

Characterization of a new p94-like calpain form in human lymphocytes

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Human circulating PBMC (peripheral blood mononuclear cells) contain three calpain isoforms distinguishable on the basis of their chromatographic properties. Two of these proteases belong to the ubiquitous calpain subfamily, corresponding to the classical μ - and m-calpain forms. The third, which shows peculiar activating and regulatory properties, is an alternatively spliced calpain 3 (p94) form. This new calpain differs from calpain 3 in that it has lost IS1 insertion and exon 15, a lysine-rich sequence regarded as a nuclear translocation signal. PBMC p94-calpain undergoes activation and inactivation without the accumulation of a low- Ca^{2+} -requiring form that is typical of the classical activation processes of μ - and m-calpain. Furthermore, it differs from the ubiquitous forms in that it displays a lower sensitivity to calpastatin.

On the basis of these selective properties, it can be postulated that PBMC p94-calpain can be activated in response to specific stimuli that are not effective on the other calpain isoenzymes. The enzyme is preferentially expressed in B- and T-lymphocytes, whereas it is poorly expressed in natural killer cells and almost undetectable in polymorphonuclear cells. This distribution might reflect the specific function of this protease, which is preferentially present in cells devoted to the production of the humoral, rather than to the cellular, immune response.

Key words: alternative splicing, Ca^{2+} -dependent proteolysis, calpain isoform, circulating white cell.

INTRODUCTION

The term 'calpain' identifies a family of intracellular thiol-proteases that have as common characteristics an absolute dependency on calcium ions and the presence of intramolecular calmodulin-like domains [1–4]. The presence in calpain of multiple EF-hand structures in both catalytic and small subunits supports this requirement. As the number of new calpains has increased, the presence of EF-hand structures has become a characteristic limited to a group of them, as other calpain-like forms without EF-hand Ca^{2+} -binding structures have been identified [1,4].

Calpains 1 and 2 (also called μ - and m-calpain respectively) are the most studied forms, together with calpain 3 (p94), which is a member of the so-called tissue-specific subfamily [5–7]. The discovery of the crystal structure of m-calpain [2,3,8–10] revealed further properties which better characterize this protease family. Although the calpain catalytic domain is highly homologous with those of other thiol proteases, such as papain or cathepsin B [6], its three-dimensional structure presents a unique feature. In native conditions [3] it is divided into two subdomains, in which disorganization of the essential catalytic residues occurs. In this condition, the enzyme is completely inactive, as the Cys catalytic residue is too far from the other functional residue, His. Binding of calcium ions to differently distributed sites causes a conformational transition which drives the subdomains together [11,12]. In human erythrocyte calpain, following the binding of calcium, achievement of the active calpain conformation precedes both dissociation of the heterodimer and autoproteolysis of domain I [13]. Dissociation and autoproteolysis have also been suggested for m-calpain as early events occurring during activation [14–16]. The two major functional differences between the two processes are reversibility in the case of a simple conformational change, and the formation of an easily activable, covalently modified protease form in the latter case. Owing to the fact that μ - and m-calpain can be involved in various cell functions [17–21], the differences observed in the activation process may be related to such specific roles.

p94-calpain differs from the ubiquitous calpains in that it has the insertion of two sequences [5,22]. The first, called IS1, is localized in the catalytic domain and contains two autolysis sites [23]. The second, called IS2, is in domain III and contains a nuclear translocation sequence, suggesting a possible localization of this protease in the nucleus [4]. Defects in the calpain 3 gene (CAPN3) are related to limb-girdle muscular dystrophy type 2A [5,24,25]. Retina [26] and lens [27] cells contain a p94-calpain isoform which differs from that found in skeletal muscle in that specific regions are deleted. Lp85, Lp82 and Rt88 are products of alternative splicing of the CAPN3 gene [26,27].

Here we report that circulating human PBMC (peripheral blood mononuclear cells) contain a new version of p94-calpain which lacks IS1 and the nuclear translocation signal localized in IS2. This protease is reversibly activated by calcium without accumulation of a low- Ca^{2+} -requiring form and is also less sensitive to calpastatin. The cellular distribution of this new calpain isoenzyme, which is highly expressed in B- and T-lymphocytes, suggests a specific intracellular role.

EXPERIMENTAL

Materials

Ficoll-Paque Plus, dextran T70, Source 15Q resin, phenyl-Sepharose resin, Sephadex G-200 resin and horseradish peroxidase-linked anti-mouse secondary antibody were purchased from Amersham Biosciences. α -cellulose, Sigmacell type 50 microcrystalline cellulose, Ca^{2+} ionophore A23187, trypsin and leupeptin were from Sigma (St. Louis, MO, U.S.A.). Calpain inhibitors I and II were from Boehringer Mannheim. Anti-calpain mAb (monoclonal antibody) 56.3 was produced as described in [28]. Recombinant rat brain calpastatin 104 (RNCST104) was produced and purified as described in [29]. Human erythrocyte calpain was purified as reported previously [30]. Human neutrophil calpain was purified as described in [31]. Calpain activator from rat skeletal muscle (UK114) was purified as reported in [32].

Abbreviations used: PBMC, peripheral blood mononuclear cells; RNCST104, recombinant rat brain calpastatin 104; mAb, monoclonal antibody.

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PBMC purification

Freshly collected buffy coats were diluted 1:3 in 0.9% NaCl (w/v), layered on to 10 ml of Ficoll-Paque Plus and centrifuged at 400 g for 30 min at 20 °C. PBMC were collected at the interface between the upper layer, containing the plasma fraction, and the lower layer, containing Ficoll-Paque Plus. PBMC were washed three times with 10 mM Hepes buffer, pH 7.4, containing 0.14 M NaCl, 5 mM KCl and 2 mg/ml glucose to remove platelets.

Neutrophil and erythrocyte purification

Neutrophil isolation is based on the procedure of Boyum [33]. Freshly collected buffy coats were diluted with 1.6% (w/v) dextran (final concentration) and left for 1 h at 25–28 °C. The sedimented erythrocytes were removed and the supernatant solution (35 ml) was layered on to 10 ml of Ficoll-Paque Plus and centrifuged at 400 g for 30 min at 20 °C. The pellet obtained was resuspended in 10 ml of 0.2% NaCl to lyse the contaminating red cells. After 30 s, 10 ml of 1.6% NaCl was added; the cells were recovered by centrifugation at 400 g for 5 min and washed three times with 10 mM Hepes buffer, pH 7.4, containing 0.14 M NaCl, 5 mM KCl and 2 mg/ml glucose.

To obtain isolated erythrocytes, freshly collected human blood was washed three times with 0.15 M NaCl. Erythrocytes were completely deprived of leucocytes and platelets, essentially following the procedure of Beutler et al. [34], by using a column containing a mixture of microcrystalline cellulose and α -cellulose (1:3, w/w).

