (Schmechel et al., 1987). All three forms of enolase are present in synaptosomes and exist in the synapse as membrane-associated proteins (Ueta et al., 2004). On leukocytes, the C-terminal lysine of alpha-enolase serves as a receptor for binding plasminogen to the cell surface, enhancing its activation (López-Alemany et al., 2003). The same role has been confirmed for neuritic cells (Nakajima et al., 1994) and the plasmin generation has been shown to be essential for nerve growth factor (NGF) induced differentiation in PC12 cells (Nakajima et al., 1994). Cleavage by cathepsin X could regulate the binding of plasminogen to neuritic cells and consequently matrix remodelling. On the

Abbreviations: LFA-1, leukocyte functional antigen-1; Mac-1, macrophage-1 antigen; NGF, nerve growth factor; PMA, phorbol 12-myristate 13-acetate.

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other hand, in cortical neurons it has been demonstrated that the C-terminal part of gamma enolase is responsible for increased survival and that the effect is specific to the gamma enolase isotype (Hattori et al., 1994).

In the present study we show that cysteine carboxypeptidase cathepsin X is able to cleave the C-terminal amino acids in alpha- and gamma-enolase and consequently, of regulating the neuritogenesis and survival of neuron cells. Our results contribute a new knowledge to clarify the contribution of inflammation and myeloid cell infiltration to the degeneration of neuronal cells as well as mechanisms in normal cell aging.

2. Materials and methods

2.1. Cell culture

KG-1 cell line is a human myeloblast cell line, derived from bone marrow aspirate obtained from male with erythroleukemia that evolved into acute myelogenous leukemia (CCL-246; ATCC, Manassas, VA). KG-1 cells differentiate to macrophage like cells, express significant amount of cathepsin X and present an adequate model to observe differentiation triggered cathepsin X translocation towards the plasma membrane. Cells were maintained in RPMI 1640 medium, Sigma (St. Louis, MO), supplemented with 2 mM glutamine, Sigma (St. Louis, MO), 2 g/l sodium bicarbonate, Riedel de Haën (St. Louis, MO) and 10% foetal bovine serum (FCS), HyClone (Logan, UT). PC12 cell line was derived from a transplantable rat pheochromocytoma. PC12 cells respond reversibly to NGF by induction of the neuronal phenotype and are used to study neuronal differentiation. Cells were cultured in DMEM supplemented with 2 mM glutamine, antibiotics and 15% horse serum.

2.2. Cathepsin X neutralizing monoclonal antibody

Specific neutralizing mouse monoclonal antibody (mAb) 2F12 against cathepsin X, isolated from human liver, was prepared from mouse hybridoma cell line as described (Kos et al., 2005). As shown by Western blot analysis and ELISA it reacted with the mature form of cathepsin X, but not with other related cathepsins such as B, L, H, S and C. The binding site for 2F12 mAb is a loop in the vicinity of the active site cleft (Obermajer et al., 2006) consisting of GEPWGERG sequence,

2.3. Two-dimensional electrophoresis, protein visualization and protein identification

KG-1 cells were resuspended in complete RPMI to a final concentration of 4×10^5 cells/ml containing 50 nM PMA, Sigma (St. Louis, MO) in 75 cm^2 culture flasks (TPP, Switzerland). Cells were allowed to differentiate to monocytes/macrophages at 37 °C with 5% CO_2 for 24 h. Non-adherent cells were washed with phosphate buffer saline pH 7.4. Cathepsin X inhibitor 2F12 mAb was added (0.5 μM) in the growth medium and cells were incubated at 37 °C for a further 24 h. The control sample contained only the growth medium and the appropriate volume of solvent in which inhibitor was diluted (phosphate buffer saline (PBS)). Afterwards, cells were washed once with PBS, harvested with PBS + 0.5% EDTA, solubilized in lysis buffer (400 mM phosphate buffer, pH 6.0, 75 mM NaCl, 4 mM EDTA-Na_2, 0.25% Triton X-100) and sonicated on ice. Cell lysate was centrifuged for 20 min, 13,000 rpm, 4 °C. The supernatant was discarded and the pellet (membrane fraction) was resuspended in lysis buffer. Samples (150 μg protein) were mixed with rehydration solution (9 M urea, 2% (w/v) CHAPS, 2% (v/v) immobilized pH gradient (IPG) buffer, 18 mM DTT, a trace of bromophenol blue) and applied on 13-cm immobilized pH 3–10 non-linear gradient (IPG) strips (GE Healthcare). Rehydration of IPG strips was carried out for 13 h employing an Immobiline Dry Strip Reswelling Tray (GE Healthcare). The rehydrated strips were then subjected to isoelectric focusing (IEF). SDS-PAGE as the second dimension was carried out with a 12% running gel on the vertical discontinuing electrophoretic system SE 600 (Hoeffer Scientific Instruments). Two simultaneously running 2-D gels were stained using SYPRO RUBY.

Spots in 2-D gels and membranes were recorded using G:BOX-HR (Syngene) and analyzed by LC–MS/MS using ESI-TRAP instrument at the Aberdeen Proteome Facility (University of Aberdeen, Scotland). The Mascot software was used to search NCBInr database (release date: 2007.01.20). The following search parameters were applied: Homo sapiens as species; tryptic digest with a maximum number of one missed cleavage. Trypsin cut C-term side of KR unless next residue is P. The peptide mass tolerance was set to ±1.7 Da and fragment mass tolerance to ±0.7 Da. Additionally, carbamidomethylation and oxidation of methionine were considered as possible modifications. Mascot protein scores of greater than 42 (spots 1 and 2) and greater than 43 (spot 4) were considered statistically significant ($p < 0.05$).

2.4. Plasminogen activation and binding assay

KG-1 cells were differentiated in a 96-well microtiter polystyrene plate (Costar, Schiphol-Rijk, NE) with 50 nM PMA (Sigma, St. Louis, MO) for 24 h. Non-adherent cells were washed with PBS. Cathepsin X neutralizing mAb 2F12 (Kos et al., 2005) was added to different concentrations in PBS and cells were incubated at 37 °C for another 30 min. The control sample contained only the PBS. For plasminogen activation, urokinase (uPA) (Sigma, St. Louis, MD) (50 pM), plasminogen (Sigma, St. Louis, MD) (500 nM) and plasmin specific fluorescent substrate DAla-Lev-Lys-AMC (Sigma, St. Louis, MD) (0.5 mM) were added to each well of the microtiter plate and plasmin formation was monitored over 1.5 h at 37 °C at 370 nm and 470 nm using a fluorescence plate reader (Safire 2, Tecan). For assessment of binding of plasminogen, differentiated KG-1 cells were incubated at 37 °C in the presence of 500 nM plasminogen in 0.1% FCS in PBS for 2 h at 4 °C. After washing, plate was incubated with goat anti-plasminogen antibody (Santa Cruz Biotechnology), followed by donkey anti-goat mAb conjugated with horseradish peroxidase (Upstate, Lake Placid, USA) at 37 °C for 1 h. The plate was washed with 0.1% FCS in PBS after every step. The substrate 3,3′,5,5′-tetramethyl-benzidine (Sigma) + H_2O_2 was used and the intensity of the signal was determined by measuring the absorbance at 450 nm after 15 min using the Rainbow microplate reader (Tecan).

2.5. Cathepsin X cleavage of model peptide of alpha- and gamma-enolase carboxyl terminal

Human procathepsin X cDNA was amplified by PCR from human cathepsin X clone (RZPD German Resource Center for Genome Research, Germany) using primers 5′-CCGCTCGAGAAAAGAGAGGCTGAAGCTGGCCTCTACTTCCGCCGG-3′ and 5′-CACTGCGGCCGCTTAAACGATGGGGTCCCCAAATGTACAGTGCTCC-3′. It was cloned and expressed in *P. pastoris* as an α-factor fusion construct as described previously (Nägler et al., 1999). Isolated procathepsin X was activated with 25 nM cathepsin L (versus 3–4 μM procathepsin X) in 50 mM sodium citrate, pH 5.5, containing 0.2 M NaCl, 1 mM EDTA and 2 mM DTT as described (Nägler et al., 1999). Cathepsins X and L were then separated with immunoprecipitation using sheep anti-cathepsin L polyclonal antibody bound to nProtein A Sepharose 4 Fast Flow (GE Healthcare, Sweden). Final sample was characterized by ELISA and Western blot.

