Changes in intracellular localization of calpastatin during calpain activation

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Localization of the two main components of the Ca^{2+} -dependent proteolytic system has been investigated in human neuroblastoma LAN-5 cells. Using a monoclonal antibody which recognizes the N-terminal calpastatin domain, it has been shown that this inhibitory protein is almost completely confined in two granule-like structures not surrounded by membranes. Similar calpastatin distribution has been found in other human and in murine cell types, indicating that aggregation of calpastatin is a general property and not an exclusive characteristic of neuronal-like cells. The existence of such calpastatin aggregates is confirmed by the kinetics of calpastatin-activity release during rat liver homogenization, which does not correspond to the rate of appearance of cytosolic proteins or to the disruption of membrane-surrounded organelles. Calpastatin distribution is affected by the intracellular increase in free Ca^{2+} , which results in

calpastatin progressively becoming a soluble protein. However, calpain is distributed in the soluble cell fraction and, in activating conditions, partially accumulates on the plasma membrane. Similar behaviour has been observed in calpastatin localization in LAN-5 cells induced with retinoic acid, suggesting that the proteolytic system is activated during the differentiation process of these cells. The involvement of calpastatin in controlling calpain activity, rather than its activation process, and the utilization of changes in calpastatin localization as a marker of activation of the system is discussed.

Key words: calpastatin-rich structures, LAN-5 neuroblastoma cells, murine erythroleukaemia (MEL) cells, PC12 human pheochromocytoma cells, rat brain.

INTRODUCTION

Calpastatin, the natural inhibitor of calpain, is present in rat brain cells in multiple forms having a different number of repetitive domains associated with the N-terminal domain (L-domain) [1,2]. In order to interact with calpastatin, calpain requires an initial conformational change primed by Ca^{2+} ions, which results in the opening of the active site [3–6]. This initial step in the activation of calpain is followed by the partial or complete autoproteolytic removal of the N-terminal segment, which includes the catalytic region. The two resulting, fully active, 78 kDa and 75 kDa protease forms can rapidly interact with calpastatin with high affinity [7].

Thus calpastatin can interact with calpain at different stages of the enzyme activation process. In the first stage, induced by conformational transition, the primary effect is to prevent autoproteolytic conversion to the fully active, low-molecular-mass forms. In the final stage, interaction with calpastatin results in the inhibition of the catalytic activity [7].

As reported by Murachi [8], many years ago, calpain and calpastatin have been considered to be both co-localized in the soluble cell fraction in a reciprocal ratio, largely in favour of the inhibitor, a situation which is difficult to reconcile with the intracellular activity of calpain. Evidence in favour of compartmentalization of calpastatin has been reported by Lane et al. [9].

In the present paper we provide evidence for a difference in the intracellular distribution of calpastatin and calpain. Accordingly, calpastatin appeared to be completely confined to two membrane-free structures, with a diameter of approx. 1 μm , localized close to nuclear invaginations. This localization was observed in LAN-5 cells and in other cell types. The existence of calpastatin-rich structures was confirmed by the kinetics of release of proteins from hepatocytes during lysis, and calpastatin activity appeared

as a soluble protein much later than the bulk of cytosolic proteins. When the intracellular concentration of Ca^{2+} was increased, and correlated with activation of the calcium-dependent proteolytic system, calpastatin underwent, as an early event, intracellular redistribution, which corresponded with a cytosolic diffusion, whereas, in a later phase, calpastatin was degraded and progressively disappeared. These data indicate a possible relationship between the activation of the Ca^{2+} -dependent proteolytic system and the intracellular localization of the main protein components, namely calpain and calpastatin.

MATERIALS AND METHODS

Cell cultures

Human neuroblastoma LAN-5 cells were cultured as reported previously [10]. Murine erythroleukemia (MEL) cells were obtained and cultured as specified previously [11]. Human pheochromocytoma cells (PC12) were purchased from the American Type Culture Collection (A.T.C.C.) (Rockville, MD, U.S.A.) and grown as described previously [12]. Human aortic smooth muscle cells (AoSMC) CC-2571 were purchased from Clonetics Corporation (Walkersville, MD, U.S.A.) and cultured as reported previously [13].