Identification of calpain isoenzymes from PBMC, erythrocytes and neutrophils

Purified PBMC (6×10^8) or neutrophils (6×10^8) were lysed by freezing and thawing in 1 ml of 50 mM sodium borate buffer, pH 7.5, containing 1 mM EDTA and 0.5 mM β -mercaptoethanol (buffer A). Purified erythrocytes (5 ml) were lysed in 25 ml of buffer A. The three different samples were centrifuged at 60 000 g for 10 min at 4 °C and the supernatant (crude extract) was loaded on to a Source 15Q column previously equilibrated in buffer A. Proteins were eluted with a linear gradient of 0–0.4 M NaCl. Calpain activity was routinely assayed as reported in [35] using 150 μ l of each fraction. Calpastatin activity was routinely assayed with 100 μ l of each fraction using human erythrocyte calpain, which has been shown to be the calpain isoform most sensitive to calpastatin inhibition [36]. Fractions containing calpain and calpastatin were pooled and loaded on to a phenyl-Sepharose column equilibrated in buffer A containing 0.3 M NaCl. Calpain activity was eluted with buffer A without NaCl. The same two steps can be inverted in order to immediately separate different calpain isoforms from calpastatin.

p94-calpain from PBMC was further purified by gel filtration on Sephadex G200 equilibrated in buffer A. One unit of calpain activity is defined as the amount required to cause the release of 1 nmol of free NH_2 groups/min under the specified conditions. One unit of calpastatin activity is the amount required to inhibit 1 unit of calpain.

Calpain detection by electrophoresis and Western blot analysis

Purified PBMC, neutrophil and erythrocyte calpains were subjected to casein zymography as indicated in [37]. SDS/PAGE was carried out according to the method of Laemmli [38]. After electrophoresis zymogram and SDS/PAGE gels were transferred to nitrocellulose membranes as indicated in [39] and probed with anti-calpain mAb 56.3.

Calcium requirement of PBMC calpains following exposure to calcium ions

Purified calpains obtained from different cell types were incubated (120 units each) in an ice bath with 50 mM sodium borate buffer, pH 7.5, in the presence of 100 μ M Ca^{2+} (μ -calpain and p94-calpain) or 400 μ M Ca^{2+} (m-calpain; final volume 0.6 ml). Aliquots of 25 μ l were collected after 0, 1, 2, 3, 5, 7 and 10 min and transferred to assay mixtures to evaluate calpain activity [35] in 5 μ M and 1 mM Ca^{2+} on samples containing μ -calpain and p94-calpain, and in 20 μ M and 1 mM Ca^{2+} on samples containing m-calpain.

Calpain and calpastatin levels in Ca^{2+} -enriched PBMC

Purified PBMC (6×10^8 cells) were diluted in 40 ml of 10 mM Hepes buffer, pH 7.4, containing 0.14 M NaCl, 5 mM KCl, 2 mg/ml glucose, 3 mg/ml BSA, 1 mM CaCl_2 and 0.1 μ M Ca^{2+} ionophore A23187 and incubated for 0, 10 and 30 min at 37 °C with gentle mixing. At the indicated times, cells were collected by centrifugation at 300 g for 5 min and lysed by freezing and thawing in 1 ml of buffer A. PBMC calpain isoenzymes and calpastatin were separated by two chromatographic steps, as indicated above. The levels of PBMC calpains and calpastatin were calculated from the area under the activity peaks.

RNA isolation, cDNA synthesis and PCR

Total RNA from different cell types (5×10^6 cells) was isolated using RNA-II nucleospin (Macherey-Nagel), and cDNAs were synthesized using the Thermoscript RT-PCR system (Invitrogen). Calpain transcripts were amplified from cDNAs using Herculase Hotstart DNA polymerase (Stratagene). To detect different calpain isoforms, three different sets of primers were chosen in the region external to the calpain catalytic domain. The oligonucleotides were: for μ -calpain (GenBank accession no. X04366), sense primer 5'-GATGGAGCTACCCGCACAGAC-3' (Sn441) and antisense primer 5'-GTGGAGGGCACCACCACATAC-3' (Asn1642); for m-calpain (GenBank accession no. NM_001748), sense primer 5'-GCCACCCGCACAGACATC-3' (Sn415) and antisense primer 5'-AAGGTGGAAGGCACGAG-AATG-3' (Asn1608); and for p94-calpain (GenBank accession no. AF127764), sense primer 5'-TTGATGGAGCCAACAGAAC-TGAC-3' (Sn559) and antisense primer 5'-GTGGAGGGCAG-GATGACG-3' (Asn1900). PCR conditions were 92 °C for 2 min, 30 cycles of 92 °C for 10 s, 55 °C for 30 s and 68 °C for 3 min and finally 68 °C for 6 min. The sequences of the resulting fragments were confirmed by the dideoxynucleotide chain-termination method [40] from both strands, using the CEQ 2000 dye terminator cycle sequencing kit (Beckman Coulter), followed by automated reading on a CEQ 2000XL DNA sequencer.

To obtain the complete sequences of μ -calpain and p94-calpain from PBMC cDNA, two sets of primers with restriction sites for *Bam*HI and *Xho*I were used. Their sequences were, for μ -calpain, sense primer 5'-AATTGGATCCCAGGATGTCG-GAGGAGAT-3' and antisense primer 5'-TTAACTCGAGCAGATGAACAGATGGTGGCT-3'; and for p94-calpain, sense primer 5'-AATTGGATCCATGCCGACCGTCATTAGCGCATC-3' and antisense primer 5'-TTAACTCGAGTCAGGCATACATGGTG-AGCTGCAG-3'. Both sequences were cloned into the pGEM-T vector (Promega).

To determine the relative levels of p94-calpain transcript in the different cell populations, equal amounts of each cDNA sample were co-amplified in the presence of primers specific for glyceraldehyde-3-phosphate dehydrogenase (sense primer