The synthetic peptides (KAKFAGRNPRNPLAK—human alpha enolase; AKYNQLMRIEEELGEEARFAGHNFRNPSVL—rat gamma enolase) of the C-terminal of alpha- and gamma-enolase were synthesized by Biosynthesis, Inc. (Denver, TX, USA). The peptides were digested at 120 μM with recombinant cathepsin X (0.75 μM) for 30 min and 1hour at 37 °C in 50 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM DTT, pH 5.5. Each sample was separated by reverse-phase HPLC using an Gemini C_{18} column (5 μm, 110 Å, 150 mm × 46 mm) (Phenomenex®) and peak fractions analyzed by Q-Tof Premier mass spectrometer (in ESI+ mode) (Waters).

2.6. Surface plasmon resonance

The interaction between plasminogen and C-terminal peptide of alpha enolase pretreated or not with recombinant cathepsin X were analyzed by surface plasmon resonance in a Biacore X system (BIAcore, Uppsala, Sweden). Plasminogen was immobilized at a concentration of 2.5 μg/ml in sodium acetate buffer, pH 5.5 at a flow rate 5 of μl/min for 10 min (6300 RFU) onto CM5 chip (BIAcore AB). Reference surface was prepared in the second flow cell by amine coupling activation followed by immediate deactivation. The chip was then washed with 5 μl of 10 mM glycine buffer (pH 9.5) at a flow rate of 30 μl/min.

For the kinetic measurements 6 different concentrations of peptide were injected for 5 min. At the end of each cycle, the surface was regenerated by one min injection of 10 mM glycine buffer (pH 9.5). The experiments were carried out in 0.04 M phosphate buffer, pH 7.4, containing 0.1 M NaCl at a flow rate of 30 μl/min. (BR-1003-98 BIAcore).

2.7. Protein quantitation by flow cytometry and ELISA

KG-1 cells were differentiated at a concentration of 4×10^5/ml in 24 well plates (Corning Costar) with 50 nM PMA for 24 h. PC12 cells were cultured on collagen precoated plates (20 μg/ml in carbonate buffer, pH 9.6, at 4 °C overnight) with 50 ng/ml NGF for 5 days. Afterwards, cells were washed with PBS to remove non-adherent cells. Adherent cells were harvested and fixed with 2% paraformaldehyde at room temperature for 30 min. Nonspecific staining was blocked with 3% BSA in PBS, pH 7.4 for 30 min. Next, KG-1 cells were incubated with goat anti-alpha enolase polyclonal antibody and PC12 cells with goat anti-gamma enolase polyclonal antibody (Santa Cruz Biotechnology) in blocking buffer for 30 min on ice. For cathepsin X staining, cells were incubated with Alexa-488 labelled mAb 2F12 for 30 min on ice. Cells were washed with 3% BSA in PBS and incubated for an additional 30 min with FITC-labelled donkey anti-goat secondary antibody. Cells were washed with PBS and analyzed with FACS Calibur (Becton Dickinson). A FITC-anti-goat-IgG or Alexa488-IgG1 was used as an isotype control.

To prepare cell lysates for analysis of the protein level of cathepsin X, PC12 cells were grown for 7 days in the presence or absence of NGF. The cells were washed with PBS, pH 7.4 and centrifuged for 5 min at 2000 rpm. The pellet was re-suspended in 50 μl of 0.05 M sodium acetate buffer, pH 6, 1 mM EDTA, 0.1 M NaCl, 0.25% Triton X-100 and stored at −80 °C. Total protein concentration was determined by the Bradford method using the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, USA). Cathepsin X ELISA was performed as reported (Kos et al., 2005).

2.8. Co-localization of alpha or gamma enolase and cathepsin X

For immunofluorescence, KG-1 cells were differentiated on glass coverslips at a concentration of 4×10^5/ml in 24 well plates (Corning Costar) with 50 nM PMA for 24 h. PC12 cells were treated with NGF (50 ng/ml) for 5 days on glass coverslips precoated with collagen (20 μg/ml). Cells were fixed with 2% paraformaldehyde at room temperature for 30 min and permeabilized with 0.05% Triton-X 100 for 10 min. Afterwards nonspecific staining was blocked with 3% BSA in PBS, pH 7.4 for 1 h. Alexa 488-labelled 2F12 mAb and goat anti-alpha or anti-gamma enolase polyclonal antibody (Santa Cruz Biotechnology) in blocking buffer were then incubated for 2 h at 37 °C. Cells were washed with PBS and incubated with Alexa 633-labelled donkey anti-goat secondary antibody. After washing with PBS the Prolong Antifade kit (Molecular Probes) was used for mounting coverslips on glass slides. Fluorescence microscopy was performed using Carl Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany). Alexa 488 and 633 were excited with an argon (488 nm) or He/Ne (633 nm) laser and emission was filtered using a narrow band LP 505–530 nm (green fluorescence) and 560 nm (red fluorescence) filters, respectively. Images were analyzed using Carl Zeiss LSM image software, version 3.0.

2.9. Live microscopy of pcDNA3/CATX-GFP transfected PC12 cells

The cDNA for procathepsin X was obtained from RZPD German Resource Center for Genome Research and amplified using the synthetic primers: 5′-GGTAGGATCCATTATGGCGAGGCGCGGG-3′ and 5′-TAACGATGGGGTCCCCAAATGTACAG-3′ and cloned into pcDNA3.1/NT-GFP-TOPO® plasmid vector (Invitrogen, USA) according to the manufacturer's protocol.

PC12 cells were placed in 24 well plate (5×10^5/ml). Cells were transfected with pcDNA3/cathepsin X-GFP construct using lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol and transfection efficiency analyzed with flow cytometry. Wells of a 96-well culture plate (Corning Costar) were coated with collagen and the PC12 cells (2×10^4/well), transfected with pcDNA3/catX-GFP, were plated in complete growth medium with 50 ng/ml NGF. Cells were tracked at 30 s intervals for 30 min. Images were taken using an Olympus IX 81 motorized inverted microscope and CellR software.

2.10. Neuronal survival assay and neuritogenesis (mAb2F12 AND Stealth™ RNAi/CATX)

Wells of a 96-well culture plate (Corning Costar) were coated with collagen. PC12 cells (2×10^4/well) were plated in either complete growth medium or serum-depleted medium. 2F12 mAb was added at different concentrations and the cells incubated for 5 days.

Silencing of cathepsin X expression was also performed with Stealth™ RNAi. The single stranded oligos were (RNA)-CCAGCGUCACGAGGAAUCAGCAUAU and (RNA)-AUAUGCUGAUUCCUCGUGACGCUGG (Invitrogen Stealth™ RNAi, Invitrogen). PC12 cells were transfected with Stealth™ RNAi-cathepsin X using lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. Cathepsin X gene silencing was confirmed with quantitative ELISA. In control experiment, Stealth™ RNAi/control (RNA)-CCATGACGCGAAGTAACCGACGTAT and (RNA)-ATACGTCGGTTACTTCGCGTCATGG were used. Next day, the cells were plated in serum-depleted medium in collagen precoated 96-well culture plates. NGF (50 ng/ml) was added.

In a similar experiment, rat gamma enolase peptide (30 C-terminal a.a.) was added at different concentrations to test its neurotrophic effect on rat PC12 cells, either in the presence or absence of recombinant cathepsin X (25 nM). The rat and the human gamma enolase C-terminal peptides differ in one amino acid (414, E or D), what should not be relevant for cathepsin X cleavage, but could affect the gamma enolase neurotrophic effect on rat cells. Cells were incubated for 5 days.

Cell survival was determined with MTS colorimetric assay (Promega, Madison, WI). Cell survival was determined by Eq. (1),

