Biologically active monomeric and heterodimeric recombinant human calpain I produced using the baculovirus expression system

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Calpain I is a heterodimeric protein that is part of a family of calcium-activated intracellular cysteine proteases presumed to play a role in mediating signals transduced by calcium. Expression of bioactive recombinant human calpain I has been achieved using the baculovirus expression system, by either co-infection with two viruses, each expressing one of the subunits, or infection with a single virus containing both subunits. The ~80 kDa catalytic subunit exhibited calcium-dependent proteolytic activity when expressed alone or with the ~30 kDa regulatory subunit. Baculoviral recombinant calpain I appeared fully active in that the catalytic subunit in unpurified cell extracts exhibited calcium-

dependent autocatalytic cleavage at the correct locus. The amount of ~80 kDa subunit accumulated at steady state was greatly increased by co-expression of the ~30 kDa subunit, suggesting a possible role for enzyme stabilization by the latter subunit. The recombinant human calpain I was purified to near homogeneity and compared with purified native human erythrocyte calpain I. The recombinant and native enzymes had equivalent inhibition constants for structurally diverse calpain inhibitors, identical calcium activation profiles, and similar specific activities, demonstrating the suitability of using the recombinant protein for studies of the native enzyme.

INTRODUCTION

Calpains are a family of non-lysosomal calcium-dependent neutral cysteine proteases represented in essentially all types of vertebrate cells [1]. Two major forms are widely distributed and are referred to as calpain I (or μ -calpain) and calpain II (or m-calpain). Both proteases are heterodimers, each consisting of ~80 kDa catalytic subunits that are the products of separate genes [2,3] and a ~30 kDa subunit that is identical in both enzymes [4,5]. Whereas free calcium is obligatory for the activation of both enzymes, calpain I is the more sensitive variant, with the isolated enzyme being half-maximally activated at low micromolar calcium concentrations, about an order of magnitude lower than those concentrations required to activate calpain II. The calcium sensitivity and specific activity of both major forms are regulated by membrane phospholipids [6–8], and calcium-induced translocation to the membrane may be part of a physiological mechanism for activation [9,10]. Recently, molecular cloning techniques have identified additional calpain isozymes with highly restricted tissue distributions, but whose proteolytic properties have not yet been characterized extensively [11,12].

Upon activation, calpains I and II produce limited cleavage of certain biologically important proteins, and in so doing may regulate key physiological and pathological processes. Among preferred calpain substrates are cytoskeletal proteins, signal-transducing enzymes, transcriptional regulatory factors, and integral membrane proteins [13,14]. Calpain activation and a concomitant limited protein degradation have been studied extensively in the nervous system in response to stimuli that raise intraneuronal calcium levels and provoke neuronal degeneration [15,16]. These studies support the hypothesis that the sustained

activation of calpain, particularly the calpain I form with a high sensitivity to calcium, provides an essential link between intraneuronal calcium overload and neuronal damage [17–19].

In view of its potential biological importance, mechanisms for the assembly, activation and regulation of calpain I are of considerable interest, but at present are only partially understood. Regions of both subunits have been implicated in phospholipid interaction and membrane translocation [8,20]. Upon activation, both subunits undergo limited autolysis that removes N-terminal domains and increases calcium sensitivity. The role of subunit autolysis in activation and the proteolytic activity intrinsic to the unmodified enzyme have been subjects of controversy [21–24], but kinetic study of calpain I indicates that autolysis of the catalytic subunit precedes substrate hydrolysis and autolysis of the ~30 kDa subunit [25]. Autolysis is insufficient to activate calpain I, since the autolysed protease still requires calcium to cleave substrates. Moreover, substrate-mimetic-type inhibitors and cysteine-modifying reagents react with the active site of calpain I only after calcium activation [26,27]. These findings indicate that calcium induces a conformational change to expose the active site, but do not elucidate its structural basis. Each of the ~80 kDa and ~30 kDa subunits possesses multiple motifs consistent for calcium binding [10], and the domains that trigger calcium-induced autolysis and activation have not been identified. Furthermore, the structural features that mediate heterodimer assembly are unknown. Finally, calpains I and II are inhibited by a co-expressed endogenous protein, calpastatin [28–30]; the domain responsible for calcium-dependent calpastatin binding has not been pinpointed.

