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Cathepsin D protease mediates programmed cell death induced by interferon-γ, Fas/APO-1 and TNF-α

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A functional approach of gene cloning was applied to HeLa cells in an attempt to isolate positive mediators of programmed cell death. The approach was based on random inactivation of genes by transfections with antisense cDNA expression libraries, followed by the selection of cells that survived in the presence of the external apoptotic stimulus. An antisense cDNA fragment identical to human cathepsin D aspartic protease was rescued by this positive selection. The high cathepsin D antisense RNA levels protected the HeLa cells from interferon-γ- and Fas/APO-1-induced death. Pepstatin A, an inhibitor of cathepsin D, suppressed cell death in these systems and interfered with the TNF-α-induced programmed cell death of U937 cells as well. During cell death, expression of cathepsin D was elevated and processing of the protein was affected, which resulted in high steady-state levels of an intermediate, proteolytically active, single chain form of this protease. Overexpression of cathepsin D by ectopic expression induced cell death in the absence of any external stimulus. Altogether, these results suggest that this well-known endoprotease plays an active role in cytokine-induced programmed cell death, thus adding cathepsin D to the growing list of proteases that function as positive mediators of apoptosis.

Keywords: cathepsin D/cell death/Fas/interferon-γ/TNF

Introduction

Recent evidence has emphasized the indispensable role of programmed cell death (PCD) in the development and maintenance of cellular homeostasis within multicellular organisms. This evolutionarily conserved process removes cells that have either finished their functional role, have the potential to be deleterious to the organism or are redundant surplus cells (Raff, 1992; Schwartz *et al.*, 1993). The identification of the genes that initiate or execute programmed cell death is a major challenge in this field (Steller, 1995; Wyllie, 1995).

Invertebrate models employing powerful genetic tools have provided the most elegant approach in isolating cell death-associated genes. Two major genes that function as positive mediators of cell death, *ced*-3 and *ced*-4, were isolated from the nematode *Caenorhabditis elegans* (Ellis *et al.*, 1991; Yuan and Horvitz, 1992; Yuan *et al.*, 1993). Subsequently, the mammaliam homologue of *ced*-3 was isolated and identified as the interleukin-1β converting enzyme (ICE). ICE is a cysteine protease that causes PCD when overexpressed in mammalian cells (Miura *et al.*, 1993). Additional members of the ICE family have been identified, including *Ich*-1/*Nedd*2 (Kumar *et al.*, 1994; Wang *et al.*, 1994), *CPP*32/*Yama* (Fernandes *et al.*, 1994, Tewari *et al.*, 1995), *Tx*/*Ich*-2 (Faucheu *et al.*, 1995) and *Mch*-2 (Fernandes *et al.*, 1995). These cysteine proteases were shown to have a central role in PCD by virtue of the findings that ectopic expression of these genes leads to cell death and blocking the activity of these proteases by specific inhibitors will suppress the apoptotic process (Tewari and Dixit, 1995). *Drosophila melanogaster* is a second important invertebrate model that has employed a powerful genetic screen in isolating cell death genes. Two novel genes, *reaper* and *hid*, that appear to be required for cell death during embryogenesis were rescued (White *et al.*, 1994; Grether *et al.*, 1995).

In mammalian systems apoptotic genes, such as *p53* and c-*myc*, were isolated and studied for many years as regulators of other cellular processes; only later was their potential function as positive mediators of cell death further revealed (Yonish-Rouach *et al.*, 1991; Evan *et al.*, 1992). This stemmed, in part, from the lack of functional strategies for the rescue of mammalian cell death-causing genes. A significant breakthrough was provided by the finding that PCD can be initiated by activation of various cell surface receptors. Among the external triggers of PCD, a group of cytokines play a crucial role, including diffusible cytokines [e.g. TNF-α, interferon-γ (IFN-γ) and TGF-β], as well as membrane bound proteins (e.g. the ligand to the Fas/APO-1 receptor) (Laster *et al.*, 1988; Trauth *et al.*, 1989; Itoh *et al.*, 1991; Lin and Chou, 1992; Novelli *et al.*, 1994; Suda and Nagata, 1994; Deiss *et al.*, 1995). These findings suggested that exposure of cell cultures to a specific cytokine may provide a well-controlled *in vitro* system for the isolation of genes that mediate PCD. Yet, it is only recently that the great potential that resides in this direction started to be exploited. One of the approaches employed the yeast two-hybrid selection system for the rescue of proteins that directly interact with a functional domain (the death domain) that constitutes the intracellular region of TNF receptor 1 and the Fas/APO-1 receptor. This led to the isolation of a few novel death domain-containing proteins that may function as critical mediators of PCD, including FADD/MORT-1, RIP and TRADD (Boldin *et al.*, 1995; Chinnalyan *et al.*, 1995; Heu *et al.*, 1995; Stanger *et al.*, 1995). A second approach employed in our laboratory consisted of a functional selection of death-causing genes from cell cultures that

were continously exposed to the killing effects of a cytokine, as detailed below.

Our functional strategy was based on random inactivation of genes via the introduction of antisense cDNA expression libraries prepared from cytokine-treated cells. The antisense cDNA library was expressed from an EBV-based episomal vector (named pTKO-1). The genes of interest were selected and cloned by virtue of the defined phenotypic change, i.e. a reduced susceptibility to cytokine-induced cell death, which resulted from inactivation of these genes (Deiss *et al.*, 1991; Kimchi, 1992). The strategy was specifically adapted for the rescue of genes that mediate the IFN-γ-induced PCD in HeLa cells (Deiss *et al.*, 1995). Cells that survived in the continuous presence of IFN-γ after transfection with the antisense cDNA library were selected and the episomal vectors they carried were rescued. Each recombinant vector was independently assayed in a second round of transfection. The plasmids that provided some growth advantage under the negative pressure of IFN-γ were then subjected to further analysis. An interferon-responsive element was introduced into the pTKO-1 vector, in order to impose high expression levels of antisense RNA during the selection process. This inducible feature of the vector was, in retrospect, very important in getting a reduction in the levels of the corresponding protein during selection by IFN-γ (Deiss *et al.*, 1995; Kissil *et al.*, 1995).

We have reported elsewhere on the isolation and characterization of three novel genes which were rescued by this strategy and which were proved to function as positive mediators of cell death. One of these genes coded for a novel Ca^{2+}/calmodulin-dependent serine/threonine kinase that carries eight ankyrin repeats and the death domain (Deiss *et al.*, 1995; Feinstein *et al.*, 1995). The other two genes directed the synthesis of a basic, proline-rich, 15 kDa protein (Deiss *et al.*, 1995) and of a 46 kDa protein that carries a P loop motif (Kissil *et al.*, 1995). The fourth antisense cDNA that was rescued by this procedure will be described in this work.