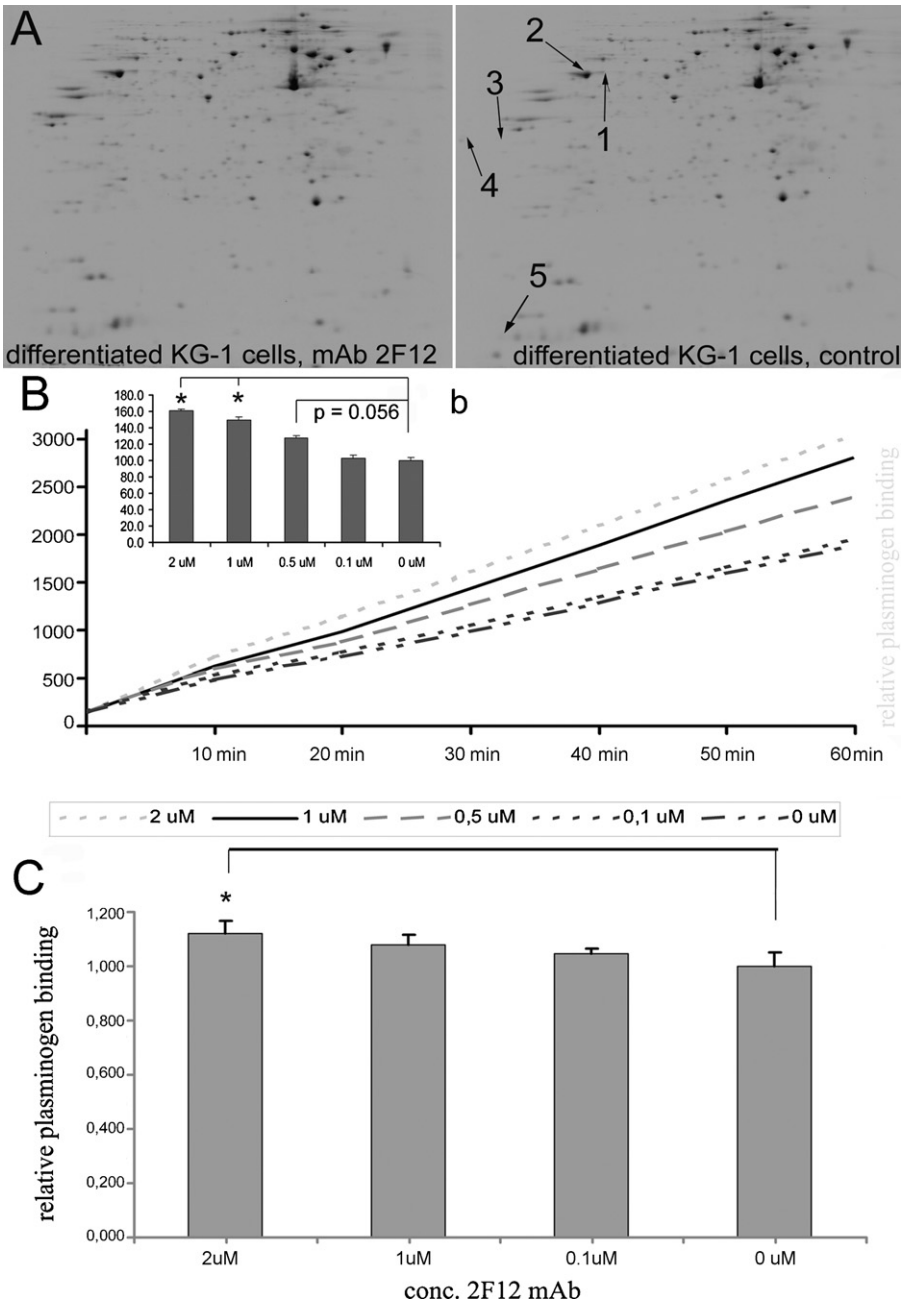

Fig. 1. Identification of proteins affected by cathepsin X proteolytic activity. (A) 2D electrophoresis of the cell membrane fraction of differentiated KG-1 cells treated or not with mAb 2F12 (1 μM) is shown and the arrows indicate five differentially expressed spots, selected for mass spectrometry analysis. (B) Plasmin generation was measured in the presence of different concentrations of 2F12 mAb and the substrate hydrolysis monitored at 2.5 min intervals at 470 nm. The kinetic profile of substrate hydrolysis is presented. (b) The insert shows the increase of plasmin generation with increasing concentrations of 2F12 mAb at 60 min. (C) Plasminogen binding to differentiated KG-1 cells in the presence of different concentrations of 2F12 mAb was detected with anti-plasminogen antibody and secondary peroxidase labelled antibody. The intensity signal of 3,3′,5,5′-tetramethyl-benzidine product was measured at 450 nm. Wells were treated in quadruplicate. Results are the means ± s.d. of at least three independent assays. $^{*}p < 0.05$ for 2F12 mAb treated cells versus control cells only.

where $A_{\text{test cells}}$ and $A_{\text{control cells}}$ are the absorbances of formazan determined for treated cells and control cells:

$$\text{Cell survival}\,(\%) = \left(\frac{A_{\text{test cells}}}{A_{\text{control cells}}}\right) \times 100. \quad (1)$$

On 1–4 consecutive days, for each treatment group, the percentage of cells bearing neurites longer than the cell diameter was enumerated in a double-blind manner by two observers. Each treatment group was seeded in four wells of a 96-well plate and each well was counted for neurites longer than the cell diameter (at least 250 cells for each treatment group in one experiment and three independent experiments were carried out). The cells were observed live at 37 °C and 5% CO_2 using an Olympus IX 81 motorized inverted microscope.

2.11. Proliferation and apoptosis detection

Wells of a 6-well culture plate (Corning Costar) were coated with collagen. To assess the proliferation, PC12 cells were labelled with CFSE (carboxy-fluorescein diacetate, succinimidyl ester) according to the manufacturer's protocol (Invitrogen) and plated (2×10^5/ml)

Table 1
Identification of proteins by LC–MS/MS searching NCBInr database for the 5 differentially expressed spots marked in Fig. 1A. Significance of differential expression was determined from two independent assays.

Sample	Sample results	Protein score	Matched peptides number	Sequence coverage (%)
1	3 Significant hits			
	1. gi|4503571 enolase 1 [Homo sapiens]	858	21	48
	2. gi|930063 neurone-specific enolase [Homo sapiens]	173	2	7
	3. |28422655 ENO1P protein [Homo sapiens]	82	4	n.a.
2	4 Significant hits			
	1. gi|62897945 enolase 1 variant [Homo sapiens]	1267	38	59
	2. gi|31170 unnamed protein product [Homo sapiens]	246	6	16
	3. gi|28422655 ENO1P protein [Homo sapiens]	170	6	n.a.
	4. gi|930063 neurone-specific enolase [Homo sapiens]	161	5	13
3	None significant hits			
4	2 Significant hits			
	1. gi|36102 Heterogeneous nuclear ribonucleoprotein A1 (helix-destabilizing protein) (single-strand RNA-binding protein) (hnRNP core protein A1)	150	2	10
	2. gi|1346343 Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1) (K1) (67 kDa cytokeratin)	44	1	1
5	None significant hits			

n.a.: data not available in the database.

in complete growth medium. 2F12 mAb was added at concentration 1 μM and cells incubated for 5 days. PC12 cells were then labelled with PI and analyzed for proliferation and cytotoxicity with two-colour flow cytometry on FACS Calibur (Beckton Dickinson). For apoptosis detection, PC12 cells were plated (2×10^5/ml) in serum-free growth medium. 2F12 mAb was added at a concentration of 0.5 μM or 1 μM and the cells incubated for 5 days. Apoptosis Detection Kit (Sigma) was used according to manufacturer's protocol and the percentages of live cells, dead cells and cells early in the apoptotic process determined by staining with annexin V FITC conjugate and propidium iodide.

2.12. Statistical analysis

The SPSS software package (release 16.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The difference between the groups was evaluated using the nonparametric two-tailed Mann–Whitney *U*-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Alpha enolase is affected by cathepsin X carboxypeptidase activity in KG-1 cells

The proteins affected by cathepsin X proteolytic activity were identified by two dimensional electrophoresis of differentiated KG-1 cells treated with cathepsin X inhibitor, with untreated cells as control. 5 spots differentially expressed in treated KG-1 cells were selected in the 2D gel (Fig. 1A). They were excised and analyzed by LC–MS/MS for protein sequence. Three of the five selected spots corresponded to plasminogen-binding proteins, the other two providing no significant hits (Table 1). Two spots were determined as enolase 1 and enolase 1 variant. The third spot was determined as a heterogeneous nuclear ribonucleoprotein A1 or cytokeratin 1. Enolase 1 (alpha enolase) was considered as one of the target proteins for cathepsin X carboxypeptidase activity, and the biological consequences of this observation were studied in more detail. Heterogeneous nuclear ribonucleoprotein A1 nor cytokeratin 1 were not studied as a potential targets of cathepsin X activity.

3.2. Plasminogen activation and binding assay

Since alpha enolase is a prominent plasminogen binding protein, we tested the role of cathepsin X in plasminogen activation on KG-1 cells by measuring plasmin generation with fluorogenic plasmin-specific substrate. Plasminogen activation was induced by adding both plasminogen and uPA to differentiated KG-1 cells. Inhibition of cathepsin X activity by a neutralizing monoclonal antibody, 2F12 mAb, increased plasmin generation by 61.3%, by comparison with control cells (Fig. 1B and b). Despite a significant increase in plasmin generation, plasminogen binding to differentiated KG-1 cells increased by only 10.6% compared to control (Fig. 1C).

