Changes in intracellular calpastatin localization are mediated by reversible phosphorylation

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We have previously reported that, in neuroblastoma LAN-5 cells, calpastatin is in an aggregated state, close to the cell nucleus [De Tullio, Passalacqua, Averna, Salamino, Melloni and Pontremoli (1999) Biochem. J. 343, 467–472]. In the present paper, we demonstrate that aggregated calpastatin is predominantly in a phosphorylated state. An increase in intracellular free \([\text{Ca}^{2+}]\) induces both dephosphorylation of calpastatin, through the action of a phosphoprotein phosphatase, and its redistribution as a soluble inhibitor species. cAMP, but not PMA-induced phosphorylation, reverses calpastatin distribution favouring its aggregation. This intracellular reversible mechanism, regulating the level of cytosolic calpastatin, could be considered a strategy through which calpain can escape calpastatin inhibition, especially during earlier steps of its activation process.

Key words: calpain regulation, cAMP-dependent phosphorylation, LAN-5 neuroblastoma cells, phosphoprotein phosphatase.

INTRODUCTION

All mammalian cells contain a soluble calcium-dependent proteolytic system, which participates in many cell functions [1–6]. It is composed of a family of thiol-proteinases, named calpains, belonging to the ubiquitous or tissue-specific subfamily [7–10]. The classical calcium dependent \(\mu\) - and \(\mu\)-calpain are regulated, in addition to fluctuations in intracellular \([\text{Ca}^{2+}]\), by a protein named calpastatin, which inhibits their proteolytic activity [1, 7, 9].

Calpastatin is normally present in the cell in large excess compared with calpains, and \(\text{in vitro}\) inhibits both the native and the auto-proteolysed form of the proteinases [11]. Calpastatin structure deduced from various mammalian cDNAs indicates that it is constituted by five domains, four of which are inhibitory repetitive domains, whereas the last one corresponding to the N-terminal region of the protein (L-domain) lacks inhibitory activity [12–15].

It is now clearly established that cells and tissues contain multiple forms of calpastatin, deriving from both post-transcriptional and post-translational modifications [16–19]. It has been reported that insertion or deletion of exons localized on the L-domain produce various forms having different specificity of inhibitory efficiency [18, 20]. Moreover, the number of inhibitory repetitive domains can also be reduced, as occurs in rat brain, which contains a low \(M_p\) calpastatin, having the L-domain associated with a single inhibitory domain [15]. Due to the fact that within the calpastatin molecule no correlation between the number of inhibitory domains and inhibitory efficiency has been observed [21], the high \(M_p\) calpastatin form could also be visualized as a precursor of free repetitive domains resulting from its proteolysis. This process may occur in order to amplify inhibitory activity required by the cells.

Furthermore, we have observed that a phosphorylation–dephosphorylation process is responsible for the modulation of calpastatin specificity in rat skeletal muscle [22], and for the inhibitory efficiency in rat brain [23]. More recently, we have also observed that, in different cell lines, calpastatin is present in an aggregated form, normally localized close to the cell nucleus [24]. Following an increase in intracellular \([\text{Ca}^{2+}]\), calpastatin is released from its association, becoming a soluble protein. This change in cell localization may be correlated with the regulation of the overall mechanism of calpain activation. In the present paper we have studied, at molecular level, the steps involved in the internal translocation of calpastatin.

We now report that cAMP-dependent phosphorylation of calpastatin is responsible for calpastatin aggregation, whereas its dephosphorylation, triggered by an increase in intracellular \([\text{Ca}^{2+}]\), determines redistribution of the inhibitor protein. The operation of both processes controls the amount of calpastatin available for calpain inhibition, and therefore the overall mechanism can be visualized as a crucial device in the regulation of the calcium-dependent proteolytic system.

MATERIALS AND METHODS

Materials

\(\text{Ca}^{2+}\) ionophore A23187, leupeptin, PMSF, dibutyryl cAMP, PMA, okadaic acid and anti-phosphoserine monoclonal antibody (mAb) were purchased from Sigma. FITC-conjugated sheep anti-mouse secondary antibody and \([\gamma-\text{P}]\text{ATP}\) were purchased from Amersham Pharmacia Biotech. Anti-calpastatin mAb 35.23 was produced as described in [21]. Human erythrocyte calpain was purified as reported previously [25]. One unit of calpain activity is defined as the amount causing the production of 1 \(\mu\)mol of acid-soluble \(\text{NH}_2\) groups in the conditions previously reported [26]. Phosphoprotein phosphatase (PPase) was purified from rat brain and assayed as described in [23]. cAMP-dependent protein kinase (PKA) and \(\text{Ca}^{2+}/\text{phospholipid-dependent protein kinase (PKC}\) were purified from rat brain as described in [23]. Human neuroblastoma LAN-5 cells were cultured in RPMI as reported previously [27].