Results

Establishment of HeLa cell lines that express the rescued antisense cDNA fragment which corresponds to cathepsin D

The gene rescue was performed as described previously (Deiss *et al.*, 1995). Briefly, HeLa cells were transfected with the antisense cDNA library and subjected to the double selection of hygromycin B and IFN-γ. After 28 days the cells that survived in the presence of IFN-γ were pooled and the episomal DNA was extracted from them and shuttled into bacteria. Each of the rescued pTKO-1 vectors was tested in a second round of transfection and those that conveyed some protection from IFN-γ-induced cell death were further analysed. One of the functional clones carried a cDNA insert of 370 bp that was 98% identical to a fragment of human cathepsin D cDNA. The fragment spanned from position 1203 to 1573 in the cathepsin D cDNA (Faust *et al.*, 1985) and was oriented in the vector in the antisense direction.

This rescued episomal vector (pTKO-1-anti-cath-D) was introduced into HeLa cells to generate a few independent hygromycin B-resistant polyclonal populations that stably express the antisense cathepsin D RNA. They were compared with hygromycin B-resistant polyclonal HeLa cell populations that were transfected, in parallel, with a control vector, pTKO1-DHFR. The cDNA insert of the pTKO-1-anti-cath-D plasmid hybridized on a Northern blot to a single endogenous 2.2 kb mRNA, as was expected for the cathepsin D mRNA transcript (Faust *et al.*, 1985). In the pTKO1-anti-cath-D-transfected cells it also hybridized to a composite antisense 1.2 kb RNA (Figure 1). The latter consisted of 370 bases of the cDNA insert and 800 bases of sequences derived from the expression cassette (Deiss and Kimchi, 1991). The antisense cathepsin D RNA levels were elevated 20-fold in response to IFN-γ. This was due to the presence of an interferon-responsive enhancer element in the pTKO1 expression vector. Consequently, a large excess of antisense over sense RNA expression was detected during the IFN-γ treatment (Figure 1). The other polyclonal cell populations that we constructed displayed a similar profile of antisense RNA expression during the IFN-γ selection (not shown).

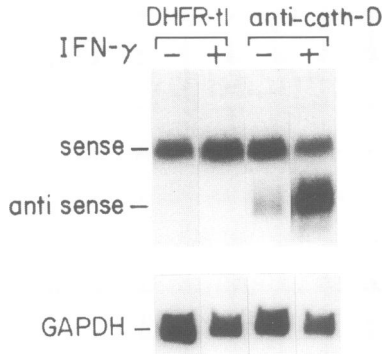

Fig. 1. Transfected HeLa cells express a large excess of antisense versus sense cathepsin D RNA. Northern analysis of total RNA (20 μg) prepared from DHFR- and anti-cath-D-transfected HeLa cells at day 4 of culture in the absence (–) or presence (+) of recombinant humam IFN-γ (1000 U/ml). The blot was probed with the cDNA fragment carried by the pTKO-1 vector. The positions of the sense and antisense cathepsin D RNAs are indicated. The GAPDH mRNA levels were used for the calibration of the RNA amounts in each slot.

Antisense cathepsin D RNA and pepstatin A protect HeLa cells from IFN-γ-induced cell death

First, the stable HeLa cells transfectants were tested for their growth sensitivity to IFN-γ. We have previously shown that parental HeLa cells displayed a biphasic pattern of response to IFN-γ, in which cells first ceased to proliferate but remained viable, followed by massive cell death with the cytological characteristics of PCD (Deiss *et al.*, 1995). One of the assays that was used to measure the antisense RNA-mediated effects was based on neutral red dye uptake into viable cells. In the absence of IFN-γ, both cell lines (DHFR- and anti-cathepsin D-transfected cells) behaved the same and displayed identical growth curves. This suggested that antisense RNA expression had no effects on normal growth of cells (Figure 2Aa). Also, the extent of reduction in neutral red dye uptake during the first 4 days, corresponding to the cytostatic effects of IFN-γ (Deiss *et al.*, 1995), was similar in both cell lines. This indicated that cathepsin D antisense RNA expression also did not interfere with the cell cycle inhibitory effects of the cytokine. The difference between the two cell