3.3. Cathepsin X cleavage of model peptides of alpha- and gamma-enolase carboxyl terminals

We analyzed the activity of cathepsin X on the carboxyl terminal peptides of alpha enolase (KAKFAGRNPRNPLAK) and its isozyme gamma enolase (AKYNQLMRIEEELGEEARFAGHNFRNPSVL). The peptides were digested by recombinant cathepsin X and each fraction separated by reverse phase HPLC and analyzed by mass spectrometry. After 30 min cathepsin X cleaved C-terminal lysine/leucine residue and, partially, the preceding alanine/valine residue. After 60 min the two C-terminal amino acids were completely cleaved (Fig. 2). Degradation stopped at this position. The results show that cathepsin X acts on enolase as a carboxymonopeptidase and cleaves C-terminal amino acids until proline residue is present in the P1 position.

3.4. Cathepsin X abolishes the binding of plasminogen to alpha enolase

Using surface plasmon resonance we tested whether the cleavage of the two C-terminal amino acids of alpha enolase by cathepsin X decreases the binding of plasminogen. Whereas the binding of undigested alpha enolase peptide to the immobilized plasminogen was concentration dependent (Fig. 3A), digested peptide showed no marked binding to plasminogen (Fig. 3B). The dissociation constant K_d was calculated from a Scatchard plot (6.61 μM) (Fig. 3C).

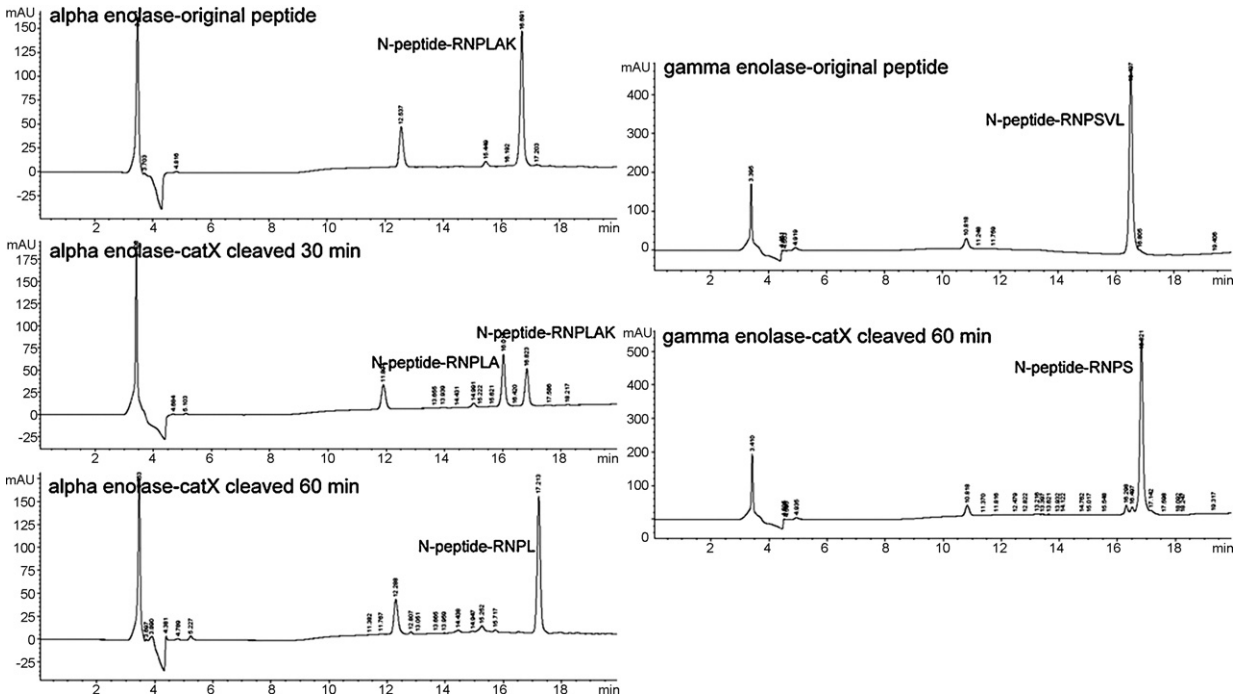

Fig. 2. HPLC analysis of the alpha- and gamma-isozyme C-terminal peptides cleaved with recombinant cathepsin X. The non-cleaved C-terminal domains (N-peptide-RNPLAK^{435} (alpha enolase) and -RNPSVL^{435} (gamma enolase)) and corresponding cleaved domains (N-peptide-RNPL^{433} and -RNPS^{433}), detected after treatment with recombinant cathepsin X for 60 min, were identified with electron-mass spectrometry in the fraction eluted between 14.0 and 16.0 min. Other peaks did not contain any cleaved peptides. After treatment with cathepsin X for 30 min, two cleaved domains were detected (N-peptide-RNPLA^{434} and -RNPL^{433}), demonstrating sequential cleavage by cathepsin X.

3.5. Protein quantification by flow cytometry and ELISA

The total protein concentration of cathepsin X was found to be higher in neuronally differentiated PC12 cells (779.0 pg/ml) than in non-differentiated (310.4 pg/ml) PC12 cells (Fig. 4A). However, its expression was about 100-fold lower than in KG-1 cells (71.06 ng/ml) or differentiated KG-1 cells (82.37 ng/ml) (Fig. 4B). The presence of cathepsin X in the medium of differentiated KG-1 cells (291 pg/ml) indicates that cathepsin X was secreted from the cells. The secretion from PC12 cells and non-differentiated KG-1 cells was lower (Fig. 4A).

The membrane contents of gamma enolase and cathepsin X were evaluated in non-differentiated and neuronally differentiated PC12 cells. Both gamma enolase and cathepsin X were observed to be increased on the plasma membrane on neuronal differentiation (Fig. 4C). In KG-1 cells, the level of alpha enolase is higher in differentiated than in non-differentiated KG-1 cells (Fig. 4D).

3.6. Co-localization of alpha enolase, gamma enolase and cathepsin X in the perimembrane region

Cathepsin X and alpha-enolase were localized on differentiated KG-1 cells and PC12 cells, using confocal immunofluorescence microscopy. As seen from the contour plot and the mask of the pixels above the threshold in both channels (blue colour), alpha-enolase and cathepsin X co-localize predominantly in the perimembrane region of differentiated KG-1 cells. However, in non-differentiated KG-1 cells the co-localization is rather weak, alpha enolase localizing predominantly in the cytosol, and cathepsin X in perinuclear vesicles (Fig. 5A).

A similar feature was observed in PC12 cells. Co-localization of cathepsin X with alpha- and gamma-enolase and is shown in non-differentiated and differentiated PC12 cells (Fig. 5B and C).

3.7. Cathepsin X translocation in PC12 cells

Translocation of cathepsin X was observed in pcDNA3/catX-GFP transfected PC12 cells on collagen 24 h after the addition of NGF. Cathepsin X is present in the lysosomes in the perinuclear region and is translocated inside the lysosomal vesicles (Fig. 5D) along the neurites. Its localization is particularly dominant in the growth zone of neurites (Fig. 5E). The results demonstrate vesicular translocation of cathepsin X to the plasma membrane (see also Supplementary Movie 1).

3.8. Cathepsin X reduces PC12 cell survival and neuritogenesis

The effects of 2F12 mAb, CA-074 (inhibitor of cathepsin B), E64 (nonspecific cysteine protease inhibitor), CLIK-148 (specific inhibitor of cathepsin L) and C-terminal gamma enolase peptide on cell survival was determined in serum-free medium for non-differentiated PC12 cells and for PC12 cells grown on collagen in the presence of NGF. The addition of 2F12 mAb significantly improved cell survival and the effect was concentration dependent. 2 μM 2F12 increased the survival 2.2-fold in the absence and 1.7-fold in the presence of NGF (Fig. 6A–D). The effect was similar to that induced by addition of serum. The addition of 1 μM 2F12 mAb to serum-enriched medium only increased survival from 2.9- to 3.0-fold. Other cysteine protease inhibitors did not show any similar effect (data not shown). Although CA-074 inhibits cathepsin B and cathepsin X (Klemencic et al., 2000), its effect on the increased PC12 cell survival was not observed. It could be that cathepsin B rather than cathepsin X was inhibited by CA-074 under our experimental conditions.