One factor that has limited molecular dissection of mechanisms for calpain assembly, activation and regulation has been the unavailability of biologically active recombinant protease. We

Abbreviations used: rhcalpain I, recombinant human calpain I; calpain inhibitor I, acetyl-Leu-Leu-Nle-H; Z, benzyloxycarbonyl; Ep-460, (2S,3S)-epoxysuccinyl-Leu-NH(CH₂)₄NH-Z; Suc-Leu-Tyr-MNA, succinyl-Leu-Tyr-4-methoxy-2-naphthylamine; MOI, multiplicity of infection.

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describe here expression, purification and characterization of proteolytically active recombinant human calpain I heterodimer (rhcalpain I) by the baculovirus system in insect cells. Additionally, the two subunits have been expressed individually in the absence of any heterodimer formation. The rhcalpain I has been compared with native human erythrocyte calpain I with respect to calcium sensitivity, specific proteolytic activity, autolysis and sensitivity to protease inhibitors.

EXPERIMENTAL

Construction of baculovirus vectors

To construct the recombinant calpain I baculoviruses, partial cDNA probes for both the ~30 kDa and the ~80 kDa subunits were prepared by PCR amplification from a human spleen λ gt10 cDNA library (no. HL1134a; Clontech, Palo Alto, CA, U.S.A.) and used to screen that library by standard techniques [31]. Phage containing most of the coding region of each subunit were isolated, and any regions not present in the isolated clones were PCR amplified from the library, sequence verified, and attached to the partial clones to produce the entire calpain coding region as designated in Figure 1(a). Both cDNAs were also modified to add the 5' untranslated sequence CCTATAAAT from the polyhedrin gene immediately 5' to the start codon of the cDNA, to change the stop codon to the baculovirus-preferred TAA [32] and to add *Bam*HI restriction sites flanking the entire cDNA (Figure 1a). These cDNAs were then used to construct the three different baculovirus transfer vectors shown in Figure 1(b). Each of the cDNAs encoding the ~30 kDa subunit or the ~80 kDa

subunit was separately inserted into the *Bam*HI site of pVL941 [33] (kindly provided by Dr. M. Summers, Texas A&M University, College Station, TX, U.S.A.) to generate viruses expressing the individual subunits. For the double-recombinant virus, the *Bam*HI fragment containing the ~30 kDa cDNA was inserted into the *Bgl*II site of pAcUW51 (Pharmingen, San Diego, CA, U.S.A.) and the ~80 kDa cDNA was inserted into the *Bam*HI site. These vectors were then used to prepare recombinant viruses using the method outlined below to express either the individual subunits separately (AcNPV-hCANP30 and AcNPV-hCANP180, expressing the ~30 kDa or the ~80 kDa calpain I subunits respectively) or both subunits in the same virus (AcNPV-hCANPI-2-5; Figure 1b).

Insect cell culture

Spodoptera frugiperda cells (Sf21) [34] were grown in suspension as described previously [35]. Some experiments were conducted with Sf21 cells grown in suspension at 27 °C in Ex-Cell 401 serum-free medium (JRH Biosciences, Lenexa, KS, U.S.A.) after gradual adaptation from the serum-containing medium.

Vector virus production

Recombinant viruses were produced by co-transfecting Sf21 cells in monolayer with 2 μ g of one of the above transfer vectors and 0.5 μ g of Baculogold linearized *Autographa californica* nuclear polyhedrosis viral DNA (Pharmingen, San Diego, CA, U.S.A.) using Insectin liposomes (InVitrogen, San Diego, CA, U.S.A.) by following the manufacturer's protocol.