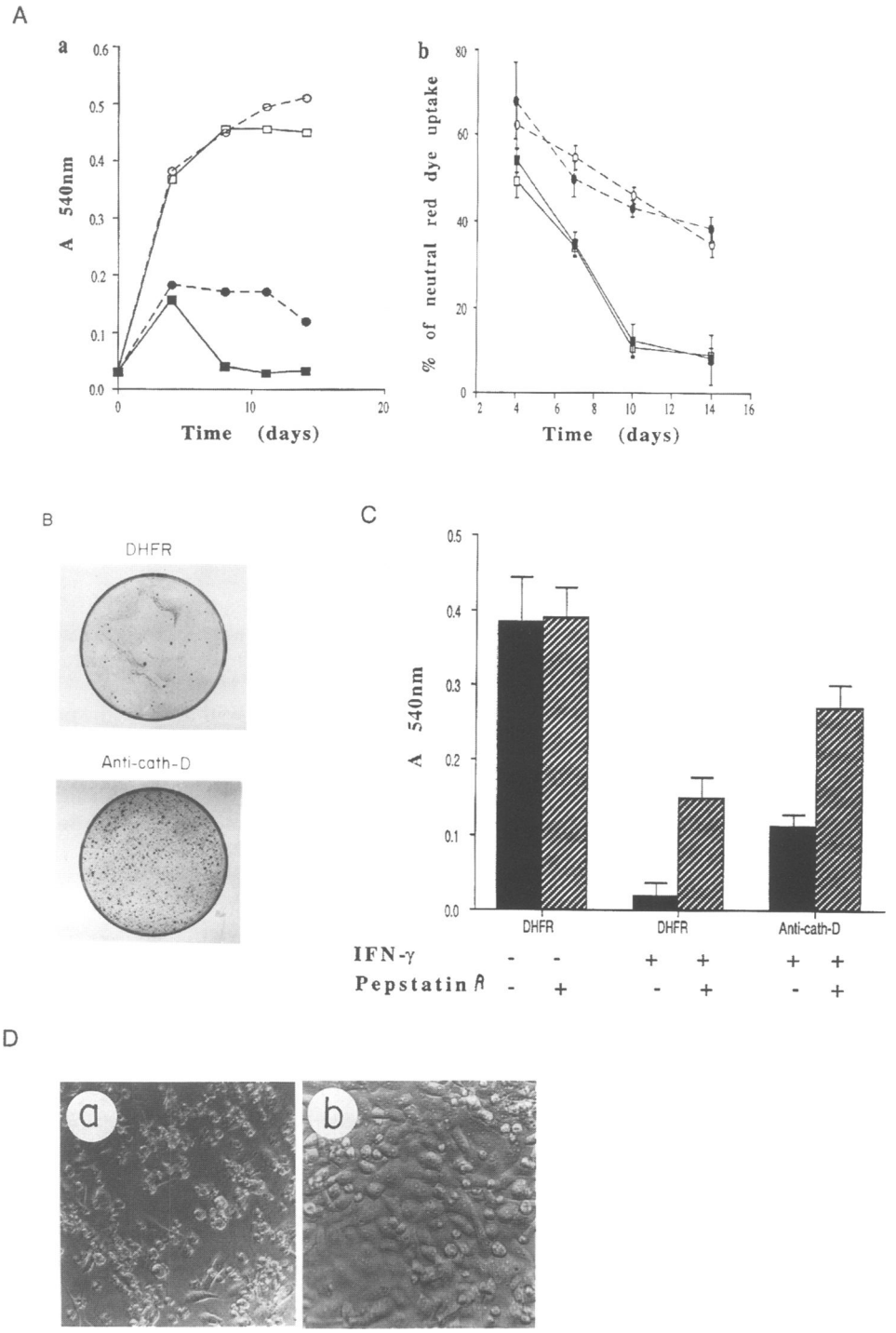

Fig. 2. Involvement of cathepsin D protease in IFN-γ-mediated cell death. (**A**) Protection from IFN-γ-induced cell death by antisense RNA expression. (a) One of the DHFR-transfected polyclonal cell populations (squares) and of the anti-cath-D-transfected polyclonal cell populations (circles) were treated with IFN-γ (1000 U/ml; filled symbols) or left untreated (open symbols). Viable cells were stained with neutral red and the dye uptake was measured at $\lambda_{540\ nm}$. Each point represents an average of quadruplicate determinations. (b) Two independent DHFR-transfected polyclonal cell populations (open and filled squares) and a pair of anti-cath-D-transfected polyclonal cell populations (open and filled circles) were treated with IFN-γ (1000 U/ml) or left untreated. The fraction of viable cells was determined by comparing neutral red dye uptake of IFN-γ-treated cells to non-treated cultures at the indicated time points. Each point represents the average ± SE of quadruplicate determinations. (**B**) Regrowth of viable cells after withdrawal of IFN-γ (1000 U/ml) from DHFR and anti-cath-D transfectants. Cells were seeded at an initial density of 10 000 cells/cm^2, treated with a combination of hygromycin B and IFN-γ (1000 U/ml) for 2 weeks, washed and stained with crystal violet 7 days later. (**C**) Protection from IFN-γ-induced cell death by pepstatin A. The HeLa cells (DHFR and anti-cath-D transfectants) were incubated for 8 days with IFN-γ (1000 U/ml) either in the presence of pepstatin A (10^{-4} M in 0.2% DMSO) or in its absence (0.2% DMSO alone). The DHFR-transfected cells were also tested for responsiveness to pepstatin A in the absence of IFN-γ. Data are given as mean ± SE neutral red dye uptake from quadruplicate samples. (**D**) Light microscopy of HeLa cells on day 8 of IFN-γ-treatment: (a) DHFR transfectants with no inhibitor; (b) anti-cath-D transfectants cultured in the presence of pepstatin A. Magnification ×200.

populations became prominent later on during the IFN-γ-induced cell death phase. In IFN-γ-treated DHFR-transfected cells dye uptake dropped from day 4 on (Figure 2Aa and Ab). Microscopic observations confirmed that this was due to massive cell death that eliminated almost all the viable adherent cells from the plates (Figure 2Da). Death was significantly (but not completely) inhibited by antisense cathepsin D RNA expression, as shown by the sustained values of the uptake (Figure 2Aa). Each of the two antisense cathepsin D RNA-expressing polyclonal populations displayed a significant increase in the fraction of cells that were stained by the vital dye during the IFN-γ-induced cell death phase (Figure 2Ab).

Another way to measure protection from cell killing consisted of counting the number of colonies that were formed after releasing the cultures from long-term treatment with the cytokine. The reduced susceptibility of the antisense-transfected cells to cell killing by IFN-γ was manifested by a 1–2 log increase in the number of cells that could form colonies following the removal of IFN-γ from treated cell cultures (Figure 2B).

To further explore the participation of cathepsin D in IFN-γ-induced PCD, the HeLa cells were incubated with pepstatin A, a specific inhibitor of aspartic proteases (Shields *et al.*, 1991). Due to the fact that cathepsin D is the major intracellular aspartic protease in cells, the outcome of the intracellular effects of this pentapeptide are commonly attributed to specific inhibition of cathepsin D activity. Pepstatin A was added to the culture medium at a final concentration of 10^{-4} M in 0.2% DMSO, in accord with previous reports in which similar incubation protocols led to effective intracellular concentrations of the drug (Shields *et al.*, 1991). Pepstatin A had no effects on growing HeLa cells that were not treated with IFN-γ. Addition of pepstatin A to the IFN-γ-treated DHFR-transfected cells inhibited, to some extent, the killing process, as reflected by the elevated values of neutral red dye uptake (Figure 2C). The highest values of dye uptake that could be measured in the presence of IFN-γ were obtained by applying the pepstatin A to the antisense cathepsin D RNA-expressing cells (Figure 2C). Microscopic examination of the IFN-γ-induced cell cultures that were protected by the double treatment (antisense RNA + pepstatin A) revealed that the majority of cells displayed the normal adherent phenotype, whereas only ~20% of the cells had a round apoptotic morphology (Figure 2Db). This further indicated that the combined reduction of both expression and activity of this endoprotease was most effective in protecting cells from IFN-γ-induced cell death. In summary, the antisense RNA and pepstatin A data suggest an active role for cathepsin D in IFN-γ-mediated PCD.