Preparation of monoclonal antibodies (mAbs)

Anti-calpastatin mAb 35.23

Recombinant RNCAS23 calpastatin (EMBL/GenBank/-DDBJ, accession number Y13591) was obtained by expression of the corresponding cDNA in *Escherichia coli* [14]. Glutathione S-transferase-RNCAS23 fusion protein was coupled with haemocyanin [15], emulsified with complete Freund's adjuvant and injected intraperitoneally into mice. Clones producing anti-

Abbreviations used: AoSMC, aortic smooth muscle cells; mAb, monoclonal antibody; MEL, murine erythroleukaemia; PC12, human pheochromocytoma cells.

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calpastatin mAbs were identified by solid phase radioimmunoassay [14] and mAb 35.23 was chosen on the basis of its recognition of native and recombinant calpastatins, including RNCast110 (EMBL/GenBank/DBJ, accession number Y13590), the form lacking any inhibitor domain. The results indicated that mAb 35.23 was directed against the calpastatin L-domain.

Anti-calpain mAb 56.3

Anti-calpain mAb was produced using, as antigen, purified human neutrophil calpain as described in [16].

Fractionated homogenization of rat liver

Freshly collected rat liver (0.5 g) was minced and suspended in 2.5 ml of 0.25 M sucrose containing 1 mM EDTA and 100 μ g/ml leupeptin (lysis medium). The sample was carefully homogenized using a Potter–Elvehjem homogenizer; a total of 4 cycles of two strokes each was performed. After each cycle, the homogenate was centrifuged at 600 *g* for 5 min, the soluble fraction was removed and an equal volume of lysis medium was added to the particulate fraction; the resulting bulk was mixed and submitted to another cycle of homogenization. The activity of cathepsin B and calpastatin was assayed in each soluble fraction.

An aliquot of the homogenate (200 μ l) obtained after each cycle was collected and centrifuged at 600 *g* for 5 min, the particulate fraction was resuspended in 500 μ l of 2% (v/v) paraformaldehyde diluted in PBS, and was used for immunofluorescence analysis to detect calpastatin granules.

To measure total calpastatin and cathepsin B activities, the homogenate was prepared using an equal amount of rat liver (0.5 g), which was homogenized with 8 strokes in a Potter–Elvehjem homogenizer and six bursts of sonication, each of 10 s duration.

Assay of calpastatin activity

Samples were heated at 100 °C for 3 min and centrifuged at 100 000 *g* for 10 min. The clear supernatants were dialysed against 50 mM sodium borate buffer, pH 7.5, containing 1 mM EDTA and 0.5 mM 2-mercaptoethanol. Calpastatin activity was then assayed using human erythrocyte calpain as described previously [17]. One unit of calpastatin activity is defined as the amount of inhibitor required to inhibit one unit of calpain enzyme activity. Human erythrocyte calpain was purified as reported previously [7].

Assay of cathepsin B activity

Cathepsin B activity was assayed using benzoyl-L-arginine- β -naphthylamide as substrate as described previously [18].

Immunofluorescence confocal microscopy

LAN-5 cells, MEL cells, PC12 cells and AoSMC were fixed with 1 ml of 2% (v/v) paraformaldehyde diluted in PBS for 30 min in an ice bath, washed twice and then permeated with PBS containing 50 mM NH_4Cl , 0.1% (v/v) saponin and 0.5% (w/v) BSA, at room temperature for 30 min. Cells were afterwards washed twice with PBS and treated with 7 μ g/ml anti-calpastatin mAb 35.23 or 8.5 μ g/ml anti-calpain mAb 56.3, both diluted in PBS containing 5% (v/v) fetal-calf serum. After incubation for 3 h at room temperature, cells were washed three times in PBS and treated with 8 μ g/ml FITC-conjugated sheep anti-mouse (Amersham Pharmacia Biotech) secondary antibody for 1 h.