Figure 1 Scheme for cloning of human calpain I subunits and construction of the recombinant baculoviruses used for expression

Both subunits of human calpain I were cloned from a human spleen cDNA library. (a) Cloning strategy, designating those regions cloned by PCR amplification and those derived from a λ cDNA library screen. Nucleotide modifications to the 5' and 3' ends of the cDNA are noted above each map. (b) Diagrammatic representation of the three recombinant calpain I baculoviruses produced for calpain I expression. The baculovirus promoter directing the expression of the particular calpain subunit is shown, being either the polyhedrin (polyHn) or the p10 very late promoters.

Characterization of proteins produced by virally infected cells

Sf21 cells were seeded in 24-well plates at 1.5×10^5 cells/cm², virus was added after cell attachment at a multiplicity of infection (MOI) of 5, and the cells were harvested 40–50 h after infection. Extracts solely for immunoblot analysis were prepared by suspending the cells in 50 mM Tris/10 mM EDTA/0.1 mM PMSF/1 μ g/ml leupeptin/0.1% NP-40, pH 7.4, followed by centrifugation at 14000 *g* for 10 min at 4 °C. Supernatants were stored at –70 °C prior to analysis. Total protein levels were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL, U.S.A.) with BSA as a standard.

To maintain enzymic activity, cells were homogenized in 50 mM Tris/50 mM NaCl/1 mM EDTA/1 mM EGTA/5 mM β -mercaptoethanol/0.1 mM PMSF/0.1% Triton X-100, pH 7.5, followed by a 10 min centrifugation at 14000 *g* at 4 °C. Supernatants were stored at 4 °C before the assay. Total protein levels of these samples were determined using the Bradford protein assay (BioRad, Melville, NY, U.S.A.) with BSA as a standard.

In vitro autolytic activation of enzymically active preparations for immunoblot analysis was carried out by incubation with or without 6.7 mM CaCl₂ for 5 min at room temperature. Reactions were terminated by the addition of 6.7 mM EDTA and SDS/PAGE gel-loading buffer, immediately followed by a 5 min incubation in boiling water.

Antibodies

Rabbit polyclonal antisera recognizing either primarily the large (~80 kDa) subunit or both subunits of human calpain I were prepared using native human calpain I purified from erythrocytes as the immunogen [36] (Ab#4 and Ab#6 respectively). Polyclonal anti-peptide serum to detect the autolytically activated form of the large subunit (~76 kDa polypeptide) was prepared as described by Saido et al. [37] using as the immunogen the synthetic peptide Leu-Gly-Arg-His-Glu-Cys, which are the first five amino acids of the N-terminus of the ~76 kDa polypeptide (Ab#34). This peptide was conjugated to keyhole limpet haemocyanin via the C-terminal cysteine using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce, Rockford, IL, U.S.A.). Immunoblot analysis of calpain polypeptides was carried out according to the method of Siman and Noszek [36], using the described antisera at a 1:1000 dilution.

Enzymic calpain assay

In vitro calpain I enzymic activity was measured in a 96-well format using a continuous fluorimetric assay. In this assay, hydrolysis of succinyl-Leu-Tyr-4-methoxy-2-naphthylamine (Suc-Leu-Tyr-MNA; Enzyme Systems Products, Dublin, CA, U.S.A.; $K_m = 0.4$ mM) was monitored by a Fluoroskan II fluorimeter (Labsystems, Helsinki, Finland). Enzyme activity was determined by measuring the calcium-dependent increase in fluorescence at 430 nm ($\lambda_{\text{excitation}} = 340$ nm) of 0.2–1 mM substrate in 0.2 ml total volume of 50 mM Tris/HCl, pH 7.5, containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM β -mercaptoethanol, 2.5% DMSO and 5 mM CaCl₂.