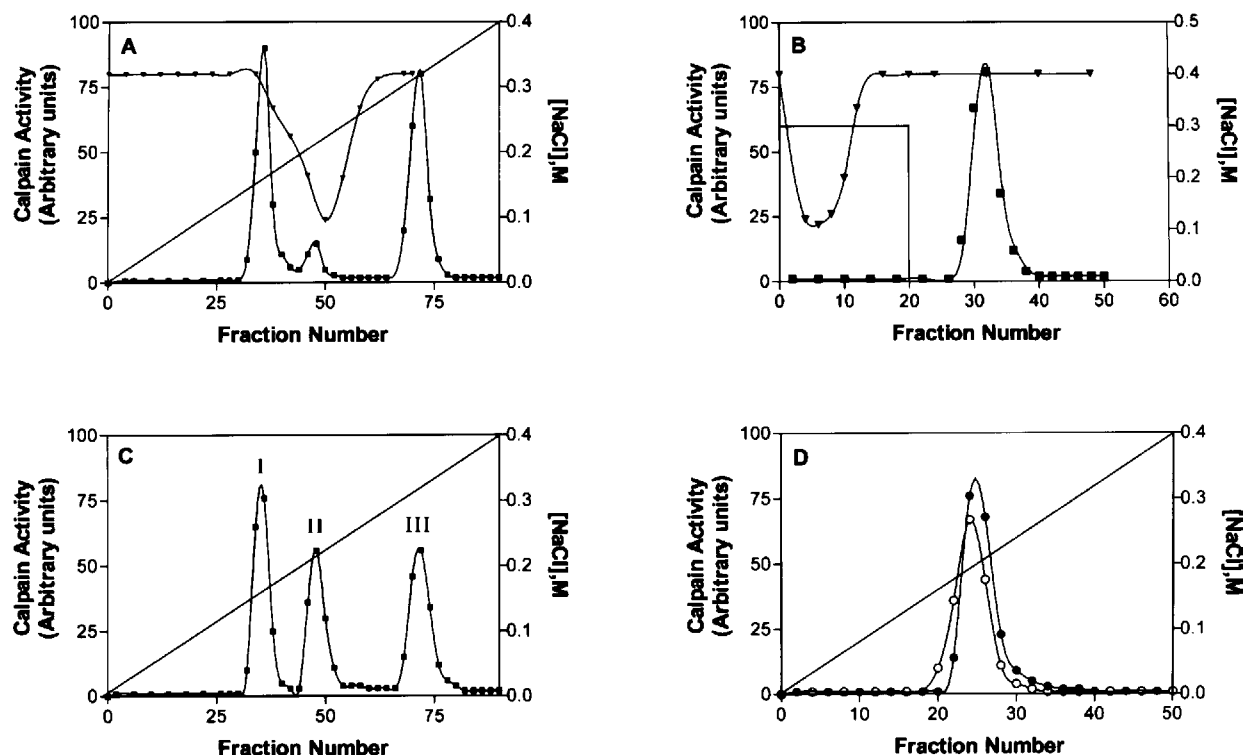


Figure 1 Calpain isoforms in PBMC, erythrocytes and neutrophils

(A) PBMC crude extract, prepared as described in the Experimental section, was loaded on to a Source 15Q column. Calpain (■) and calpastatin (▼) activities were assayed. (B) Fractions 42–65 were pooled and subjected to hydrophobic chromatography on phenyl-Sepharose. (C) PBMC or (D) erythrocyte (●) or neutrophil (○) crude extracts were first subjected to phenyl-Sepharose chromatography; the peak containing calpain activity was subsequently loaded on to Source 15Q resin. I, II and III in (C) represent calpain activity peaks, see the text for details. Calpain and calpastatin activities were measured as described in the Experimental section. On all panels the straight line denotes NaCl concentration.

5'-ACGACCCCTTCATTGACC-3' and antisense primer 5'-TGC-TTCACCACCTTCTTG-3') and for p94-calpain (Sn559 and Asn1900; see above). PCR conditions were the same as those used to detect the different calpain isoforms present in PBMC.

Amino acid sequence of p94-calpain from PBMC

PBMC p94-calpain samples were subjected to SDS/PAGE (7.5% gels), transferred to nitrocellulose and stained with Ponceau S. The calpain band was cut, destained and digested with trypsin as indicated in [41]. The resulting peptides were separated by reversed-phase HPLC on a C_2/C_{18} column (μ RPC C_2/C_{18} SC2.1/10; 1 × 100 mm; Amersham Biosciences) equilibrated in 0.1% trifluoroacetic acid. Absorbed peptides were eluted with a linear gradient of acetonitrile (from 0 to 70%) and fractions of 0.05 ml were collected. Separated tryptic peptides were directly applied to a Beckman LF 3000 protein sequencer. Their amino acid sequences were compared with human μ -calpain (GenBank accession no. CAA27881.1), human p94-calpain (GenBank accession no. ADD28253.1) and human m-calpain (GenBank accession no. NP_001739.1).

RESULTS

Calpain isoforms in human circulating leucocytes

In crude extracts of PBMC, following ion-exchange chromatography (Figure 1A), two major peaks of Ca^{2+} -dependent activity

were detected. These eluted at ionic strengths of about 0.16 and 0.32 M NaCl respectively. No proteolytic activity was detectable in the absence of Ca^{2+} in the assay mixture, indicating that these enzymes belong to the calpain family proteases. In between the two major peaks, at approx. 0.21 M NaCl, a third small peak of Ca^{2+} -dependent proteolytic activity was identified. In these fractions, calpastatin, the natural calpain inhibitor, was also eluted (see Figure 1A).

To separate calpain from calpastatin, these fractions were collected and loaded on to phenyl-Sepharose. As shown in Figure 1(B), calpastatin is not retained by the resin, whereas calpain activity is eluted following washing without NaCl. If this hydrophobic step is performed on PBMC crude extracts before ion-exchange chromatography, three calpain activity peaks can be detected, eluting at the same salt concentrations (Figure 1C). These data indicate that PBMC contain three calpain forms distinguishable on the basis of their chromatographic properties. For comparison, human erythrocyte and neutrophil lysates were subjected to the same chromatographic steps. As shown in Figure 1(D), in both cases a single calpain form was identified, emerging from the column at an NaCl concentration corresponding approximately to that of the second PBMC peak of calpain (see Figure 1C).

Three calpain forms were also detected when PBMC crude extracts were subjected to zymogram analysis in the presence of Ca^{2+} . As shown in Figure 2(A), three different digestion regions were identified, indicating the presence of three distinct Ca^{2+} -dependent enzymes. No digestion was found in the same position when zymogram analysis was performed in the absence of calcium

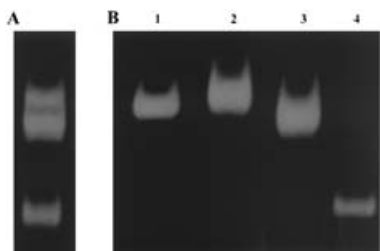


Figure 2 Zymogram analysis on calpains isolated from PBMC and erythrocytes

(A) A sample of PBMC crude extract (4 μ l; see the Experimental section) was loaded on to 12% casein-containing gel. After electrophoresis, the gel was incubated overnight at 25 °C in the presence of 5 mM CaCl_2 . (B) PBMC calpains (2 units), separated by ion-exchange chromatography (see Figure 1C), were subjected to zymography, as in (A): lane 2, calpain peak I; lane 3, calpain peak II; lane 4, calpain peak III. Digestion bands were revealed by destaining the Coomassie Brilliant Blue-stained gel. For comparison, a sample of human erythrocyte lysate (20 μ l) was also processed (lane 1).