Separation of calpastatin I and II in LAN-5 cells and their purification

Confluent human neuroblastoma LAN-5 cells, grown in a T-175 cm\(^2\) Falcon flask (\(\approx 15 \times 10^6\) cells), were collected and

Abbreviations used: mAb, monoclonal antibody; PKA, cAMP-dependent protein kinase; PKC, \(\text{Ca}^{2+}/\text{phospholipid-dependent protein kinase; PPase, phosphoprotein phosphatase.}\)

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Figure 1  Intracellular localization and chromatographic properties of calpastatin in untreated and calcium ionophore-treated human neuroblastoma LAN-5 cells

(A) The heated homogenate from untreated LAN-5 cells (≈ 15 x 10⁶) was applied to a DE-53 chromatographic column and the separation of calpastatin I and II was obtained as described in the Materials and methods section. Inset: human neuroblastoma LAN-5 cells, grown on glass slides, were fixed and double stained with anti-calpastatin mAb 35.23 (green fluorescence) and propidium iodide (red fluorescence) as described in the Materials and methods section.

(B) LAN-5 cells (≈ 15 x 10⁶ cells) were incubated in RPMI medium (which contains 0.42 mM CaCl₂) in the presence of 0.5 μM calcium ionophore, at 37 °C for 30 min. After exposure to the ionophore, cells were washed, collected and lysed as described in the Materials and methods section. Heated homogenate was prepared and ion-exchange chromatography was performed in order to separate calpastatin I and II. Inset: cells grown on glass slides were incubated with ionophore as in (A), then washed twice with PBS and finally treated for confocal microscopy analysis as described in the Materials and methods section. Scale: 0.4 cm = 0.5 μm.

Figure 1 legend

In vitro phosphorylation of calpastatin I and II by PKA or PKC
Calpastatin I and II, purified from LAN-5 cells as described above, were phosphorylated by PKA or PKC in the conditions previously reported [23].

Immunofluorescence confocal microscopy
LAN-5 cells grown on glass slides were washed three times with PBS and fixed in an ice bath for 30 min with 1 ml of 2 % (v/v) paraformaldehyde diluted in PBS. Detection of calpastatin was achieved by performing confocal analysis as described in [24], using anti-calpastatin mAb 35.23 as primary antibody, and a FITC-conjugated sheep anti-mouse as secondary antibody.

Immunoprecipitation of calpastatin and analysis of its phosphorylation state
Confluent LAN-5 cells (≈ 15 x 10⁶ cells) were washed three times with PBS, suspended in 1 ml of 20 mM Tris/HCl buffer (pH 7.6), containing 0.5 % Nonidet P40, 2.5 mM EDTA, 2.5 mM EGTA, 0.15 M NaCl (buffer A), 0.1 mg/ml leupeptin, 1 mM PMSF and 20 μg/ml aprotinin, and lysed by sonication (four bursts of 10 s each at 0 °C). After centrifugation at 100000 g for 15 min, the supernatant was collected, precleared by incubation with 15 μl of Protein G–Sepharose at 4 °C for 15 min, then incubated with 3 μg of anti-calpastatin mAb 35.23 at 4 °C for 2 h. To increase the amount of immunoprecipitated calpastatin, the mixture was further incubated with 2 μg of anti-mouse IgG at 4 °C for 1 h. Protein G–Sepharose (15 μl) was then added to the mixture and incubated at 4 °C for 2 h. The Sepharose beads were pelleted and washed five times with 1 ml of buffer A. To remove the contamination of immunoglobulin chains, the immunoprecipitate of each sample was dissolved in 50 mM sodium acetate.
buffer (pH 4.5) to dissociate the immunocomplexes, and the solutions were heated for 2 min at 100 °C. Heat-denatured immunoglobulin chains were removed by centrifugation at 100 000 g for 15 min, and calpastatin, completely resistant to this treatment, was subjected to 8 % SDS/PAGE, followed by immunoblotting. Phosphorylated calpastatin forms were recognized using an anti-phosphoserine monoclonal antibody and the immunoreactive material was detected by a peroxidase-conjugated secondary antibody [28] developed with an ECL® detection system (Amersham Pharmacia Biotech).

RESULTS

Comparison between calpastatin chromatographic properties and its intracellular localization

Calpastatin activity, present in untreated growing human neuroblastoma LAN-5 cells, is separated by ion-exchange chromatography on DE-53, into two peaks named calpastatin I and II, based on their elution volume. As shown in Figure I(A), calpastatin II is the predominant form, representing approx. 60 % of total inhibitory activity. For the evaluation of calpastatin activity, human erythrocyte calpain has been used, since it is a proteinase highly sensitive to calpastatin inhibition, independent of the modifications to the inhibitor protein [23]. In these conditions, the measurement of calpastatin activity is directly correlated with its actual amount. Using the monoclonal antibody mAb 35.23, directed against the L-domain of rat brain calpastatin, we have observed that, in untreated human neuroblastoma LAN-5 cells, calpastatin is localized in two fluorescent spots close to the nucleus (Figure 1A, inset), a localization similar to that previously seen in other cell types [24]. Very low diffused fluorescence is detectable in these cells, indicating that the largest fraction of calpastatin is confined to an aggregated state and not free in the cytosol.