Regulation of cathepsin D protein expression and processing during IFN-γ-induced PCD

In the DHFR-transfected control cultures, as well as in the parental HeLa cells, the cathepsin D mRNA levels were induced 3- to 4-fold by IFN-γ. The elevation in cathepsin D mRNA persisted in cells for a few days (Figure 3). The effects of IFN-γ on the expression and processing of cathepsin D protein were then analysed on immunoblots. A typical change in the relative abundance of the different cathepsin D forms was detected in the

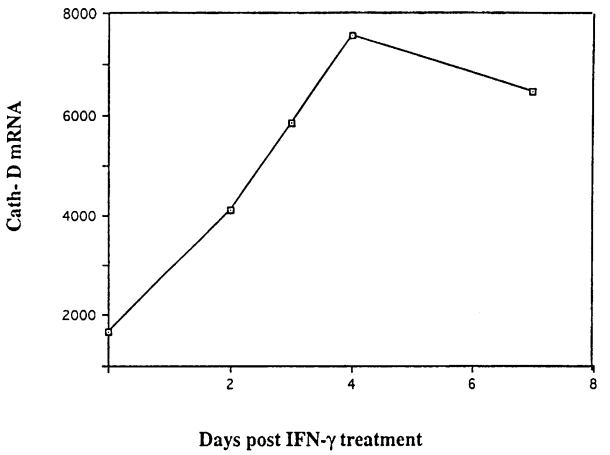

Fig. 3. Cathepsin D mRNA expression in the DHFR-transfected cells at different time points after IFN-γ treatment. Values were derived from densitometry of Northern blots containing total RNA (20 μg) and probed with the cDNA fragment carried by the pTKO-1 vector. The GAPDH mRNA levels were used for the calibration of the RNA amounts in each slot and values of densitometric scanning were corrected accordingly.

treated cells (Figure 4A and B). The 48 kDa form of cathepsin D, usually detected in trace amounts in untreated HeLa cells, gradually accumulated to high levels between days 4 and 7 of IFN-γ treatment. In contrast, the steady-state levels of the 30 kDa form were not increased (Figure 4B) and in some experiments were even reduced at the late time points (Figure 4A). The 48 kDa cathepsin D is a proteolytically active, single chain form often found in pre-lysosomal vesicles. It is normally targeted to lysosomes, where it is further processed into the double chain form (30 and 14 kDa) of the enzyme (Fujita *et al.*, 1991; see also the scheme in Figure 4C: note that the monoclonal antibodies used in Figure 4 are directed against an epitope in the heavy 30 kDa chain). The unusual accumulation of the 48 kDa form, therefore, suggested that normal processing of the protease was interrupted during IFN-γ-mediated cell death. In addition, on days 5–7 of IFN-γ treatment the levels of the 48 kDa precursor were 8- to 10-fold higher than the levels of the 30 kDa form before treatment (Figure 4A). The increased steady-state levels of cathepsin D proteins could result, at least partially, from RNA elevation.

It is noteworthy that in some, but not all, experiments the intracellular levels of the 52 kDa preprocathepsin D form also increased after IFN-γ treatment of the parental cells (Figure 4A). Traces of the 52 kDa form were also found in the culture medium, but no effects of IFN-γ on the levels of this secreted form were detected (not shown).

The prominent IFN-γ-mediated elevation of cathepsin D protein and accumulation of the intermediate forms were both prevented in the HeLa polyclonal cell population expressing the antisense RNA (Figure 4B; the calculated values were 8.2- and 1.1-fold increase in the cathepsin D protein forms by IFN-γ for DHFR and anti-cathepsin D transfectants respectively). A few independently generated antisense-expressing polyclonal populations were examined and none of them displayed elevated levels of cathepsin D in response to IFN-γ. These findings, therefore, confirmed that the large excess of antisense over sense RNA during IFN-γ selection effectively reduced the total

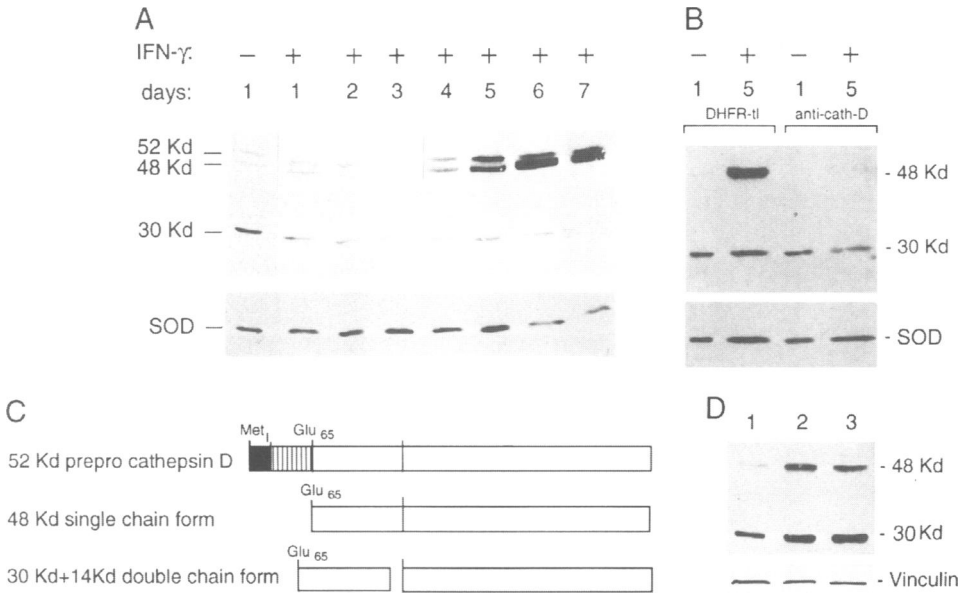

Fig. 4. Regulation of expression and processing of cathepsin D protease by IFN-γ and TNF-α. (**A** and **B**) Immunoblot analysis of cathepsin D forms before and after treatment with IFN-γ (1000 U/ml). Cell lysates were prepared at the indicated time points from parental HeLa cells (A) and from DHFR and anti-cath-D transfectants (B). Samples of 300 μg were fractionated on SDS–PAGE (12%), blotted to nitrocellulose and detected using the ECL system (Amersham). The sizes of cathepsin D forms are shown. The same blots were reacted with polyclonal antibodies generated against copper/zinc superoxide dismutase (SOD) to correct for possible differences in protein amounts in each slot. (**C**) A scheme that depicts the different steps of cathepsin D processing as previuosly reported for rat cathepsin D (Fujita *et al.*, 1991). (**D**) Immunoblot analysis of cathepsin D forms before (lane 1) and after treatment of U937 cells with TNF-α (lanes 2 and 3, 24 and 48 h respectively).

levels of cathepsin D protein, as was expected. The question as to why the residual levels that continued to be expressed in these IFN-γ-treated cells did not accumulate as intermediate forms of cathepsin D is still open.