Staining of chromatin was carried out by incubating fixed cells with 2 μ g/ml propidium iodide for 5 min [19]. Cells were mounted on coverslips with FluoroGuard anti-fade reagent (Bio-Rad Laboratories) before analysis. Images of the samples were collected by confocal microscopy using a Bio-Rad MRC1024 instrument (krypton/argon laser) on a Nikon Diaphot 200, using a planapochromat $\times 60$ oil-immersion objective with numerical aperture 1.4 [see Figures 1 (lower panel), 2, 4, 5 and 6], or a planapochromat $\times 40$ objective with numerical aperture 0.75 [see Figures 1 (upper panel) and 3]. The excitation/emission wavelengths were 488/522 nm for fluorescein-labelled antibodies and 488–568/605 nm for propidium iodide-stained chromatin.

Treatment of LAN-5 cells with the calcium ionophore A23187 or retinoic acid

LAN-5 cells, grown on glass slides, were incubated at 37 °C for increasing times with 1 μ M A23187 or with 1 μ M retinoic acid, both diluted in RPMI medium. After incubation, the cells were washed twice with PBS and treated for confocal microscopy analysis as described in the above section.

RESULTS

Intracellular localization of calpastatin

mAb 35.23, directed against the L-domain of calpastatin forms, was used to identify the cellular distribution of calpastatin in nerve cells.

Permeabilized human neuroblastoma LAN-5 cells were incubated with mAb 35.23 and the immunocomplex was stained with a second fluorescein-labelled antibody directed against mouse immunoglobulins. As shown in Figure 1(A, upper panel), in growing, untreated LAN-5 cells, fluorescent spots are clearly visible in each cell with moderate or no fluorescence detectable in the cytosol. Pretreatment of the mAb solution with purified calpastatin completely abolished the appearance of the spots (Figure 1B, upper panel). Micropictures at higher magnification (Figure 1, lower panel) showed that aggregation of calpastatin is localized as two granular structures, not surrounded by membranes, in a nuclear invagination; calpastatin is almost absent in the nucleus and in the cytosol. This suggests that most of intracellular calpastatin is localized in specific structures rather than distributed in a diffused form in the cytosol.

To determine whether this calpastatin compartmentalization is a general property, other human and murine cell types were stained with mAb 35.23. As shown in Figure 2, the cell distribution of calpastatin observed in LAN-5 cells was identical in MEL cells, PC12 and AoSMC. In each of these cell types two calpastatin fluorescent spots were visible as the unique localization of this protein inhibitor. Electron microscopic ultrastructural analysis revealed that calpastatin was confined in membrane-free granules of 1 μ m in diameter, indicating its possible association with specific intracellular structures (results not shown). Further studies are in progress to identify the type of organization involved.

In order to define the compartmentalization of calpastatin, rat liver, a tissue which is easily disrupted by homogenization, was used. To establish if calpastatin is particulate, a sample of minced liver was submitted to an increasing number of strokes in a homogenizer and, after each cycle of 2 strokes, calpastatin activity and total soluble proteins released were measured. As shown in Figure 3, more than 80% of the total soluble proteins were extracted from hepatocytes after the first round of homogenization, whereas calpastatin activity was released with a different kinetics, and in the highest amount after the second

Figure 1 Localization of calpastatin in human neuroblastoma LAN-5 cells

Upper panel: (A) Human neuroblastoma LAN-5 cells were fixed and double stained with anti-rat brain calpastatin mAb 35.23 (green fluorescence) and propidium iodide (red fluorescence) as described in the Materials and methods section. The image was obtained at 488–568 nm excitation and 522 nm and 605 nm emission. (B) Before immunostaining, anti-calpastatin mAb 35.23 was preincubated with excess of purified recombinant RNCAT23 calpastatin. The white bar indicates 50 μ m. Lower panel: magnification of the images shown in the upper panel using a planapochromat $\times 60$ oil-immersion objective with numerical aperture 1.4. The bar indicates 5 μ m.