In these cell conditions, the calcium-dependent proteolytic system is considered relatively quiescent. To establish if calpain activation, promoted by an intracellular [Ca²⁺] increase, also produces changes in calpastatin properties, we have treated LAN-5 cells with the Ca²⁺ ionophore A23187. For these experiments relatively mild conditions were chosen in order to prevent
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Figure 5 Changes in intracellular localization of calpastatin following treatment of LAN-5 cells with dibutyryl cAMP and PMA

(A) Upper panel: untreated control LAN-5 cells. (B) Upper panel: LAN-5 cells incubated for 30 min at 37 °C in the presence of 20 μM dibutyryl cAMP. (C) Upper panel: LAN-5 cells incubated for 30 min at 37 °C in the presence of 100 ng/ml PMA. (A) Lower panel: LAN-5 cells exposed for 30 min at 37 °C to 0.5 μM calcium ionophore. (B) Lower panel: LAN-5 cells exposed for 30 min at 37 °C to 0.5 μM calcium ionophore and then incubated 30 min at 37 °C in the presence of 20 μM dibutyryl cAMP. (C) Lower panel: LAN-5 cells exposed for 30 min at 37 °C to 0.5 μM calcium ionophore and then incubated 30 min at 37 °C in the presence of 100 ng/ml PMA. After treatment, calpastatin localization was determined as described in the Materials and methods section. Scale: 0.5 cm = 0.5 μm.

degradation of calpastatin [24]. In fact, comparing Figure 1(A) with Figure 1(B), total calpastatin activity is not affected by this cell treatment. In these conditions, peak I is now the predominant form, containing more than 60% of total activity. At the morphological level, calpastatin is no longer exclusively confined in aggregates, but a large quantity is detectable as diffused fluorescence in the cell-soluble fraction (Figure 1B, inset).

Taken together, these data suggest that an intracellular increase in [Ca^{2+}] promotes calpastatin redistribution in the cell, inducing a molecular modification altering the chromatographic properties of the inhibitor molecule.

Reversible phosphorylation of purified LAN-5 cells’ calpastatin

Previous work [23] has suggested that calpastatin can be phosphorylated in vitro by various protein kinases. Now, we have exposed calpastatin II purified from LAN-5 cells to a PPase isolated from rat brain. Following this treatment, calpastatin II (Figure 2) acquires properties similar to those of calpastatin I, since following ion-exchange chromatography on DE-53, it elutes earlier in a position typical of calpastatin I. This latter inhibitor form is unaffected by PPase treatment (results not shown).

To establish if this treatment causes only the removal of phosphate groups from the inhibitor protein, without modifying other characteristics, we have submitted PPase-treated calpastatin II both to SDS/PAGE (Figure 2, inset) and to phosphorylation by PKA and PKC (Figure 3). Exposure of purified calpastatin II to PPase does not produce changes in mobility of calpastatin bands, nor in the ratio between the two forms present in this preparation. Furthermore, PPase-treated calpastatin II incorporates approx. 3 times more [\(^3\)P]phosphate groups than the untreated form, reaching values similar to those of calpastatin I. In these in vitro experiments, PKA and PKC are slightly different in promoting phosphorylation of calpastatin; these data indicate that, following exposure of calpastatin to PPase, sites recognized by both kinases are available.

In vivo phosphorylation of calpastatin

The in vitro experiments suggest that dephosphorylation of calpastatin II is responsible for the changes in its chromatographic properties, as well as in its intracellular localization.

To provide evidence that this process is operating also in intact cells, we have exposed LAN-5 cells to okadaic acid, a compound known to inhibit PPase activity [29]. As shown in Figure 4, the interconversion of calpastatin forms, promoted by a rise in intracellular [Ca^{2+}], is almost completely prevented by the presence of okadaic acid. In fact, following the addition of this PPase inhibitor, the level of phosphorylated calpastatin II remains unchanged.
Correlation in vivo between calpastatin phosphorylation and its intracellular localization

To establish whether in vivo stimuli promoting phosphorylation cause changes in intracellular calpastatin localization, we have exposed LAN-5 cells to dibutyryl cAMP or to PMA, in order to induce the activation of PKA and PMA-stimulated PKC isoenzymes [30].