The addition of gamma enolase C-terminal peptide also markedly increased cell survival (Fig. 6E and F). When recombinant cathepsin X was added, however, the effect of the peptide decreased by 19.8% in the presence or 91.0% in the absence of NGF (Fig. 6E and F). Thus, the C-terminal part of gamma enolase exerts

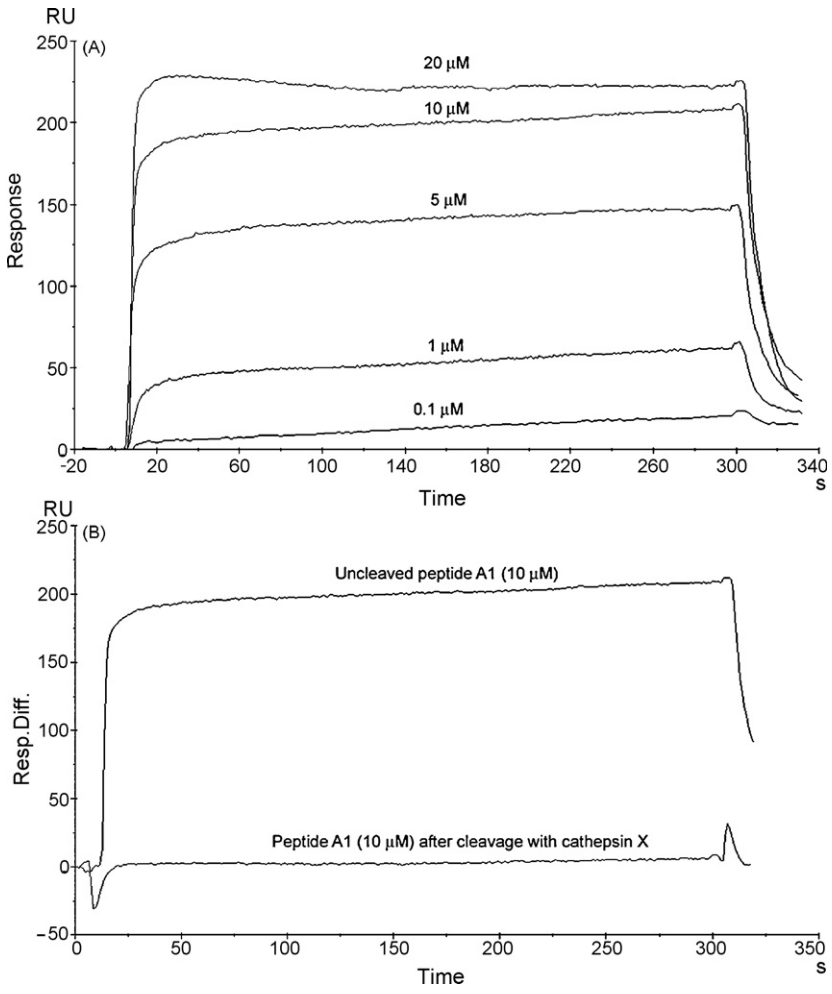

Fig. 3. Surface plasmon resonance analysis of alpha enolase C-terminal domain interaction with plasminogen immobilized on a CM5 sensor chip using BIAcore X system. (A) Different concentrations of alpha enolase C-terminal were injected over the plasminogen-immobilized surface at a flow rate of 5 μl/min in 1% PBST running buffer at 25 °C. (B) Pretreatment of alpha enolase C-terminal peptide with recombinant cathepsin X abolished interaction with immobilized plasminogen.

a pro-survival role on PC12 cells, which is abolished by the proteolytic activity of cathepsin X, particularly in the absence of growth factors in serum-free medium.

However, after inhibition of cathepsin X activity not only cell survival of PC12 cells in serum-free medium was increased, but also the neurite length distribution pattern was changed. In the presence of NGF, 2F12 mAb increased the number of cells with neurites longer than the cell diameter by 2.2-times (Fig. 7A).

A similar effect on neuritogenesis was observed also in cells with decreased expression of cathepsin X, caused by Stealth™ RNAi/catX. Two RNAi sequences of 25 nucleotides were chosen for silencing cathepsin X. In this case the number of cells bearing extended neurites was 2.8-fold higher than in the control (Fig. 7B). In the absence of NGF, inhibition of cathepsin X still initiated spontaneous neurite outgrowth, but the number of neurite bearing cells was low.

3.9. Effect of cathepsin X inhibition on apoptosis or proliferation of PC12 cells

The increase of PC12 cell survival following inhibition of cathepsin X by 2F12 mAb was also demonstrated by flow cytometry analysis. After 48 h, the percentage of apoptotic cells was decreased by 52.1% by the inhibition of cathepsin X (Fig. 8A). The percentage of propidium iodide (PI) positive cells was also significantly reduced (Fig. 8B). The percentage of proliferating PI^{neg} cells, as observed with CFSE fluorescence intensity, was decreased (Fig. 8C), particularly in the absence of NGF, showing that the inhibition of cathepsin X, like the presence of NGF, leads to a differentiation stimulus.

4. Discussion

In the present study, inhibition of cathepsin X has been shown to promote neurite outgrowth in PC12 cells and to increase their survival. The effect is attributable to preservation of the neurotrophic activity of the C-terminal end of alpha- and gamma-enolase on the plasma membrane of PC12 cells. Since cathepsin X is a lysosomal protease, it could exert some important functions intracellularly as well, affecting neuronal survival as shown for other cathepsins in neuronal cells. However in the present study, we disscuss the importance of C-terminal end of enolase isozymes, that are shown to be cleaved by cathepsin X.

HPLC analysis and subsequent electrospray mass spectrometry revealed that cathepsin X is capable to sequentially eliminate two C-terminal amino acids in both isozymes. Cathepsin X acts primarily as a carboxypeptidase and, unlike another cysteine carboxypeptidase, cathepsin B, does not exhibit endopeptidase activity (Guncar et al., 2000). Cathepsin X was initially suggested to act as a carboxymonopeptidase (Nägler et al., 1999), later its carboxydipeptidase activity was also reported (Klemencic et al., 2000; Therrien et al., 2001). Recent publications demonstrate again a specific monocarboxypeptidase activity of cathepsin X (Devanathan et al., 2005;