Cathepsin D aspartic protease mediates APO-1/Fas and the TNF-α-induced PCD

We studied whether cathepsin D protease is also involved in other apoptotic systems triggered by the activation of cell surface receptors that differ from the IFN-γ receptor. The different HeLa cell transfectants were treated with the agonistic anti-APO-1 monoclonal antibody, in order to determine whether cathepsin D mediates Fas/APO-1-induced apoptosis. The parental and DHFR-transfected cells were efficiently killed by anti-Fas/APO-1 antibodies. Cell death exhibited features characteristic of apoptosis, similar to the IFN-γ effects. By 40 h ~70% of the cells rounded up and detached from the plates (not shown) and the uptake of neutral red dye was reduced accordingly (Figure 5A). The killing required a short pretreatment of the cells with a low dosage of IFN-γ (50 U/ml), which had no effect by itself on cell viability. The low dosage of IFN-γ sensitized the cells to killing by the agonistic antibody, due to elevation of Fas/APO-1 expression (Itoh *et al.*, 1991; further morphological and molecular details on the system will be published elsewhere). Expression of antisense cathepsin D RNA, or alternatively the addition of pepstatin A to the culture medium of the DHFR-transfected cells, substantially suppressed Fas/APO-1-mediated cell death, resulting in an increased fraction of viable cells (Figure 5A). The latter indicated that cathepsin D is essential for Fas/APO-1-induced PCD.

We also found that pepstatin A interfered with the apoptotic process triggered in U937 histiocytic lymphoma cells by tumour necrosis factor-α (TNF-α) (Wright *et al.*, 1992). The killing in this system was very rapid and characterized by typical nuclear changes, such as chromatin condensation followed by fragmentation. DAPI staining of U937 nuclei indicated that 6 h after TNF-α administration approximately a third of the cell population already contained nuclei with typical fragmented chromatin (Figure 5B). Addition of pepstatin A to the culture showed a significant reduction in the number of fragmented nuclei (Figure 5B). Interestingly, the earlier step of chromatin condensation seemed less susceptible to the effect of pepstatin A. These data indicated that cathepsin D endoprotease also mediates some critical steps along the apoptotic pathway which leads to U937 cell death.

Examination of the pattern of cathepsin D expression in TNF-α-treated U937 cells revealed that it shared a few common features with the HeLa cell system. The total levels of cathepsin D proteins were significanly increased. Moreover, the proteolytically active 48 kDa intermediate form accumulated in TNF-α-treated U937 cells, indicating that, again, processing into the double chain form was interrupted (Figure 4D). Yet, in contrast to the HeLa cell system, this conversion was not completely blocked and a mild increase in the 30 kDa form was detected as well. These data suggest a common pattern of changes in the expression/processing of cathepsin D protein in a few apoptotic systems.

Ectopic expression of cathepsin D is not compatible with cell viability

The outcome of overexpression of cathepsin D was directly measured in HeLa cells by co-transfections with the *lacZ* gene, used as a marker of gene expression. Cathepsin D was driven by the tetracycline-repressible promoter (Gossen and Bujard, 1992) and the β-galactosidase gene