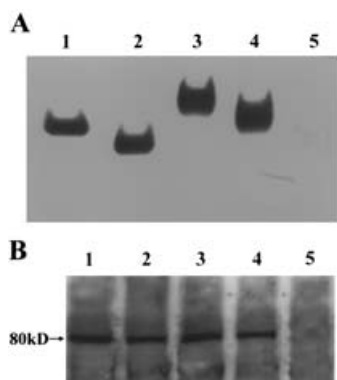


Figure 3 Immunoblot analysis on purified PBMC, erythrocyte and neutrophil calpains

(A) PBMC calpains (2 units), separated by ion-exchange chromatography (see Figure 1C), were loaded on to 12% casein-containing gel, transferred to a nitrocellulose sheet and probed with anti-calpain mAb 56.3. Lane 3, calpain peak I; lane 4, calpain peak II; lane 5, calpain peak III. For comparison, samples of purified erythrocyte (lane 1) and neutrophil calpain (2 units each; lane 2) were also considered. (B) The same samples were loaded on to SDS/PAGE (12% gels) and then subjected to Western-blot analysis as described in (A).

ions (results not shown). As shown in Figure 2(B), the bands identified directly in PBMC crude extracts correspond to the digestion bands of the three isolated calpains (Figure 2B, lanes 2–4) separated as described in Figure 1. As expected, when a sample of human erythrocyte lysate is subjected to the same zymogram analysis, a single digestion band is observed (Figure 2B, lane 1).

To establish whether the bands identified by zymogram analysis contained calpain, as well as the activity peaks eluted from ion-exchange chromatography, Western-blot analysis was also carried out using anti-calpain mAb 56.3. As shown in Figure 3(A), two of the three forms of PBMC calcium-dependent proteases are recognized by this antibody (Figure 3A, lanes 3 and 4). The third enzyme, which shows the highest mobility in zymography, does not interact with mAb 56.3, an antibody directed against the μ -calpain class. Calpains purified from human erythrocytes (lane 1) and human neutrophils (lane 2), both belonging to the μ -calpain subfamily, are recognized by this mAb. The same samples, when subjected to SDS/PAGE and Western-blot analysis using mAb 56.3, revealed a single band at a molecular mass of 80 ± 3 kDa, a

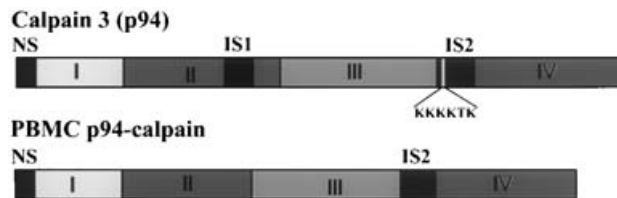


Figure 4 Comparison of the domain structures of p94-calpain and PBMC p94-calpain cloned protein

The amino acid sequence of PBMC p94-calpain deduced from its cDNA was compared with the amino acid sequence of p94-calpain (accession no. AAD28253.1). The whole transcript corresponding to PBMC p94-calpain was amplified, cloned and sequenced as described in the Experimental section. Roman numerals refer to the domains in the calpain catalytic subunits. NS, N-terminal subdomain.

value in agreement with the molecular mass of the large catalytic subunit of calpain (Figure 3B).

Identification of PBMC μ - and p94-calpain sequences

To determine the molecular differences among these calpain isoenzymes, we amplified their cDNA prepared from PBMC. We chose three sets of oligonucleotides to specifically identify and amplify cDNAs for μ -, m- and p94-calpain. Surprisingly, p94-calpain, in addition to μ - and m-calpain, was detected in PBMC. The complete sequences of both μ -calpain and p94-calpain from PBMC were cloned and identified.

The sequence of PBMC μ -calpain deduced from its cDNA is almost identical to that of μ -calpain from skeletal muscle, differing only at positions corresponding to residues 270 and 349, in which Lys and Glu have been replaced by Met and Val respectively. Another modification occurs at position 121, corresponding to an Ile residue, in which the third codon base T is replaced by C, with no change in the coded amino acid residue.

The sequence of PBMC p94-calpain corresponds to a variant of calpain 3 (p94) in which a stretch of 144 bp, corresponding to 48 amino acids, localized in the IS1 region, is deleted. Furthermore, a small Lys-rich sequence composed of 18 bp, present in the IS2 insertion, is also lacking (Figure 4). This enzyme presents the typical calpain structure, particularly in the domain containing the active site and at the level of the calcium-binding calmodulin-like region. Deletions of specific p94 regions have also been observed in the calpains expressed in eye tissues [26,27].

To pick out the p94-calpain form among those detected in PBMC, we analysed the amino acid sequences of the tryptic fragments obtained from the three purified enzymes. We found that one of the tryptic peptides recovered from PBMC calpain peak I (see Figure 1C) has the sequence TAAEPR, which corresponds to residues 13–18 of p94-calpain in the N-terminal region of the enzyme. This peptide is present in neither μ - nor m-calpain. A second tryptic fragment, having the sequence DFFLYNASK, is identical to peptide 523–531 of p94-calpain. This calpain fragment, which is in proximity to the active site, is also present in μ -calpain (residues 463–471), but Tyr and Lys residues are replaced in μ -calpain by Ala and Arg, respectively. These results indicate that calpain activity recovered under peak I (see Figure 1C) contains a p94-like calpain.

Catalytic properties of calpains isolated from PBMC

To further characterize PBMC calpains, we determined their calcium requirement. As shown in Figure 5, PBMC p94-calpain

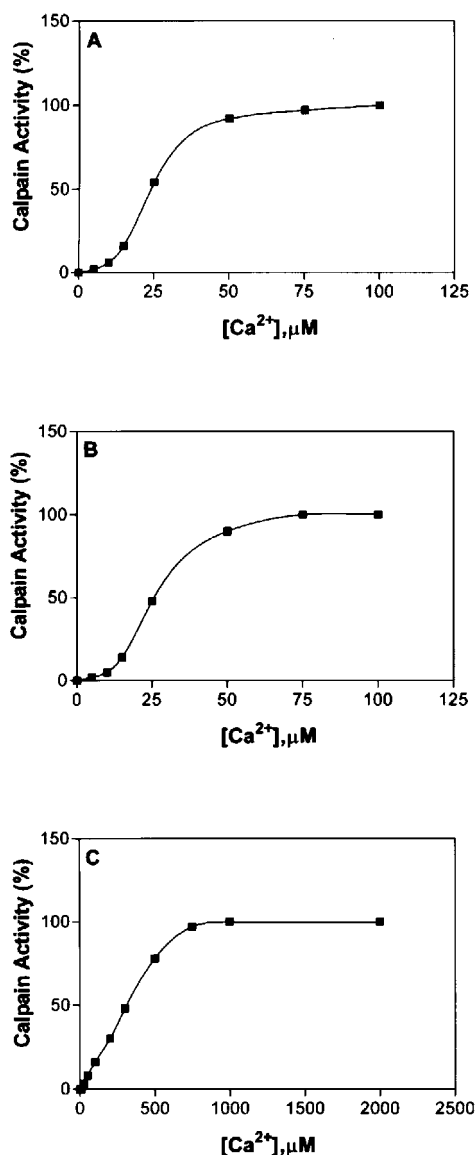


Figure 5 Ca²⁺ requirement of calpains isolated from PBMC

Calpains (5 units) isolated by ion-exchange chromatography (see the Experimental section and Figure 1C) were assayed for 10 min at 25 °C in the presence of increasing calcium concentrations. Calpain activity was determined as described in the Experimental section. (A) PBMC p94-calpain, (B) calpain peak II, (C) calpain peak III.