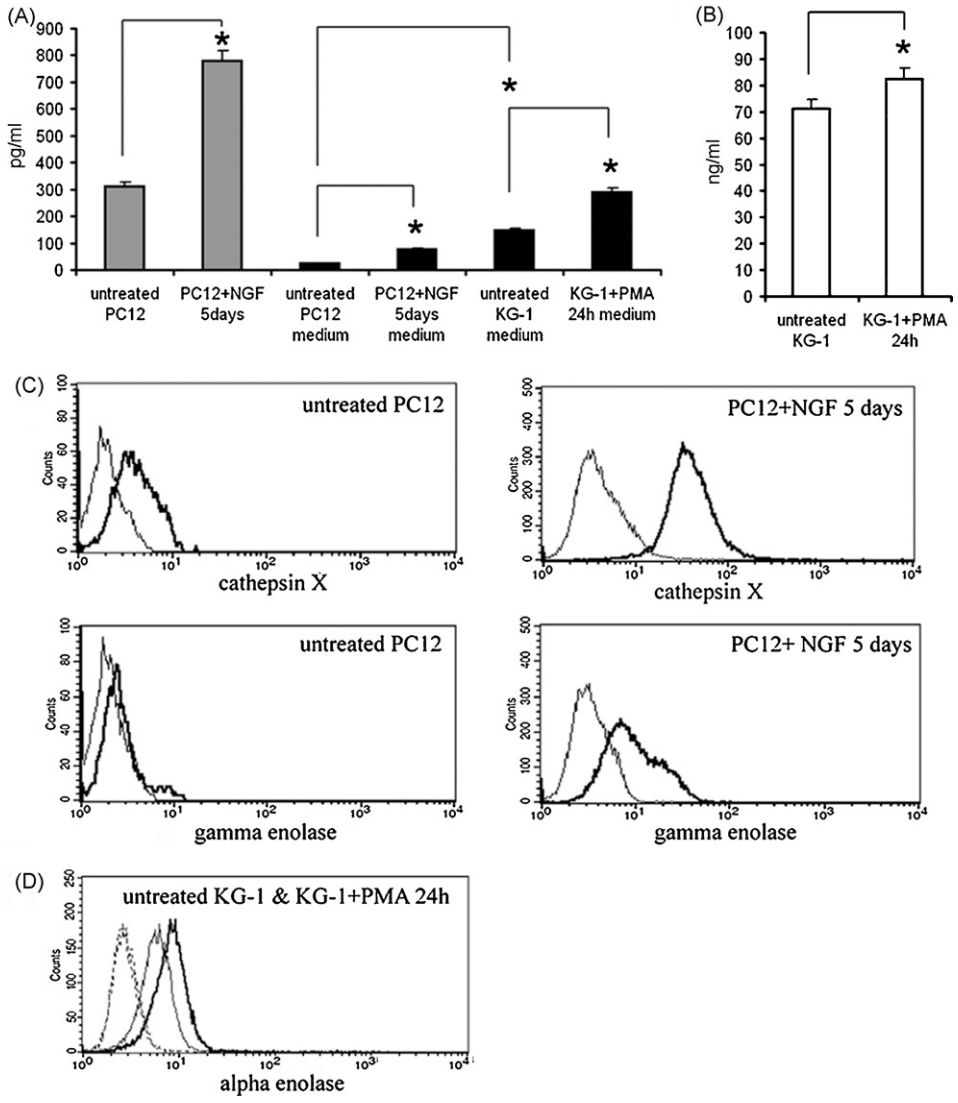

Fig. 4. Expression of cathepsin X, alpha- and gamma-enolase in PC12 and KG-1 cells. (A and B) Protein levels of active cathepsin X in cell lysates and cell culture conditioned medium were determined by ELISA, using 3B10 and 2F12 mAb as capture and detection antibody, respectively. The active cathepsin X protein level in KG-1 cells is about 100-fold higher than in PC12 cells. Differentiation increases the levels of active cathepsin X. (C) Flow cytometric analysis of non-permeabilized PC12 cells untreated or treated with NGF (50 ng/ml) for 5 days. Thin solid line represents isotype control and thick line the expression of cathepsin X or gamma enolase,. The membrane expression is increased on treatment with NGF. (D) Flow cytometric analysis of alpha enolase membrane expression in non-permeabilized KG-1 cells treated (thick solid line) or not (thin solid line) with PMA (50 nM) for 24 h. Broken thin line represents isotype control. $^{*}p < 0.05$.

Puzer et al., 2005). Devanathan et al. (2005) showed that cathepsin X exhibits broad S2, S1 and S1′ specificities, excluding proline in one of these positions. In particular, peptides with proline in S2 position show minor proteolysis, whereas peptides with proline in position S1 and S1′ show no hydrolysis at all (Devanathan et al., 2005). Our study is in agreement with their results, confirming that cathepsin X acts as a monocarboxypeptidase, since the C-terminal amino acid of enolase peptide after cathepsin X digestion was either alanine/valine or leucine/serine. Moreover, the hydrolysis stopped completely after reaching proline in the P1 position.

The C-terminal parts of the two isozymes, neuron specific enolase (γγ) and non-neuronal enolase (αα) have been shown to exert a functional role on the plasma membrane of neuronal cells. Hattori et al. (1994) demonstrated that C-terminal region of neuron specific enolase promotes survival of rat embryonic neocortical neurons. The neurotrophic effect was detected at relatively low dose—20 nM. The C-terminal part of alpha enolase in the neuronal plasma membrane was shown to bind plasminogen (Nakajima et al., 1994). Plasminogen binding was shown to enhance the neurite outgrowth of neocortical explants (Nagata et al., 1993) and plasmin generation to enhance NGF-induced neuritogenesis in PC12 cells (Jacovina et al., 2001).

The differences in the C-terminal amino acids of gamma and alpha enolase could be crucial for their specific biological effects. By eliminating the C-terminal lysine in alpha enolase that acts as a plasminogen receptor on the cell surface, cathepsin X was shown to abolish the binding of plasminogen as evident from surface plasmon resonance study. The biological effect of this alpha enolase cleavage was revealed by the co-localization of alpha enolase and cathepsin X on the plasma membrane of differentiated KG-1 cells. In KG-1 monocytes, cathepsin X is present in perinuclear endolysosomal compartments and does not co-localize with alpha enolase, however differentiation triggers its translocation to the plasma membrane (Obermajer et al., 2006), where it may interact with enolase. The inhibition of cathepsin X by 2F12 Mab led to the presence of larger amounts of alpha enolase in the membrane fraction of differentiated KG-1 cells. It is likely that enolase is internalized after the proteolytic cleavage with cathepsin X. The

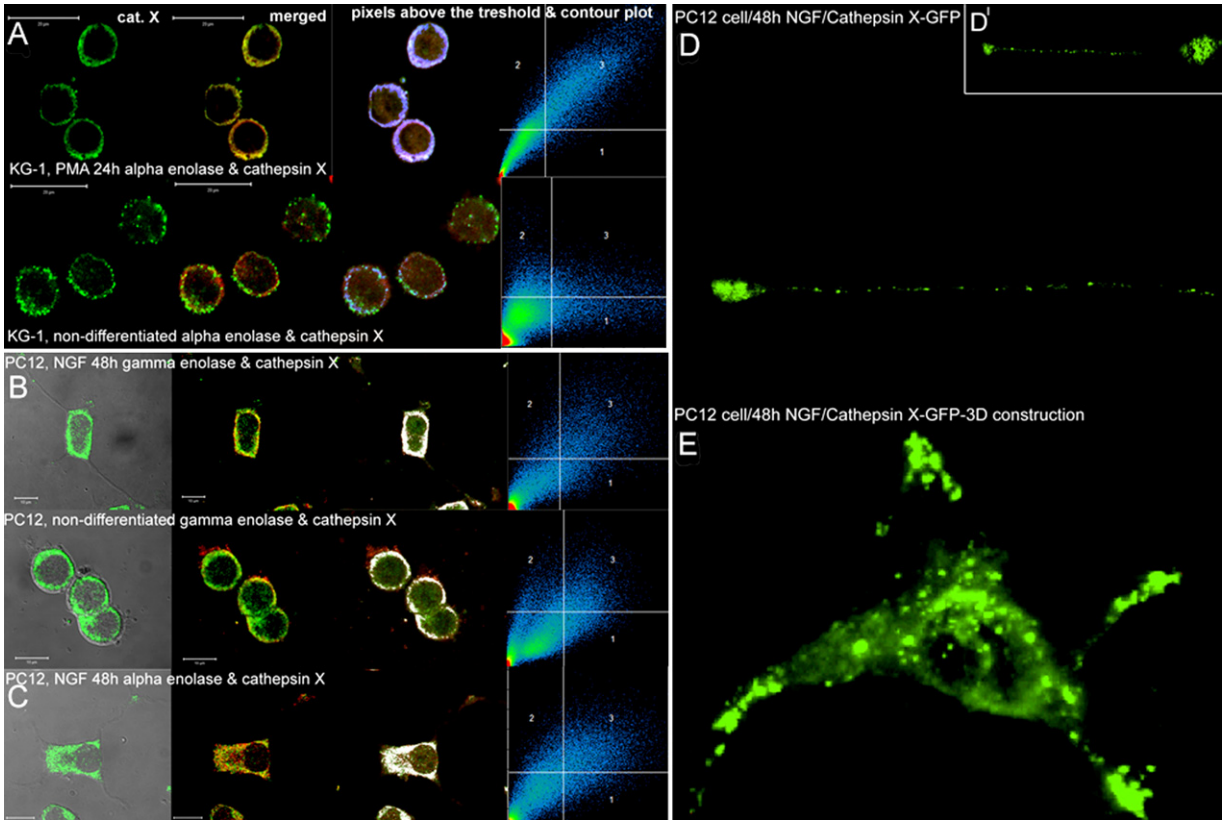

Fig. 5. Localization of cathepsin X in KG-1 and PC12 cells. (A–D) Co-localization of cathepsin X (green fluorescence, Alexa Fluor 488) and alpha- and gamma-enolase (red fluorescence, Alexa Fluor 633) in KG-1 cells and PC12 cells. (A) In non-differentiated KG-1 cells cathepsin X and alpha enolase do not co-localize. However upon 24 h differentiation with PMA (50 nM) cathepsin X and alpha enolase are co-localized, mainly at the perimembrane region. (B) In PC 12 cells cathepsin X and gamma enolase are localized at the perimembrane region, but NGF treatment induces a polarized neuronal phenotype, where cathepsin X and gamma enolase are co-localized predominantly at the neurite growth cones. (C) A similar co-localization profile was obtained for alpha enolase and cathepsin X in NGF treated PC12 cells. Fluorescent dyes were imaged sequentially in all co-localization experiments in a frame-interlace mode to eliminate cross talk between the channels. The threshold level for this display was set to one third of the maximal brightness level. The pixels above the threshold in both channels (blue to white colour) and the contour plot are shown for images demonstrating co-localization. Scale bars represent 20 μm for A and 10 μm for B and C. (D and E) Live microscopy of pcDNA3/catX-GFP transfected PC12 cells. (D′) PC12 cell transfected with pcDNA/catX-GFP and stimulated with 50 ng/ml NGF for 24 h is shown in the insert. (D) In enlarged view vesicles containing cathepsin X-GFP trafficking inside neurite can be seen. (E) 3D reconstruction of pcDNA3/catX-GFP transfected PC12 cell demonstrates dense localization of cathepsin X in growing cones of neurites. See Appendix BSupplementary Movie 1. Images were taken using an Olympus IX 81 motorized inverted microscope and CellR software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

preserved C-terminal lysine by cathepsin X inhibition accounts for the markedly enhanced plasminogen activation. This supports the previously proposed role of alpha enolase in plasminogen activation (López-Alemany et al., 2003; Das et al., 2007). The consequent plasminogen activation, enhanced by alpha enolase, could initiate plasmin-mediated effects. As shown in this study, plasmin generation in differentiated KG-1 cells was markedly enhanced by cathepsin X inhibition, whereas the plasminogen binding to the cell surface was not affected as much. As shown previously, alpha enolase is responsible for the majority of cell-dependent plasmin activation on Nalm6 cells, despite the presence of numerous other plasminogen-binding proteins on the cell surface (López-Alemany et al., 2003). While plasminogen may bind to different cell-surface proteins, the functional consequences of the cathepsin X action might be distinct. If alpha enolase is a predominant binding protein, the cleavage of its C-terminal dipeptide by cathepsin X eliminates the crucial plasminogen activating site on the cell surface, thereby markedly affecting plasmin-mediated effects.