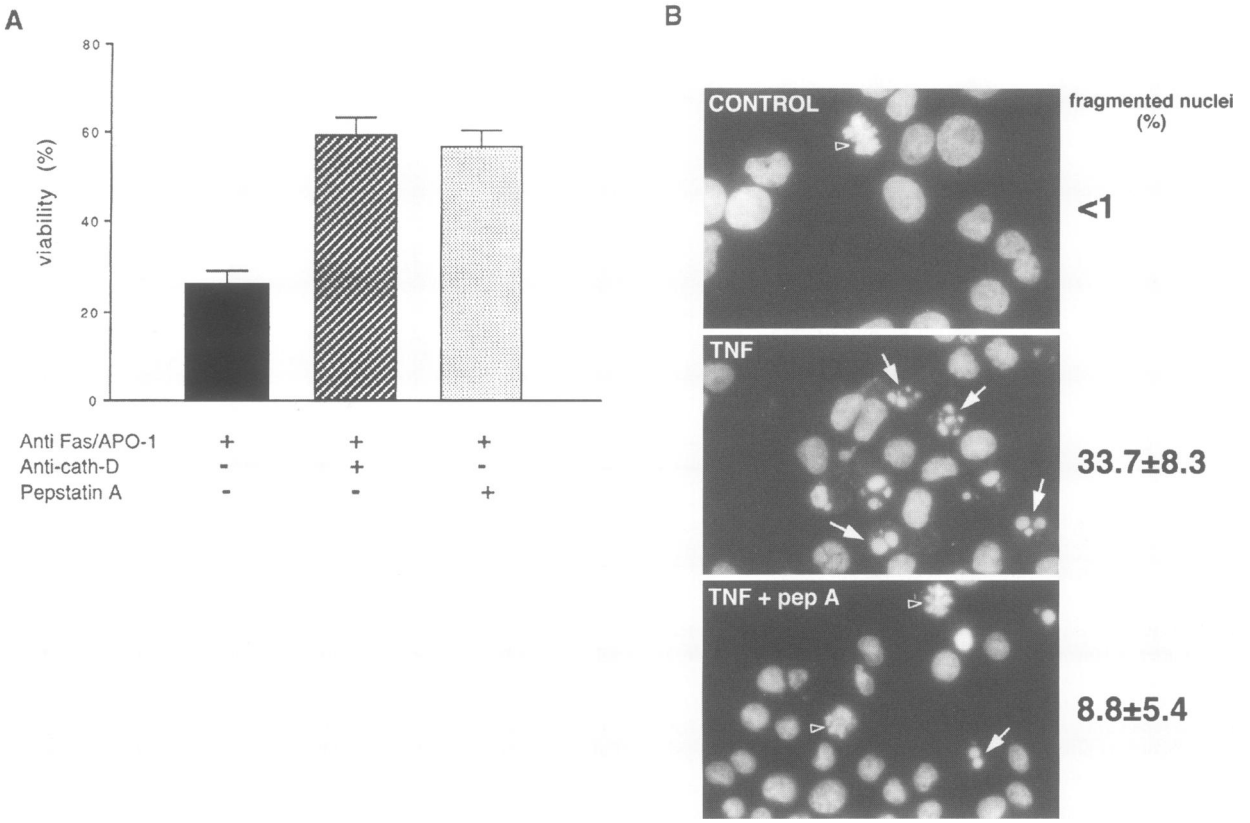

Fig. 5. Involvement of cathepsin D protease in Fas/APO-1- and TNF-α-mediated cell death. (**A**) Suppression of Fas/APO-1-mediated cell death by anti-cathepsin D RNA or by pepstatin A. The HeLa cells (DHFR and anti-cath-D transfectants, 20 000 cells/microtiter well) were exposed to anti-APO-1 antibodies for 40 h as described in Materials and methods. Pepstatin A (10^{-4} M in 0.2% DMSO) was added where indicated to the DHFR transfectants 20 h before their exposure to the anti-APO-1 antibodies. Viability was assessed by neutral red assay in quadruplicate samples; results are expressed as percentage dye uptake at the end of each treatment out of the total uptake in the corresponding control wells, which were not exposed to the antibodies (100% viability). (**B**) Pepstatin A interferes with TNF-α-induced apoptotic cell death in U937 cells. The cells were seeded at a density of 2×10^5 cells/ml 24 h after their preincubation with pepstatin A (10^{-4} M in 1% DMSO) or with DMSO alone. Where indicated, TNF-α (100 U/ml, 10 ng/ml) was added and 6 h later samples were cytospun on glass slides and stained with DAPI (0.5 μg/ml; Sigma). Microscopy was performed under fluorescent light conditions. Magnification ×1000. Nuclei with fragmented chromatin are indicated by arrows; empty arrowheads point to mitotic nuclei. Data are presented as the percentage ± SE of cells with a fragmented nuclear morphology. For each condition a minimum of 400 cells in 14 separate fields were scored.

was driven by the CMV constitutive promoter. The morphology of *lacZ*-containing blue cells was determined 48 h post-transfection, in cultures which were maintained in the absence of tetracycline to allow cathepsin D transcription/translation. It was found that 70% of the *lacZ*-containing cells displayed a round apoptotic phenotype upon co-transfections with cathepsin D, whereas co-transfections with the control tetracycline vector displayed a background of <20% apoptotic cells (Figure 6A–C). In order to further quantitate the effects of ectopic expression of cathepsin D on cells, in a second independent approach co-transfections were performed with vectors expressing the secreted alkaline phosphatase (SEAP) instead of *lacZ*. In these experiments we measured the outcome of tetracycline withdrawal on SEAP activity released by transfectants carrying the cathepsin D gene. We found that activation of cathepsin D by tetracycline withdrawal significantly reduced the SEAP activity secreted into the culture medium around 48 h post-transfection, as compared with the values obtained from the same population maintained in the presence of tetracycline (Figure 6D). In contrast, tetracycline withdrawal had no effect on SEAP activity released by control cultures co-transfected with the empty vector.

Discussion

Cathepsin D has multiple functions in different cellular compartments. The best known function is the classical lysosomal activity that participates in the proteolysis of endocytosed proteins. In addition, cathepsin D has an endosomal activity that is involved in the proteolytic activation of secreted proteins, such as growth factors and hormones (Diment *et al.*, 1989). It also has an extracellular activity, carried by the secreted 52 kDa form, which has been implicated in controlling the migration of metastatic cells (Mignatti and Rifkin, 1993) and which could also be involved in the control of cell migration during normal development. This endoprotease has been implicated in some pathological disorders, such as degenerative brain changes, connective tissue diseases and muscular dystrophy (Matus and Green, 1987; Kenessey *et al.*, 1989; Johnson *et al.*, 1991). In some of these disorders abnormal proteolytic activity of cathepsin D was correlated with increased degradation of cytoskeletal elements such as MAP-2. Suprisingly, the phenotype of recently described mice deficient for cathepsin D reflected only a small portion of these multiple functions. The latter may exclus-