Figure 2 Comparison of calpastatin intracellular localization among different cell types

Cells were fixed and immunostained with anticalpastatin mAb 35.23 as described in the Materials and methods section. (A) MEL cells, (B) LAN-5 cells, (C) AoSMC, (D) PC12.

cycle of homogenization. Cathepsin B, used as a marker of vesicle-confined enzymes, was virtually absent in the soluble fractions obtained from the first round of homogenization and became progressively and increasingly detectable after 3–4 cycles of homogenization, which coincided with the partial disruption of the lysosomal vesicles. Thus, overall, these findings are consistent with the presence of intracellular calpastatin in association with a membrane-free structure. These conclusions are further supported by confocal microscopic analysis (Figure 3, inset) showing that, after the first round of homogenization (inset a), calpastatin was still detectable in the form of fluorescent spots in the particulate material, whereas it was almost undetectable after 3 cycles of homogenization (inset b).

Changes in calpastatin distribution following calpain activation

To allow better correlation of the localization of calpastatin with its function, the activation of calpains in LAN-5 cells was induced by means of an increase in free Ca^{2+} promoted by treatment with the calcium ionophore A23187. Figure 4(A) shows that this treatment promoted two modifications with respect to the intracellular localization of calpastatin. At shorter incubation times, calpastatin is also found in the cytosol, whereas, with longer incubation, both soluble and particulate calpastatin decrease. After 120 min of exposure, more than 80 % of the fluorescent calpastatin aggregates disappear. To investigate if these modifications in cell distribution were accompanied by changes in the level of calpastatin activity, the calpain inhibitory activity in cells exposed to calcium ionophore for increasing times was measured. As shown in Figure 4(B), calpastatin activity remained constant for the first 20 min of treatment and then progressively decreased, reaching 35–40 % of the initial level after 120 min. This loss of calpastatin activity produces an imbalance in the regulation of the proteolytic system and could be involved in promoting the 'pathological' function of calpains.

Figure 3 Appearance of soluble calpastatin during liver homogenization

Freshly collected rat liver was homogenized as described in the Materials and methods section. After each cycle of homogenization, the sample was centrifuged and the soluble fraction and particulate fraction were collected. Protein content (hatched bars), calpastatin activity (filled bars) and cathepsin B activity (dotted bars) were measured in the soluble fraction obtained after the indicated number of homogenization cycles. The values are the means $\pm$ S.D. of three different rat liver preparations. Inset: an aliquot of the particulate fraction obtained after each cycle was fixed and immunostained with anti-calpastatin mAb 35.23 as described in the Materials and methods section. (a) Calpastatin-rich structures detected in the particulate fraction after 1 cycle of homogenization. (b) Calpastatin-rich structures detected in the particulate fraction after 3 cycles of homogenization. The inset micrographs are representative of 20 different fields examined.

Changes in calpastatin localization following induction of LAN-5 cell differentiation by retinoic acid

In order to verify whether the changes in intracellular distribution of calpastatin could be related to a physiological stimulation of the cell, LAN-5 cells were treated with retinoic acid. Retinoic acid is known to induce differentiation of these cells, indicated by the generation of long, neurite structures projecting from the cell body. As shown in Figure 5, following exposure of LAN-5 cells to retinoic acid for various times, calpastatin is progressively displaced from its aggregated form and becomes a soluble protein. After 15 h of incubation, when cell differentiation was almost complete, calpastatin was barely detectable. Thus the overall changes in the cell distribution and the cell content of calpastatin were similar in LAN-5 cells, whether induced by treatment with retinoic acid or promoted by an increase in intracellular Ca^{2+} (compare Figure 4 and Figure 5). These data suggest that this intracellular redistribution of the calpain inhibitor may be relevant to the occurrence of a physiological process, in our case represented by cell differentiation.

Figure 4 Changes in calpastatin distribution following calpain activation

(A) LAN-5 cells were grown on glass slides and treated with 1 μM calcium ionophore A23187 as described in the Materials and methods section. At the indicated times the cells were fixed, immunostained with anticalpastatin mAb 35.23 and examined by confocal microscopy. (B) LAN-5 cells (5×10^6) were incubated with 1 μM A23187, harvested at the indicated times and lysed by sonication (six bursts of 10 s) in 1 ml of 0.25 M sucrose containing 1 mM EDTA. The samples were then centrifuged at 100 000 g and calpastatin activity in the supernatant was assayed as described in the Materials and methods section.