In PC12 cells the induced plasmin formation enhanced by alpha enolase has been proposed to increase neuritogenesis (Nakajima et al., 1994; Jacovina et al., 2001). Neuritogenesis, an important indicator of neuronal differentiation in PC12 cells (Greene and Tischler, 1976), requires matrix remodelling and the role of plasmin in this process is important since it cleaves extracellular proteins such as fibronectin, laminin and thrombospondin and activates other proteolytic systems involved in matrix degradation. We have shown, by live microscopy of PC12 cells, that inhibition of cathepsin X increases neuritogenesis and changes the neurite length distribution profile. The formation of neurites was much more potentiated when neutralizing 2F12 mAb and NGF were added to the cells. In this case neurites grew in 24–48 h to a length which could be seen for NGF alone after 72–90 h. It is interesting that the 2F12 mAb or Stealth^{TM} RNAi/catX initiated neurite outgrowth with different characteristics from those induced by other neurite promoting factors, and increased the number of PC12 cells with monopolar neurites longer than the diameter of the cell body. The biological effects of cathepsin X inhibition are very similar to those of the undefined cysteine protease with ALLNaI activity shown to have the role, in early phase, of neurite extension, as proposed by Saito and Kawashima (1988). This protease was suggested to regulate specific membranous proteins needed for neurite initiation, localized at the cell surface or at the cytoplasmic side of the cell membrane (Saito and Kawashima, 1989; Saito et al., 1990). The apparent molecular weight of this undefined cysteine protease was 33 kDa (Saito et al., 1992), which corresponds to that of active cathepsin X.

pcDNA3/catX-GFP transfected PC12 cells clearly show that cathepsin X is localized in the endolysosomal compartments near the nucleus and in growing cones of neurite extensions. Cathepsin

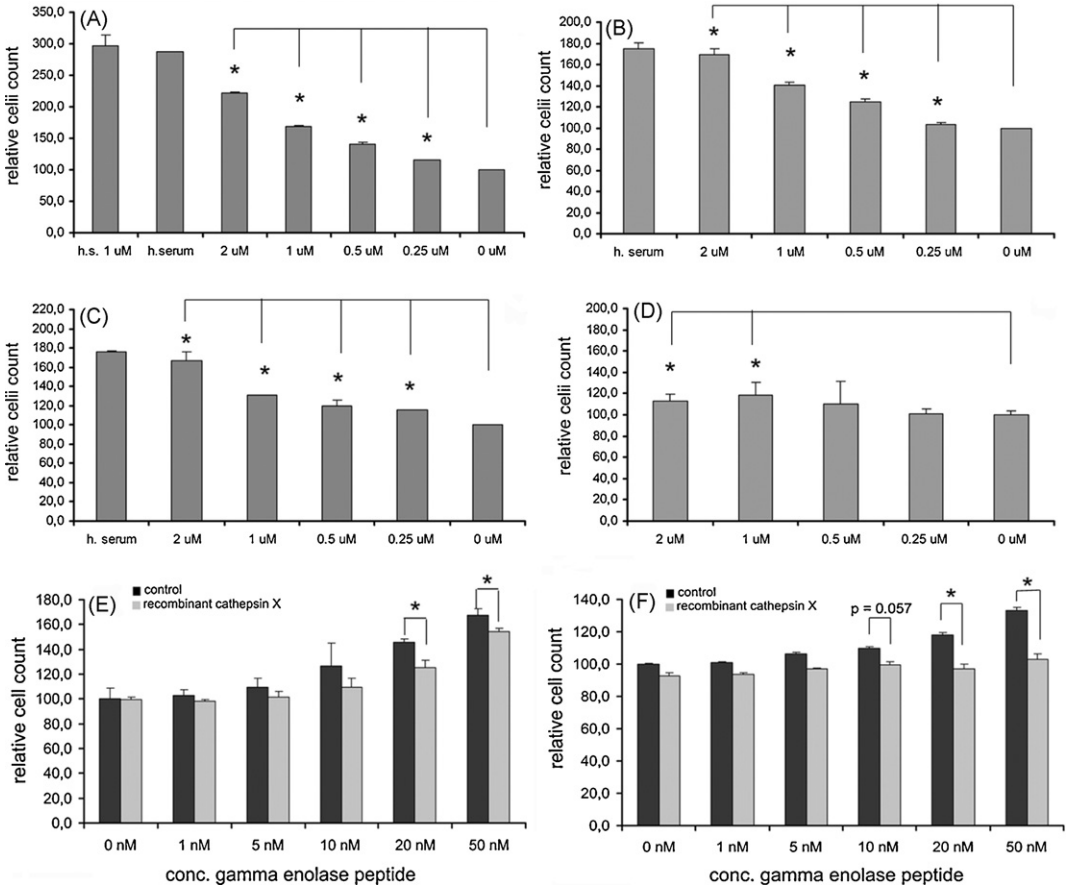

Fig. 6. Preserved gamma enolase C-terminal end increases survival of PC12 cells. (A–D) PC12 cells were cultured in serum-free medium in the presence of different concentrations of cathepsin X neutralizing mAb 2F12 in the absence (A and B) or presence (C and D) of NGF (50 ng/ml) on plastic (A and C) or collagen-coated (B and D) surface for 5 days. Inhibition of cathepsin X increased survival of PC12 in a concentration dependent manner. Survival of PC12 cells in the presence of horse serum (h.s.) is given for comparison. Cells were treated in quadruplicate. Results are the means ± s.d. of at least three independent assays. (E and F) PC12 cells were cultured in the presence of increasing concentrations of C-terminal gamma enolase peptide in the presence (E) or absence (F) of NGF (50 ng/ml). Addition of recombinant cathepsin X significantly reduced the neurotrophic effect of gamma enolase peptide. Cells were treated in quadruplicate. Results are the means ± s.d. of at least three independent assays. $^{*}p < 0.05$. Scale bars, 100 μm.

X is translocated to the plasma membrane of the growing cones inside the compartments, as revealed by live microscopy. Expression, as well as the plasma membrane localization of cathepsin X, is increased on stimulation with NGF, as demonstrated by ELISA and flow cytometry. Confocal microscopy of PC12 cells shows co-localization of enolase isozymes and cathepsin X on the plasma membrane. The release of cathepsin X from the cells was confirmed by ELISA and is in agreement with the extracellular lysosomal release of other cathepsins (Roshy et al., 2003; Liuzzo et al., 1999) and with the transport of endogenous procathepsin X to the plasma membrane, its accumulation in vesicles at lamellipodia and partial association with the cell surface (Lechner et al., 2006). Cathepsin X may bind to heparan sulphate proteoglycans (Nascimento et al., 2005) on the cell surface, where it could perform the cleavage of the C-terminal dipeptide of enolase isozymes. The mechanism of alpha or gamma enolase translocation, release and membrane attachment remains unknown, however, for several other intracellular proteins such as plasminogen receptors (annexin II (Hajjar et al., 1994), actin (Duandi and Ganz, 1996), and cytokeratin (Gonias et al., 2001)), the presence on plasma membrane has been demonstarted despite the lack of cleavage signal sequences or transmembrane domains, For gamma enolase, specific binding to cell surface was shown, however its receptor has not been identified yet (Hattori et al., 1995).