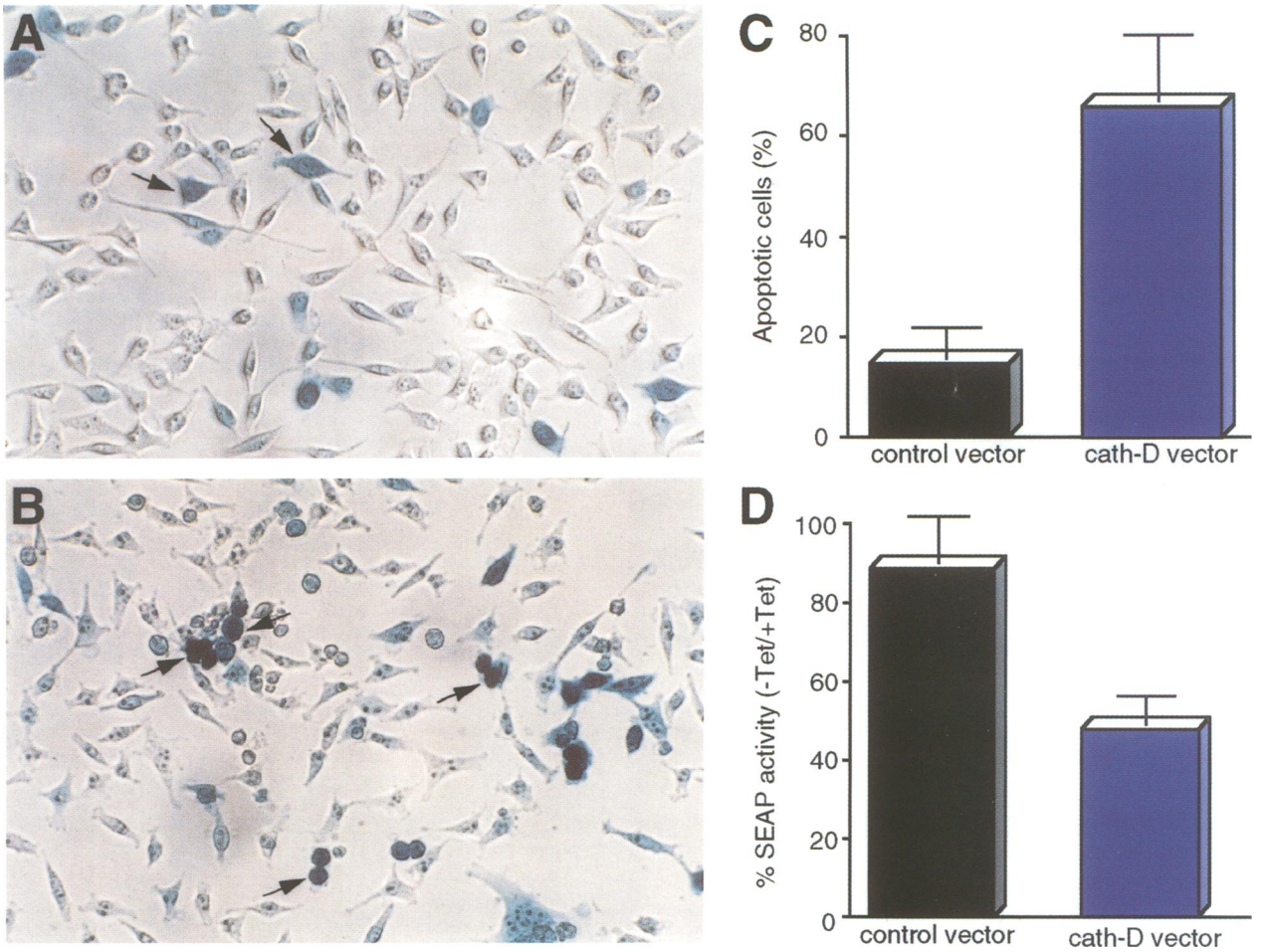

Fig. 6. Ectopic expression of cathepsin D reduces cell viability. (**A** and **B**) X-Gal staining of HeLa cells co-transfected with *lacZ* (driven by the CMV promoter) and either with the cathepsin D cDNA (driven by a tetracycline-repressible promoter) or with the control vector. In both cases cells were cultured in the absence of tetracycline and stained after 48 h with X-Gal solution for 3 h. Light microscopic micrographs are shown. Magnification ×200. Examples of normal blue stained cells (A) and of apoptotic blue stained cells (B) are indicated by arrows. (**C**) The frequency of blue cells with an apoptotic rounded morphology was assessed by counting 800 total blue cells from eight different fields coming from duplicate transfections (described in A and B). (**D**) Assessment of secreted alkaline phosphatase (SEAP) in the growth medium of HeLa cells co-transfected with SEAP and with either the control vector or the above mentioned cathepsin D vector; each transfection was divided into two plates, one of which was immediately supplemented with tetracycline (1.5 μg/ml). SEAP activity secreted into the growth medium during the last 5 h of incubation was determined 48 h after transfections. Data on SEAP activity were obtained in duplicate from four experiments. The values give the percentage of SEAP activity measured in the absence of tetracycline out of total activity produced in the presence of tetracyline.

ively refer to functions which were rate limiting and not compensated for by other mechanisms. Thus the homozygous knockout mice exhibited progressive atrophy of the intestinal mucosa, probably due to cell migration abnormalities. They also displayed a profound destruction of lymphoid cells, probably due to deficiencies in the endosomal processing of secreted death-protective growth factors. Lysosomal bulk proteolysis, however, was maintained in these mice, suggesting that the main well-known function of this protease was masked by some compensatory mechanisms (Saftig *et al.*, 1995).

A novel facet in the function of cathepsin D is documented here for the first time, using cell cultures of epithelial and haematopoietic origin that have been exposed to a well-defined external stress. We propose that cathepsin D mediates a regulated type of programmed cell death, initiated by various cytokines. The first clue was provided by the rescue of a cathepsin D cDNA fragment as a functional element in a genetic screen set to isolate positive mediators of IFN-γ-induced apoptosis in HeLa cells. This suggested that cathepsin D must be a rate limiting step in the commitment or execution of PCD, at least in the model cell system in which the gene rescue was performed. The involvement of this protease in cell death was then further established and extended to other cytokines, including Fas/APO-1 ligand and TNF-α, with the help of a second functional tool, i.e. the cathepsin D pentapeptide inhibitor. It was found that the inactivation of cathepsin D by pepstatin A protected cells from cytokine-induced cell death in a fashion similar to the protection conferred by antisense RNA expression. In HeLa cells the combined reduction of both expression and activity of this endoprotease was most effective in protecting cells from IFN-γ-induced cell death. The cathepsin D connection to cell death was then further supported by the finding that expression and processing of the endogenous protease were both regulated during PCD. Either the increased protein expression levels *per se*

or the abnormal accumulation of the intermediate active form of cathepsin D could be relevant to the process. Finally, another line of experiments showed that ectopic expression of the cathepsin D gene induced the death of HeLa cells in the absence of any external stimulus. The latter data are consistent with a role of cathepsin D as a positive mediator of programmed cell death, but definitely do not provide by themselves independent evidence, due to the non-specific effects that could accompany the overexpression of a protease.

It will be interesting to determine in the future the precise intracellular localization of cathepsin D during cell death. For example, to find out whether it continues to be localized in vesicular compartments or may be released into the cytosol. The block in processing into the double chain form of cathepsin D could result from alterations in targeting to lysosomes during cell death or from some leakage into the cytoplasm from endosomal vesicles. Determination of the intracellular localization of cathepsin D will be important for studying the protein substrates during the death process. Also, it will be interesting to define the spectrum of apoptotic systems in which cathepsin D functions as a positive mediator. For instance, the involvement of cathepsin D could be restricted to apoptotic systems which are exclusively triggered by the interaction of cytokines with their cognate receptors or, alternatively, may be a common target of several external and internal stimuli. Systems in which cell death is triggerd by p53 activation, DNA damage agents, steroids, cytokine deprivation or deregulated c-*myc* should be examined in this respect.

Members of the ced-3/ICE family of cysteine proteases have thus far been implicated in various Fas/APO-1-mediated apoptotic systems (Enari *et al.*, 1995; Los *et al.*, 1995). This emerged from the finding that transfections with *crmA*, a cowpox viral gene that inhibits the ICE protease, protected cells from Fas/APO-1-mediated cell death. In addition, the tetrapeptide YVAD, which specifically inhibits the ICE family of proteases, also interfered with Fas/APO-1-mediated programmed cell death. The relevance of other proteases in some well-defined apoptotic processes, e.g. granzyme B, which mediates cell death by cytotoxic T lymphocytes (Heusel *et al.*, 1994), has also been previously documented. Obviously the functional position of cathepsin D with respect to the ICE-like proteases along the Fas/APO-1-induced apoptotic pathways must be pursued in the future. Altogether, we propose that this aspartyl protease should be added to the growing list of proteases that mediate programmed cell death.