Production and purification of rhcalpain I

To produce rhcalpain I for purification, suspension cultures of serum-free-adapted Sf21 cells at $1.5\text{--}2 \times 10^6$ cells/ml in ExCell 401 (JRH Biosciences, Lenexa, KS, U.S.A.) were infected at an MOI of 2.0 with AcNPV-hCANPI-2-5 and harvested 40–42 h after infection by centrifugation at 228 *g*. Pelleted cells were resuspended in Hepes buffer (10 mM Hepes, 2 mM EDTA,

2 mM EGTA, 5 mM β -mercaptoethanol, pH 7.5), with 0.1 mM PMSF, 5 μ M pepstatin A and 10 μ g/ml aprotinin added just before use, then broken open with 10–12 strokes in a Dounce homogenizer (Wheaton, Millville, NJ, U.S.A.). This extract was centrifuged at 2100 *g* for 10 min, followed by re-centrifugation of the supernatant solution at 38700 *g* for 1 h. The supernatant solution from the latter centrifugation step was subjected to a 30–45% ammonium sulphate precipitation. The resulting protein pellet was resuspended in Hepes buffer with 10 mM NaCl, dialysed overnight against the same buffer, then loaded onto a Q-Sepharose Fast Flow column (Pharmacia, Piscataway, NJ, U.S.A.) pre-equilibrated with that buffer. After washing with Hepes buffer with 10 mM NaCl, rhcalpain I was eluted from the column with Hepes buffer containing 200 mM NaCl. Fractions containing rhcalpain I in this and subsequent steps were pooled on the basis of SDS/PAGE and enzymic activity. The salt concentration of the pool was increased to 500 mM with a 5 M stock of NaCl before loading on to a phenyl-Sepharose CL-4B column (Pharmacia, Piscataway, NJ, U.S.A.) pre-equilibrated with Hepes buffer containing 500 mM NaCl. After washing with the equilibration buffer, the bound protein was eluted with Hepes buffer (no NaCl). The ionic strength of the pooled fractions (measured as specific conductance) was adjusted to 2.5 mMhos by the addition of Hepes buffer. The material was then loaded on to a Mimetic Red 2 column (American International Chemical, Natick, MA, U.S.A.) pre-equilibrated with Hepes buffer containing 10 mM NaCl. After washing the column with the equilibration buffer, the bound protein was eluted in a single step with Hepes buffer containing 60 mM NaCl.

Purification of human erythrocyte calpain I

Calpain I was purified from human red blood cells using a modification of the method described by Lee et al. [38]. All chromatographic media used in this procedure were from Pharmacia (Piscataway, NJ, U.S.A.). Packed red blood cells were washed three times in 0.85% saline, lysed in Buffer A (20 mM Tris/HCl, 1 mM EDTA, 1 mM EGTA, 5 mM β -mercaptoethanol, pH 7.5) containing 1 mM PMSF and 10 μ M pepstatin A, then centrifuged for 1 h at 23400 *g*. The supernatant was fractionated on a Q-Sepharose Fast Flow column using a 50–300 mM NaCl linear gradient in Buffer A. Fractions containing calpain were detected by immunoblot analysis, pooled, then concentrated by ammonium sulphate precipitation (30–45%). The precipitate was resuspended in buffer A with 50 mM NaCl, dialysed overnight against Buffer A with 50 mM NaCl, then applied to a S300 gel filtration column equilibrated in the same buffer. The salt concentration of the pooled S300 eluant was increased to 500 mM, loaded on to a phenyl-Sepharose CL-4B column equilibrated with buffer A with 500 mM NaCl, then eluted with a linear 500–0 mM NaCl gradient. Pooled peak fractions were dialysed against buffer A with 25 mM NaCl and refractionated on a Q-Sepharose Fast Flow column using a linear 50–300 mM NaCl gradient in buffer A. Final purity was estimated to be 85% by Coomassie-Blue-stained SDS/PAGE. Native calpain purified through the Q-Sepharose step was used for studies of specific activities and calcium sensitivities. Material purified through the S300 step or further was used for inhibitor analyses.