At a morphological level, treatment with dibutyryl cAMP of LAN-5 cells previously exposed to the calcium ionophore A23187 (Figure 5B, lower panel) determines the reappearance of the aggregated calpastatin spots, which were almost undetectable following an increase in intracellular [Ca²⁺] (Figure 5A, lower panel). Exposure of LAN-5 cells to PK (Figure 5C, lower panel) does not promote significant aggregation of calpastatin as compared with the effect of dibutyryl cAMP. Hence, PKA-induced calpastatin phosphorylation is the signal involved in the aggregation of the inhibitor molecules. This different behaviour of calpastatin, observed when PKA and PKC are activated, suggest that phosphorylation of calpastatin on different sites induces different effects on the inhibitor protein.

These data are further supported by the levels of calpastatin I and II measured in these stimulated cells (Figure 6) following ion-exchange chromatography. Treatment with dibutyryl cAMP has little or no effect on control cells; whereas in Ca²⁺ enriched cells, cAMP induces the reappearance of calpastatin II, in an amount comparable with that of untreated cells.

Taken together, these in vivo experiments point out that PKA-phosphorylation and PPhase-dephosphorylation are directly involved in determining the cellular localization of calpastatin, as well as the amount of soluble calpastatin available for calpain inhibition. The changes in the phosphorylation state of calpastatin occurring in vivo, following an intracellular increase in [Ca²⁺], or by cAMP-induced activation of PKA, were also observed by Western-blot analysis performed on immuno-precipitated calpastatin, detected with an anti-phosphoserine antibody. To avoid interferences in the results caused by heavy and light immunoglobulin chains (50 kDa and 25 kDa respectively), these contaminating proteins were removed from the immunoprecipitates, as indicated in the Materials and methods section. As shown in Figure 7 (lane 1), the two calpastatin bands, corresponding to the high and low Mₐ forms expressed in these cells, contain phosphoserine residues in control untreated cells. As expected, following treatment with the calcium ionophore A23187, the same bands become less intense (Figure 7, lane 2), indicating a loss of phosphate groups. The decrease in the extent of phosphorylation was calculated, by scanning the exposed film, to be approx. 60% of total. Dibutyryl cAMP alone (Figure 7, lane 3), or added to ionophore-treated cells (lane 4), induces a large increase in the intensity of two phosphorylated calpastatin bands, which become similar or slightly more intense than those of control cells. No appreciable cross-reactive bands were visible, when anti-phosphothreonine or -phosphotyrosine antibodies were used (results not shown).

DISCUSSION

In many cells and tissues calpastatin is present in multiple forms. This fact is considered as a cellular response required for the inhibition of many calpain forms, differently localized and activated [1,7,9,10]. Although this hypothesis is still valid, post-translational modification of calpastatin appears to be a prior step involved in the complex mechanism regulating the calcium-dependent proteolytic system.

We have reported previously that calpastatin from rat skeletal muscle and from rat brain can be phosphorylated in vitro by both PKC and PKA. This modification was interpreted as the mechanism modulating calpastatin specificity [22] or inhibitory efficiency [23]. More recently we have also observed that in different cell lines calpastatin is present in an aggregated state.
localized close to the nucleus [24]. Intracellular increases in [Ca\textsuperscript{2+}], a condition triggering calpain activation, induce the liberation of calpastatin molecules from aggregates, becoming soluble.

We have now identified in LAN-S cells biochemical signals inducing intracellular aggregation or cytosolic diffusion of calpastatin. In fact, PKA-catalysed phosphorylation of calpastatin is the primary event causing aggregation, whereas an increase in [Ca\textsuperscript{2+}], condition triggering activation of the proteolytic system, results also in the disaggregation of the inhibitor molecules, through the action of a PPase activity.

This conclusion is supported by the following experimental evidence: (i) the \textit{in vivo} effect of okadaic acid, (ii) the \textit{in vitro} effects of the PPase, (iii) the \textit{in vivo} effect of dibutyryl cAMP and of increase in [Ca\textsuperscript{2+}] and (iv) the identification of phosphorylated calpastatin by an anti-phosphoserine antibody.

Even if PKC is able to phosphorylate calpastatin \textit{in vitro}, unlike PKA, it does not induce intracellular aggregation. This differential effect of PKC and PKA is due to the phosphorylation of calpastatin on different sites [31]. Moreover, in rat skeletal muscle, PKC phosphorylation was shown to promote changes in the functional properties of calpastatin. On the basis of these observations, it could be speculated that PKA or PKC phosphorylation promotes different responses in calpastatin molecules; the former is addressed to define calpastatin intracellular phosphorylation promotes different responses in calpastatin molecules. On the basis of these observations, it could be speculated that PKA or PKC phosphorylation promotes different responses in calpastatin molecules; the former is addressed to define calpastatin intracellular localization, the latter to control the inhibitor efficiency. This PKA-promoted phosphorylation process regulates the amount of soluble calpastatin available for calpain inhibition.

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