Materials and methods

Cell lines and cytokine treatment

The HeLa human epithelial carcinoma cells were grown in DMEM (BioLab) and the U937 human histiocytic cells in RPMI (BioLab). Growth medium was supplemented with 10% fetal calf serum (BioLab), 4 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Recombinant human interferon-γ (3×10^7 U/ml) was purchased from PeproTech. Recombinant TNF-α (5×10^6 U/ml) was a kind gift of Cetus Corporation. Hybridoma supernatant of anti-APO-1 antibodies (5 μg IgG_3/ml; Trauth *et al.*, 1989) was kindly provided by P.H.Krammer. HeLa cells were first pretreated with 50 U/ml IFN-γ for 20 h and were then exposed to the anti-APO-1 antibody at 38°C for an additional 40 h (50 ng/ml).

For DAPI staining, U937 cells were cytospun on glass slides and fixed with 3% paraformaldehyde. The cells were washed in phosphate-buffered saline (PBS) and stained with DAPI (0.5 μg/ml; Sigma) for 10 min, washed in PBS, drained and mounted in Mowiol.

Rescue of antisense cDNAs and transfections

Transfection of the antisense cDNA library into HeLa cells was done by the standard calcium phosphate technique. The pTKO-1 vectors carrying antisense cDNA fragments were isolated as described (Deiss and Kimchi, 1991; Deiss *et al.*, 1995). A few secondary polyclonal HeLa cell populations expressing the cathepsin D antisense cDNA fragment from the pTKO-1 vector were generated. This was performed by transfection of subconfluent monolayers of HeLa cells with 40 μg of the corresponding plasmid (named pTKO-1-anti-cath-D). HeLa cells were transfected in parallel with a control vector, pTKO-1-DHFR (Deiss and Kimchi, 1991). Pools of 10^4 independent stable clones were generated from each transfection. The stable transfectants were continously grown in the presence of 200 μg/ml hygromycin B (Calbiochem).

Neutral red dye uptake assay

The HeLa cells were cultivated in 96-well microtiter plates at an initial number of 15 000 or 20 000 cells/well and were treated with either IFN-γ or anti-APO-1 antibodies respectively or were left untreated. Where indicated, pepstatin A (pepA) (Sigma) or DMSO were added to the culture medium. The culture medium and drugs were replaced every 3–4 days. Viable cells were stained with neutral red (Sigma) as detailed before (Wallach, 1984). The dye uptake was measured in quadruplicates at $\lambda_{540\ nm}$ using an automated Micro-Elisa auto-reader.

RNA analysis

Total cellular RNA was extracted using Tri-Reagent™ (Molecular Research Center Inc.). Samples of 20 μg total RNA were processed on Northern blots as previously described in detail (Yarden and Kimchi, 1986). DNA fragments used as probes were purified from agarose gels with the Geneclean kit (BIO 101 Inc.). The fragments were labelled with 5 μCi [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) using a Random Priming kit (Boehringer).

Protein analysis

Cells were extracted in RIPA (10 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate and 5 mM EDTA) containing a mixture of protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotenin, 100 μg/ml leupeptin, 1.5 μg/ml pepstatin A, 2 μg/ml antipain, 2 μg/ml chymostatin, 0.1 mM $NaVO_3$ and 0.1 mM NaF). Protein concentration was determined using a protein assay reagent (Bio-Rad). Aliquots of 300 μg of the cell lysates were fractionated by SDS–PAGE (12%). The proteins were then electroblotted onto a nitrocellulose membrane and blots were incubated in blocking solution [10% skimmed milk and 0.05% Tween-20 (Sigma) in PBS] for 2 h at room temperature and then reacted with antibody-containing solution for 18 h at 4°C. The washed membranes were incubated with peroxidase-conjugated second antibodies, either goat anti-mouse IgG [IgG(H+L) chains; Jackson Immuno Research Laboratories] at a 1:10 000 dilution or protein A conjugated to horseradish peroxidase (Amersham) at a 1:10 000 dilution. Detection of the bound antibodies was carried out using ECL detection reagents (Amersham). The anti-cathepsin D monoclonal antibodies (EURO/DPC UK) were used at 1:5 dilution; these antibodies recognize an epitope in the 30 kDa heavy chain. Polyclonal antibodies against copper/zinc superoxide dismutase were used at a 1:250 dilution. These antibodies were kindly provided by Y.Groner.

Transient transfections

Cathepsin D cDNA insert (2176 bp, a *Sal*I–*Eco*RI fragment containing the full-length coding sequences and flanking non-coding regions; see Faust *et al.*, 1985) was subcloned into the tetracycline-controlled expression vector (pSBC-TtA) (Dirks *et al.*, 1993). The vector (40 μg) was transiently transfected into a HeLa cell clone (HtTA-1) that expresses the tetracycline transactivator gene by the standard calcium phosphate technique (2×10^5 cells were seeded in 9 cm plates 18–20 h prior to transfection). An empty tetracycline promoter-containing vector was used as a control in the assays. In order to exclusively follow the transfected cells, these constructs were co-transfected with either the CMV–β-galactosidase fusion (Clontech) or with the SEAP gene expressed from the SV40 promoter (the pSBC-2 vector) (Dirks *et al.*, 1993). The molar ratio was 6:1 in favour of the tetracycline vectors. Each transfection was divided into two plates, one of which was

immediately supplemented with tetracycline (1.5 μg/ml). All the enzymatic activities were assessed 48 h after transfections.

β-Galactosidase staining and determination of SEAP activity

To detect *lacZ* expression, cells were fixed with 3% paraformaldehyde for 5 min, rinsed twice with PBS and stained for 3 h in X-Gal buffer containing 77 mM Na_2HPO_4, 23 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 1 mg/ml X-Gal, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$. The reaction was stopped with 70% ethanol. Photography was under phase microscopy using Kodak Ektachrome 160T. For the SEAP activity assay the medium of transfected cells was changed 5 h before assay. Aliquots of 100 μl medium were removed from the transfected plates and heated at 65°C for 5 min. The medium was then clarified by centrifugation at 14000 *g* for 2 min. The medium aliqouts were adjusted to 1× SEAP assay buffer containing 2 M diethanolamine, pH 9.8, 1 M $MgCl_2$ and 20 mM L-homoarginine. Aliquots of 20 μl 120 mM *p*-nitrophenylphosphate dissolved in water were then added to each mixture. The reaction mixtures were then incubated for 30 min at 37°C. The hydrolysis of *p*-nitrophenylphosphate was measured at 405 nm.

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