and calpain peak II (see Figure 1C) are characterized by a similar calcium requirement, having a $K_{0.5}$ between 20 and 30 μM Ca²⁺, in the same range of concentration reported for both human erythrocyte and neutrophil calpain [31,42]. PBMC calpain recovered under the third activity peak (Figure 1C) requires a much higher calcium concentration to express catalytic activity (Figure 5C); $K_{0.5}$ is in the range 0.2–0.3 mM and V_{max} is reached at 0.6–0.8 mM Ca²⁺. These catalytic properties are consistent with those of a calpain isoform belonging to the m-subfamily. These observations also explain why it is not recognized by mAb 56.3 [28].

It is well known that, after exposure to Ca²⁺, μ- and m-calpain forms undergo autodigestion, a process that is followed by the loss of catalytic activity. This step is accompanied by the transient accumulation of a low-molecular-mass 75-kDa form, which displays a greatly reduced calcium requirement [42]. To characterize

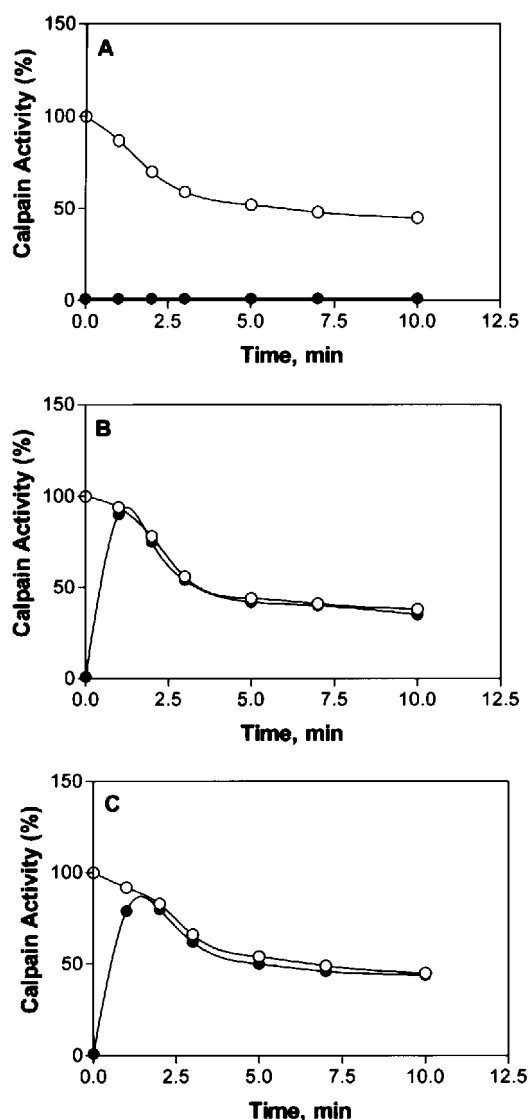


Figure 6 Ca²⁺ requirement of PBMC calpains following exposure to calcium ions

(A) PBMC p94-calpain and (B) PBMC μ-calpain activity were assayed in 5 μM Ca²⁺ (●) and 1 mM Ca²⁺ (○) after incubation in 100 μM CaCl₂ for the indicated times (see also the Experimental section). (C) PBMC m-calpain activity was assayed in 20 μM Ca²⁺ (●) and 1 mM Ca²⁺ (○) after exposure to 400 μM CaCl₂ for the indicated times. All data shown represent at least three experiments with identical results.

these processes, we exposed PBMC calpains to calcium and followed both the loss of total activity and, by measuring their activity at micromolar [Ca²⁺], the appearance of a low-Ca²⁺-requiring form. As shown in Figure 6(A), when PBMC p94-calpain is exposed to 100 μM Ca²⁺, approx. 50% of total activity is lost after 10 min of incubation. However, this autoinactivation process is not accompanied, at any time of incubation, by the appearance of a stable low-Ca²⁺-requiring form. The enzyme recovered from such incubation shows a very low activity at micromolar [Ca²⁺] and a requirement for this metal ion identical to that of the untreated protease. By contrast, PBMC μ-calpain (Figure 6B) shows a very low activity in the presence of 5 μM Ca²⁺ in the native condition (zero time of the experiment) but, following exposure to 100 μM Ca²⁺, its activity in 5 μM Ca²⁺ rapidly increases; after approx.

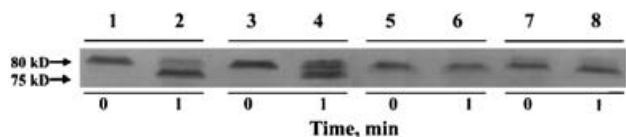


Figure 7 Molecular modifications in calpains exposed to Ca^{2+} ions

Purified calpains (10 units) were incubated in 50 mM sodium borate buffer, pH 7.5, containing 100 μM Ca^{2+} (final volume, 60 μl) in an ice bath. Aliquots (25 μl) were collected at the indicated times and subjected to SDS/PAGE (8.5% gels). The gel was transferred to nitrocellulose and probed with anti-calpain mAb 56.3. Lanes 1 and 2, erythrocyte calpain; lanes 3 and 4, neutrophil calpain; lanes 5 and 6, PBMC p94-calpain; lanes 7 and 8, PBMC μ -calpain.

Table 1 Effect of natural and synthetic inhibitors on calpains isolated from circulating cells

Calpains were isolated from the indicated sources as described in the Experimental section. RNCAS104 was expressed in *Escherichia coli* and purified as described in [29]. Calpain activity (5 units) was assayed as described in the Experimental section, in the presence of increasing concentrations of the inhibitors indicated. IC_{50} refers to the concentration required to promote 50% calpain inhibition. The data shown are the means \pm S.D. from three independent experiments.