Localization of calpain following activation of the calcium-dependent proteolytic system

Direct evidence for the above conclusion and a demonstration that the components of the proteolytic system are really localized

Figure 5 Changes in calpastatin distribution following induction of cell differentiation

LAN-5 cells grown in RPMI medium on glass slides were treated with 1 μ M retinoic acid at 37 °C. After incubation the cells were fixed, immunostained with mAb 35.23 and examined by confocal microscopy. (A), untreated cells; (B), (C) and (D), cells incubated for 4, 8 and 15 h respectively.

Figure 6 Changes in calpain localization following enzyme activation

MEL cells were (A, B) untreated, or (C, D) incubated at 37 °C for 15 min with 1 μ M A 23187 and were fixed and immunostained with anti-calpain mAb 56.3 as described in the Materials and methods section. (A) and (C), confocal micrographs obtained at 488–568 nm excitation/522 and 605 nm emission. (B) and (D), images obtained at 488 nm excitation/522 nm emission.

in different cell compartments was obtained in experiments to analyse the cell distribution of calpain. As shown in Figure 6, using an anti-calpain mAb it was established that, in resting MEL

cells, calpain is localized in the cytosolic compartment (Figures 6A and 6B), whereas, following an increase in intracellular free Ca^{2+} , the proteinase becomes partially associated with the plasma membrane (Figures 6C and 6D). These results demonstrate that, in untreated cells, calpain and calpastatin are localized in different compartments. Furthermore, on the basis of this distribution, calpastatin seems to be preferentially involved in controlling those calpain forms released from membranes following activation by autoproteolysis [7].

DISCUSSION

The localization of the Ca^{2+} -dependent proteolytic system which comprises calpain and its natural inhibitor, calpastatin, both reported to be in the same soluble cell fraction, has always posed the question of how the system operates with the enzyme and its inhibitor so closely situated. A tentative explanation has been based on the hypothesis that each component, following rapid and brief activation of the system, could translocate to a different cell compartment, thus allowing calpain to express its limited proteolytic activity; whereas, at longer activation times calpastatin could interact with active calpain to prevent uncontrolled and unspecific degradation. This overall hypothesis was based mainly on earlier observations indicating that calpain is preferentially activated on the inner face of the plasma membrane but, once converted into the autoproteolysed, active form, diffuses as a soluble protein into the cytosol.

In the present report, we show that in neuroblastoma LAN-5 cells, as well as in three other cell types, calpastatin is mostly localized in structures close to nuclear invaginations. Ultrastructural analysis and studies of the release of calpastatin during lysis of hepatocytes indicate that these calpastatin-rich structures are granules, without surrounding membranes, which become disrupted by mechanical shock or by sonication.

In contrast, calpain has been confirmed to be located diffusely in the cytosol. We also report that, following an increase in $[\text{Ca}^{2+}]$, calpain partially aggregates with the plasma membrane, whereas calpastatin apparently changes its cellular distribution and becomes progressively widespread in the cytosol. These results clearly indicate that, in the early stages of $[\text{Ca}^{2+}]$ -related cell stimulation, calpain, because of its different cell localization with respect to calpastatin, can rapidly move to the inner surface of the cell membrane and accordingly interact with Ca^{2+} and with positive modulators and thereby become an active proteinase. If the activating conditions persist, calpastatin, which has now diffused into the cytosol, is co-localized with calpain and can thereby modulate calpain activity. At longer times of activation, calpastatin is progressively degraded by calpain and this may constitute a transition from a physiological process to a pathological process in which uncontrolled protein degradation takes place.

In our opinion, this difference in the localization of the components of the Ca^{2+} -dependent proteolytic system is a strategy for ensuring that the rapid cellular responses effected by calpain can be efficiently curtailed by calpastatin and, at the same time, reduces the possibility of non-specific proteolytic degradation of cellular components by calpain.

Further experiments are in progress to identify these protein complexes *in vivo* and to clarify these calpastatin-rich structures.

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