Gamma enolase, in contrast to alpha enolase, has no C-terminal lysine or the ability to bind plasminogen, however, its C terminal part also exhibits high neurotrophic potential. Its C-terminal peptide strongly increased survival of PC12 cells in a concentration dependent manner, an effect that was eliminated by the addition of recombinant cathepsin X. The effect was most pronounced in serum free medium in the absence of NGF, in contrast to neuronally differentiated PC12 cells. Withdrawal of trophic support, either by serum deprivation of proliferating neuroblast-like cells or by NGF/serum removal from neuronally differentiated cells, leads to their apoptotic death (Batistatou and Greene, 1991). NGF withdrawal similarly triggers death of sympathetic neurons in vivo (Gorin and Johnson, 1979). By analogy, our results demonstrate that the presence of serum and/or NGF diminishes the increased survival mediated by gamma enolase peptide. This shows that the intact C-terminal part of gamma enolase may act as a neurotrophic factor. The specific expression of gamma enolase in neuronal cells implies such a role, a suggestion that has so far not been appropriately investigated. Inhibition of cathepsin X with 2F12 mAb profoundly enhanced cell survival, decreased the number of apoptotic cells and induced differentiation, observed as reduced PC12 cell proliferation similar to that induced by NGF. Inhibitors for other cysteine proteases, such as CA-074 and CLIK148 or general cysteine protease inhibitor E-64 were not effective, therefore, cathepsin X rather than other cathepsins is involved in this process.

In conclusion, we have identified a new mechanism of cathepsin X activity in neuronal cells. As a carboxypeptidase, cathepsin X cleaves the terminal dipeptides of alpha- and gamma-enolase,

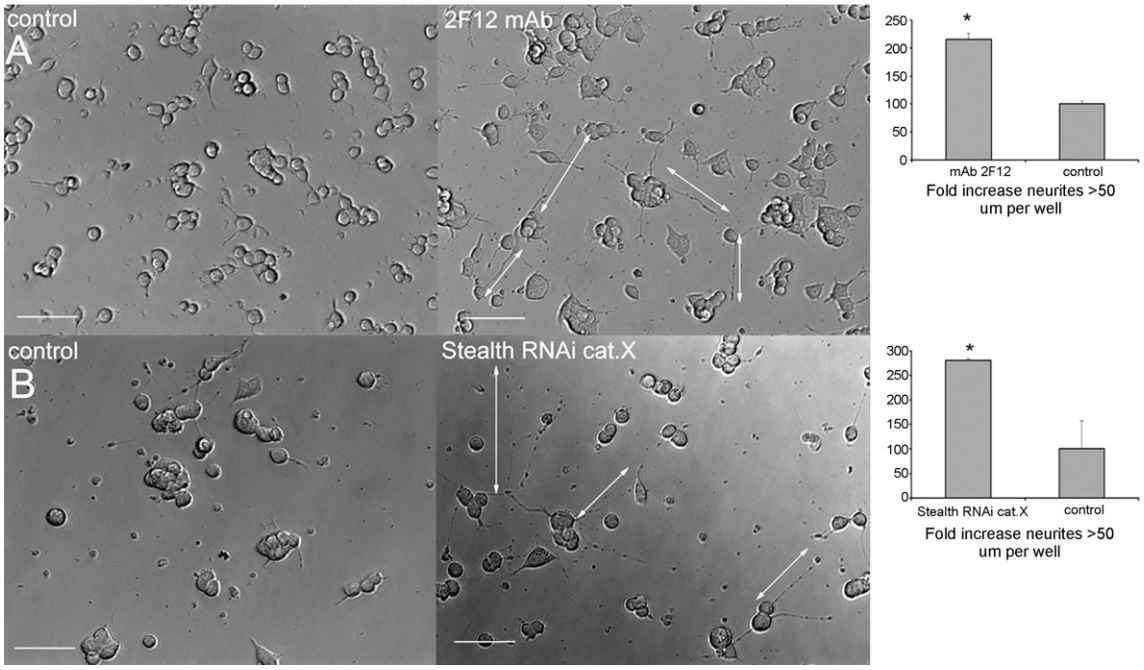

Fig. 7. Inhibition of cathepsin X changes the neurite length distribution profile of PC12 cells. Inhibition of cathepsin X was accomplished either with 2F12 mAb treatment or transient transfection with Stealth™ RNAi/catX. Afterwards, cells were placed onto a collagen-coated surface in the presence of NGF (50 ng/ml) and the percentage of cells bearing neurites longer than the cell diameter was scored in each well of a 96-well plate. Results are the means ± s.d. of at least three independent assays. Representative DIC images of control, mAb 2F12 treated, Stealth™ RNAi/catX and control transfected PC12 cells are shown. Arrows indicate neurites longer than the cell diameter. $^{*}p < 0.05$ for 2F12 mAb or Stealth™ RNAi/catX treated cells versus control cells only. Bars, 50 μm.

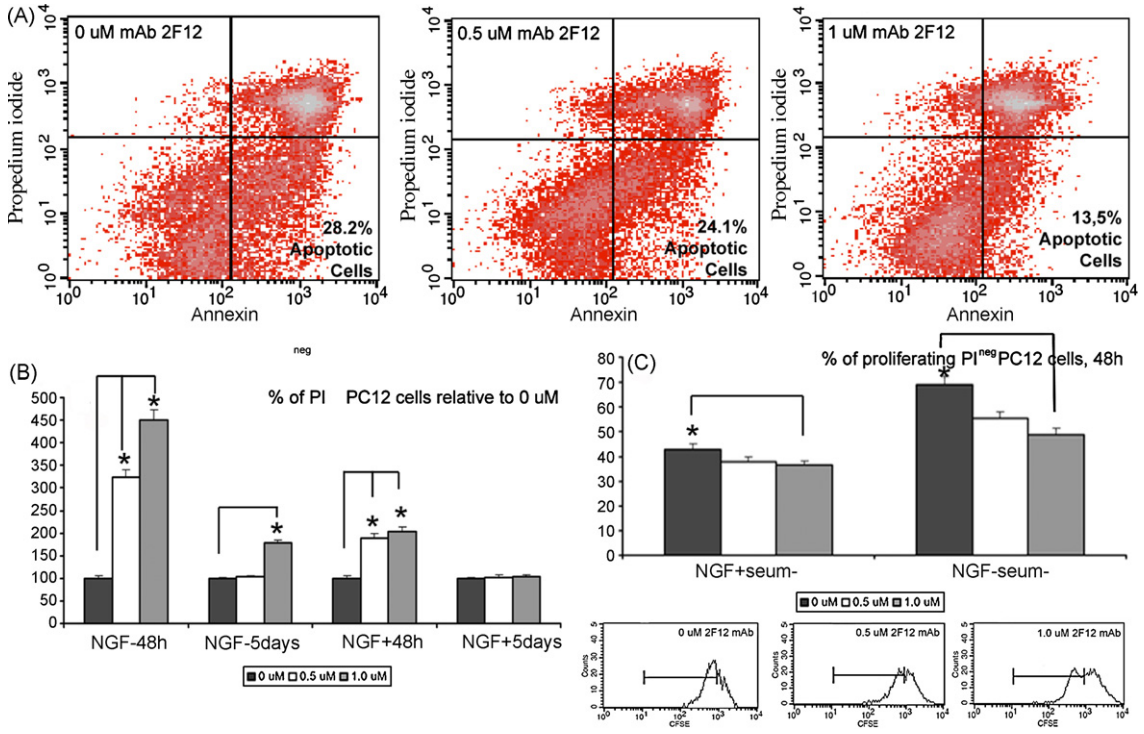

Fig. 8. Effect of cathepsin X inhibition on proliferation, apoptosis and cell survival of PC12 cells. (A) PC12 cells were cultured in serum-free medium in the absence or presence of 0.5 and 1.0 μM mAb 2F12. After 24 h they were harvested, labelled with annexin-FITC and PI and analyzed by flow cytometry. The quadrant threshold was set according to control PC12 cells grown in complete medium and PC12 cells, treated with TPCK inhibitor (100 μM) for 5 h. (B) PC12 cells were grown in the presence or absence of NGF (50 ng/ml) for 48 h and 5 days. Afterwards, they were harvested and labelled with PI and the percentage of PI^{neg} cells relative to control cells was determined with flow cytometry. (C) PC12 cells were labelled with CFSE reagent and cultured in the presence or absence of NGF (50 ng/ml) for 48 h. Proliferation threshold was set according to CFSE labelled PC12 cells analyzed immediately after the labelling. The percentage of proliferating cells of the PI^{neg} population is given. Results are the means ± s.d. of at least three independent assays. $^{*}p < 0.05$.

eliminating the neurotrophic activity of these two isozymes. The reverse effect of cathepsin X inhibition was powerful relative to the low expression of cathepsin X in neuronal cells compared to myeloid cells. Hence, precise regulation of its activity is crucial for proper neuron function. The importance of this newly recognized mechanism in different neurodegenerative disorders needs to be evaluated with a focus on the role of cathepsin X in neuroinflammatory related cognitive decline.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2009.02.019.

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