Calpain form	Inhibition (IC_{50} ; μM)			
	Leupeptin	Calpain inhibitor I	Calpain inhibitor II	RNCAS104
PBMC p94-calpain	2 ± 0.5	0.25 ± 0.05	0.25 ± 0.05	3.6 ± 0.07
PBMC μ -calpain	2 ± 0.5	0.2 ± 0.05	0.2 ± 0.05	0.48 ± 0.07
Erythrocyte calpain	2.25 ± 0.5	0.2 ± 0.05	0.25 ± 0.05	0.5 ± 0.06
Neutrophil calpain	2 ± 0.5	0.3 ± 0.05	0.25 ± 0.05	0.6 ± 0.07

1 min of incubation, it is identical to that measured in 1 mM Ca^{2+} , the $[\text{Ca}^{2+}]$ necessary to evaluate calpain V_{max} . The accumulation of this low- Ca^{2+} -requiring form is accompanied by an autolytic process which produces the loss of half of the total activity in 10 min. A similar behaviour was observed in PBMC m-calpain (Figure 6C), for which a low- Ca^{2+} -requiring form is also produced during the autolysis process. In this case, too, a 50% loss of total activity was observed after 10 min Ca^{2+} exposure.

Hence, the two PBMC calpains belonging to the μ -calpain subfamily show different behaviours when exposed to calcium ions. To better characterize this difference, these two enzymes were analysed by Western blotting using mAb 56.3. As expected (Figure 7), after a 1-min exposure to calcium ions, the native 80-kDa catalytic subunit in calpains from erythrocytes (Figure 7, lanes 1 and 2) and neutrophils (Figure 7, lanes 3 and 4), and in μ -calpain from PBMC (Figure 7, lanes 7 and 8), was replaced by a low-molecular-mass form with a mobility corresponding to 75 kDa. An intermediate 78-kDa form can be transiently produced during autolysis. However, when PBMC p94-calpain (Figure 7, lanes 5 and 6) is exposed to calcium, no accumulation of low-molecular-mass forms is detected. These observations, which are in agreement with the different catalytic properties described in Figure 6, further support the concept that the activation process of these two PBMC calpains is different.

We also studied their sensitivity to natural and synthetic inhibitors. For comparison, we tested human erythrocyte and neutrophil calpains. All four calpain forms are equally inhibited by leupeptin and calpain inhibitors I and II, with the IC_{50} values differing slightly from one isoform to another (Table 1). However, the sensitivity to recombinant calpastatin (RNCAS104) is signi-

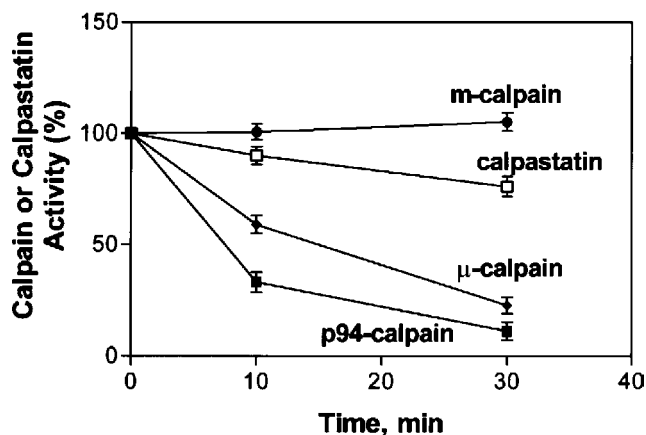


Figure 8 Calpain and calpastatin levels in Ca^{2+} -enriched PBMC

Purified PBMC (6×10^8 cells) were incubated for the indicated times in 1 mM CaCl_2 (final concentration) in the presence of 0.1 μM A23187 (see the Experimental section). Cells were lysed and crude extracts, prepared as described previously, were subjected to Source 15Q and phenyl-Sepharose chromatography. Each data point represents the mean \pm S.D. from three independent experiments.

ficantly lower for PBMC p94-calpain than for the other μ -calpains considered, as this protease shows an IC_{50} 5–6-fold higher than that of the other isoforms. This lower sensitivity to calpastatin inhibition might reflect a different regulatory system operating on this calpain form. However, p94- and μ -calpain from PBMC, together with human erythrocyte calpain, are equally sensitive to both phospholipids [43] and UK114 protein [32], which are known to reduce markedly the calcium requirement of μ -calpain isoforms. Hence, the same activating agents are effective on PBMC p94-calpain, promoting expression of its catalytic activity at physiological $[\text{Ca}^{2+}]$.

Role of p94-calpain in PBMC

To determine whether this newly identified calpain exerts a function in circulating mononuclear cells, we examined its activation *in vivo*, following an increase in intracellular $[\text{Ca}^{2+}]$.

Although it is very difficult to identify the specific activation of a single calpain form in cells expressing multiple protease isoforms, we monitored PBMC p94-calpain autodegradation as a marker of its activation. As shown in Figure 8, the level of p94-calpain activity is reduced by 65% after 10 min of incubation with the Ca^{2+} ionophore A23187. A similar behaviour, albeit less intense (40%), was also observed for μ -calpain. No appreciable loss of m-calpain activity was detected, whereas the calpastatin level was slightly reduced (10%). In these conditions, μ -calpain isoforms are preferentially activated, as indicated by their partial inactivation. Moreover, the decrease in calpastatin levels confirms the activation of this calpain [44]. In these experimental conditions, m-calpain does not yet seem to be significantly involved, as its level is unchanged.

PCR analysis performed on the different cell types isolated from PBMC revealed that PBMC p94-calpain is mostly expressed in T- and B-lymphocytes, whereas it is poorly detectable in natural killer cells and monocytes (Figure 9). No amplification of p94-calpain was detectable in neutrophil cDNA in these conditions. This selective distribution might be related to a specific function of this protease isoform in cells involved in the immune response.

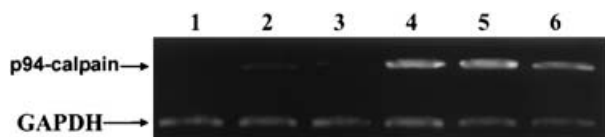


Figure 9 Detection of calpain 3 transcript in different blood cell populations

Equal amounts of cDNA from neutrophils (lane 1), natural killer cells (lane 2), monocytes (lane 3), B-lymphocytes (lane 4), T-lymphocytes (lane 5) and PBMC (lane 6) were subjected to 30 cycles of amplification as described in the Experimental section, using primers for both p94-calpain and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). After PCR, the samples were loaded on to a 1% agarose gel. The amplification products were visualized by ethidium bromide staining. The different blood cell populations isolated to homogeneity were kindly provided by Professor C. Mingari.

DISCUSSION

We report that, in addition to the classical ubiquitous μ - and m-calpain, PBMC contain a third Ca^{2+} -dependent protease in an amount comparable with that of μ -calpain. This newly isolated calpain isoform is characterized by catalytic properties similar to those of μ -calpain, but differs in its activation process and regulation mechanism. Having sequenced the cDNA coding for this protein, we observed that it is not a member of the ubiquitous calpain family, as might be suggested by its catalytic properties. Clearly, it is produced by alternative splicing of the CAPN3 gene, coding for p94-calpain [5,22,45], by the removal of exons 6 and 15. Exon 6 corresponds to the IS1 insertion, which is localized in catalytic subdomain IIb, whereas exon 15, containing five Lys residues, is a nuclear import sequence [1,4]. Since the role of this sequence in p94-calpain has not yet been well identified, we have no information about the effect of its absence on this PBMC calpain form. However, it can be postulated that this modification may be necessary to present the protease to cell compartments other than the nucleus.

The expression of different calpain isoforms due to alternative splicing has also been found in retina and lens [7,46], producing active protease forms that are probably adapted to selective roles. Lens calpain lacks both IS1 and IS2, and retina calpain lacks IS2; both enzymes are devoid of a nuclear translocation signal. We have demonstrated that this mechanism also operates in PBMC, in which it produces a new, differently spliced, p94-calpain. These data suggest that alternative splicing may occur in different cells or tissues to produce new and specific calpain forms that are better adapted to selective functions.

PBMC p94-calpain also displays typical catalytic and regulatory properties, which are distinguishable from those of μ - and m-calpain. Once activated, this calpain isoenzyme does not produce any low- Ca^{2+} -requiring form, suggesting that its activated state is reached through a reversible Ca^{2+} -induced conformational transition followed, as for many other proteases, by inactivating autolysis. The absence of the low- Ca^{2+} -requiring form in the activation mechanism of this p94-calpain could explain why, in this case, calpastatin is not so efficient. As the calpain inhibitor is particularly effective against the low- Ca^{2+} -requiring forms of calpain [47], it is reasonable to think that calpastatin is more functionally active on those calpain isoenzymes that accumulate autolysed forms active at low physiological $[\text{Ca}^{2+}]$.

A striking aspect of the presence of this new calpain form in circulating cells is seen in its peculiar distribution in the various white cell types. Indeed, PBMC p94-calpain is preferentially expressed in B- and T-lymphocytes, rather than in natural killer cells or neutrophils. This expression pattern, together with the indication that the enzyme becomes activated in cells following an

increase in free Ca^{2+} and that it is not consistently affected by an excess of intracellular calpastatin, strongly suggests the involvement of the enzyme in the multiple functions promoted in B and T lymphocytes during the development of the humoral immune response. The virtual absence of this p94-like calpain in killer cells and phagocytes suggests that it is not required for their specific functions. Further experiments are in progress to clarify these aspects.

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REFERENCES

- Huang, Y. and Wang, K. K. W. (2001) The calpain family and human disease. *Trends Mol. Med.* **7**, 355–362
- Strobl, S., Fernandez-Catalan, C., Braun, M., Huber, R., Masumoto, H., Nakagawa, K., Irie, A., Sorimachi, H., Bourenkow, G., Bartunik, H. et al. (2000) The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 588–592
- Dutt, P., Spriggs, C. N., Davies, P. L., Jia, Z. and Elce, J. S. (2002) Origins of the difference in Ca^{2+} requirement for activation of μ - and m-calpain. *Biochem. J.* **367**, 263–269
- Sorimachi, H., Ishiura, S. and Suzuki, K. (1997) Structure and physiological function of calpains. *Biochem. J.* **328**, 721–732
- Sorimachi, H., Imajoh-Ohmi, S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y. and Suzuki, K. (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and μ -types. Specific expression of the mRNA in skeletal muscle. *J. Biol. Chem.* **264**, 20106–20111
- Sorimachi, H., Ishiura, S. and Suzuki, K. (1993) A novel tissue-specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca^{2+} -binding domain. *J. Biol. Chem.* **268**, 19476–19482
- Nakajima, T., Fukuiage, C., Azuma, M., Ma, H. and Shearer, T. R. (2001) Different expression patterns for ubiquitous calpains and Capn3 splice variants in monkey ocular tissues. *Biochim. Biophys. Acta* **1519**, 55–64
- Imajoh, S., Aoki, K., Ohno, S., Kawasaki, H., Sugihara, H. and Suzuki, K. (1988) Molecular cloning of the cDNA for the large subunit of the high- Ca^{2+} -requiring form of human Ca^{2+} -activated neutral protease. *Biochemistry* **27**, 8122–8128
- Reverter, D., Sorimachi, H. and Bode, W. (2001) The structure of calcium-free human m-calpain: implications for calcium activation and function. *Trends Cardiovasc. Med.* **11**, 222–229
- Elce, J. S., Hegadorn, C. and Arthur, J. S. (1997) Autolysis, Ca^{2+} -requirement and heterodimer stability in m-calpain. *J. Biol. Chem.* **272**, 11268–11275
- Moldoveanu, T., Hosfield, C. M., Lim, D., Elce, J. S., Jia, Z. and Davies, P. L. (2002) A Ca^{2+} switch aligns the active site for calpain. *Cell* **108**, 649–660
- Reverter, D., Strobl, S., Fernandez-Catalan, C., Sorimachi, H., Suzuki, K. and Bode, W. (2001) Structural basis for possible calcium-induced activation mechanisms of calpains. *Biol. Chem.* **382**, 753–766
- Dainese, E., Minafra, R., Sabatucci, A., Vachette, P., Melloni, E. and Cozzani, I. (2002) Conformational changes of calpain from human erythrocytes in the presence of Ca^{2+} . *J. Biol. Chem.* **277**, 40296–40301
- Pal, G. P., Elce, J. S. and Jia, Z. (2001) Dissociation and aggregation of calpain in the presence of calcium. *J. Biol. Chem.* **276**, 47233–47238
- Suzuki, K. and Sorimachi, H. (1998) A novel aspect of calpain activation. *FEBS Lett.* **433**, 1–4
- Nakagawa, K., Masumoto, H., Sorimachi, H. and Suzuki, K. (2001) Dissociation of m-calpain subunits occurs after autolysis of the N-terminus of the catalytic subunit and is not required for activation. *J. Biochem. (Tokyo)* **130**, 605–611
- Wang, K. K. W. (2000) Calpain and caspase: can you tell the difference? *Trends Neurosci.* **23**, 20–26
- Carragher, N. O. and Frame, M. C. (2002) Calpain: a role in cell transformation and migration. *Int. J. Biochem. Cell. Biol.* **34**, 1539–1543
- Carafoli, E. and Molinari, M. (1998) Calpain: a protease in search of a function? *Biochem. Biophys. Res. Commun.* **247**, 193–203
- Lu, T., Xu, Y., Mericle, M. T. and Mellgren, R. L. (2002) Participation of conventional calpains in apoptosis. *Biochim. Biophys. Acta* **1590**, 16–26
- Azam, M., Andradi, S. S., Sahr, K. E., Kamath, L., Kuliopulos, A. and Chisti, A. H. (2001) Disruption of the μ -calpain gene reveals an essential role in platelet function. *Mol. Cell. Biol.* **21**, 2213–2220

- 22 Kinbara, K., Shoichi, I., Tomioka, S., Sorimachi, H., Jeong, S. Y., Amano, S., Kawasaki, H., Kolmerer, B., Kimura, S., Labeit, S. and Suzuki, K. (1998) Purification of native p94, a muscle specific calpain, and characterisation of its autolysis. *Biochem. J.* **335**, 589–596
- 23 Rey, M. A. and Davies, P. L. (2002) The protease core of the muscle-specific calpain p94 undergoes Ca^{2+} -dependent intramolecular autolysis. *FEBS Lett.* **532**, 401–406
- 24 Richard, I., Bronx, O., Allamand, V., Fougerousse, F., Chiannikulchai, N., Bourg, N., Brenguier, L., Devaud, C., Pasturaud, P., Roudaut, C. et al. (1995) Mutations in the proteolytic enzyme calpain 3 causes limb-girdle muscular dystrophy type 2A. *Cell* **81**, 27–40
- 25 Ono, Y., Shimada, H., Sorimachi, H., Richard, I., Saido, T. C., Beckmann, J. S., Ishiura, S. and Suzuki, K. (1998) Functional defects of a muscle-specific calpain p94 caused by mutations associated with limb-girdle muscular dystrophy type 2A. *J. Biol. Chem.* **273**, 17073–17078
- 26 Azuma, M., Fukiage, C., Higashine, M., Nakajima, T., Ma, H. and Shearer, T. R. (2000) Identification and characterisation of a retina-specific calpain (Rt88) form rat. *Curr. Eye Res.* **21**, 710–720
- 27 Ma, H., Fukiage, C., Azuma, M. and Shearer, T. R. (1998) Cloning and expression of mRNA for calpain Lp82 from rat lens: splice variant of p94. *Invest. Ophthalmol. Vis. Sci.* **39**, 454–461
- 28 Pontremoli, S., Melloni, E., Damiani, G., Salamino, F., Sparatore, B., Michetti, M. and Horecker, B. L. (1988) Effects of a monoclonal anti-calpain antibody on responses of stimulated human neutrophils. Evidence for a role for proteolytically modified protein kinase C. *J. Biol. Chem.* **263**, 1915–1919
- 29 Averna, M., De Tullio, R., Salamino, F., Melloni, E. and Pontremoli, S. (1999) Phosphorylation of rat brain calpastatins by protein kinase C. *FEBS Lett.* **450**, 13–16
- 30 Michetti, M., Salamino, F., Tedesco, I., Averna, M., Minafra, R., Melloni, E. and Pontremoli, S. (1996) Autolysis of human erythrocyte calpain produces two active enzyme forms with different cell localisation. *FEBS Lett.* **392**, 11–15
- 31 Pontremoli, S., Sparatore, B., Salamino, F., Michetti, M., Sacco, O. and Melloni, E. (1985) Reversible activation of human neutrophil calpain promoted by interaction with plasma membranes. *Biochem. Int.* **11**, 35–44
- 32 Melloni, E., Michetti, M., Salamino, F. and Pontremoli, S. (1998) Molecular and functional properties of a calpain activator protein specific for μ -isoforms. *J. Biol. Chem.* **273**, 12827–12831
- 33 Boyum, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. *Scan. J. Clin. Lab. Invest.* **21** (suppl. 97), 77–89
- 34 Beutler, E., West, C. and Bluma, K. G. (1976) The removal of leukocytes and platelets from whole blood. *J. Lab. Clin. Med.* **88**, 328–333
- 35 Pontremoli, S., Melloni, E., Viotti, P. L., Michetti, M., Salamino, F. and Horecker, B. L. (1991) Identification of two calpastatin forms in rat skeletal muscle and their susceptibility to digestion by homologous calpains. *Arch. Biochem. Biophys.* **288**, 646–652
- 36 Salamino, F., Averna, M., Tedesco, I., De Tullio, R., Melloni, E. and Pontremoli, S. (1997) Modulation of rat brain calpastatin efficiency by post-translational modifications. *FEBS Lett.* **412**, 433–438
- 37 Simon, J., Arthur, C., Mykles, D. L. and Donald, L. (2000) Calpain zymography with casein or fluorescein isocyanate casein. In *Calpain Methods Protocols* (Elce, J. S., ed.), pp. 109–116, Humana Press, Totowa, NJ
- 38 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 39 Towbin, J., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- 40 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- 41 Stone, K. L. and Williams, K. (1993) Enzymatic digestion of protein and HPLC peptide isolation. In *A Practical Guide to Protein and Peptide Purification for Microsequencing*, 2nd edn (Matsudaia, P., ed.), Academic Press, San Diego
- 42 Pontremoli, S. and Melloni, E. (1986) Regulation of Ca^{2+} -dependent proteinase of human erythrocytes. In *Calcium and Cell Function*, vol. 6 (Cheung, W. Y., ed.), pp. 159–183, Academic Press, Orlando, FL
- 43 Pontremoli, S., Melloni, E., Sparatore, B., Salamino, F., Michetti, M., Sacco, O. and Horecker, B. L. (1985) Role of phospholipids in the activation of the Ca^{2+} -dependent neutral proteinase of human erythrocytes. *Biochem. Biophys. Res. Commun.* **129**, 389–395
- 44 De Tullio, R., Averna, M., Salamino, F., Pontremoli, S. and Melloni, E. (2000) Differential degradation of calpastatin by μ - and m-calpain in Ca^{2+} -enriched human neuroblastoma LAN-5 cells. *FEBS Lett.* **475**, 17–21
- 45 Herasse, M., Ono, Y., Fougerousse, F., Kimura, E., Stockholm, D., Beley, C., Montarras, D., Pinset, C., Sorimachi, H., Suzuki, K. et al. (1999) Expression and functional characteristics of calpain 3 isoforms generated through tissue-specific transcriptional and post-transcriptional events. *Mol. Cell. Biol.* **19**, 4047–4055
- 46 Fukiage, C., Nakajima, E., Ma, H., Azuma, M. and Shearer, T. R. (2002) Characterization and regulation of lens-specific calpain Lp82. *J. Biol. Chem.* **277**, 20678–20685
- 47 Melloni, E., Michetti, M., Salamino, F., Minafra, R. and Pontremoli, S. (1996) Modulation of the calpain proteolysis by calpastatin and phospholipids. *Biochem. Biophys. Res. Commun.* **229**, 193–197

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