Enzyme inhibition analysis

All inhibition analyses were performed using 0.2 mM Suc-Leu-Tyr-MNA substrate with approximately 10 nM enzyme. Enzyme concentration was determined by active site titration [39] using either *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane

(E-64) or (2*S*,3*S*)-epoxysuccinyl-Leu-NH(CH₂)₄NH-Z (where Z is benzyloxycarbonyl) (Ep-460). For reversible inhibitors, 40-fold-concentrated stock solutions in DMSO or enzyme assay buffer (calpastatin domain I only) were added to an enzyme/substrate mixture prior to initiating the reaction with 5 mM CaCl₂. Inhibition of enzyme activity was calculated as the percentage decrease in the rate of substrate hydrolysis in the presence of inhibitor (v_i) relative to the rate in its absence (v_o). Comparison between v_o and v_i was made within the linear range for substrate hydrolysis. The K_i was calculated as $IC_{50}/(1 + [S]/K_m)$ [40]. For irreversible inhibitors, second-order rate constants for inactivation were determined by analysis of progress curves obtained in the presence of substrate and inhibitor [41]. Reactions were performed in single cuvettes, with the increase in fluorescence recorded continuously by a Perkin-Elmer LS50B spectrofluorimeter (Norwalk, CT, U.S.A.) and were monitored until there was no further product generated in inhibitor-containing assays. Inhibitor concentrations were at least 10-fold greater than the ~ 10 nM enzyme concentration in all cases. Values of k_{obs} , the pseudo first-order rate constant for inactivation, were calculated from plots of fluorescence versus time by non-linear regression (Sigma Plot) to the exponential eqn. (1) [42]:

$$y = Ae^{-k_{obs}t} + B \quad (1)$$

where y is the fluorescence at time t (F_t), A is the amplitude of the reaction $F_0 - F_\infty$, and B is the maximal amount of product formed when the enzyme is completely inactivated (F_∞). The apparent second-order rate constant for inactivation was calculated as $(k_{obs}/[I]) \times (1 + [S]/K_m)$, correcting for the effect of substrate on the inactivation rate.

Preparation of calpain inhibitors

Acetyl-Leu-Leu-Nle-H (calpain inhibitor I) was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Purified recombinant human calpastatin domain I was obtained from Calbiochem (La Jolla, CA, U.S.A.). In the following procedures, organic extracts were dried over MgSO₄. Chromatography was performed on flash silica gel.

Ep-460 [43]

Benzyl chloroformate (9.5 ml) was added over 15 min to 1,4-butanediamine (6.33 g) in chloroform (80 ml) at 0 °C, resulting in immediate precipitation. After 1 h at 0 °C and 12 h at 20 °C, water (30 ml) and conc. HCl were added to pH ~ 1 . The chloroform layer was extracted with 2×10 ml 1 M HCl. The combined acidic layers, made basic with 10 M NaOH (pH ~ 12), were extracted with CH₂Cl₂ to afford H₂N(CH₂)₄NH-Z (1.71 g, 11 % yield). NMR δ (p.p.m.) [(²H)chloroform] 1.15 (2 H, broad s), 1.52 (4 H, m), 2.72 (2 H, t), 3.23 (2 H, q), 5.03 (1 H, broad s), 5.11 (2 H, s), 7.36 (5 H, s).

1,3-Dicyclohexylcarbodi-imide (1.59 g) was added to a solution of *t*-butoxycarbonyl-Leu-OH-H₂O (1.92 g), H₂N(CH₂)₄NH-Z (1.71 g) and 1-hydroxybenzotriazole-H₂O (1.19 g) in CH₂Cl₂ (40 ml) at 0 °C. After 1 h at 0 °C and 17 h at 20 °C, the filtrate was rinsed with 1 M HCl, water, satd. Na₂CO₃ and satd. NaCl to give *t*-butoxycarbonyl-Leu-NH(CH₂)₄NH-Z (3.38 g, 100 % yield) as an off-white solid (m.p. 85–87 °C). NMR δ (p.p.m.) [(²H)chloroform] 0.94 (6 H, 2d), 1.45 (9 H, s), 1.54 (4 H, m), 1.68 (3 H, m), 3.22 (4 H, m), 4.05 (1 H, m), 4.98 (1 H, broad s), 5.12 (2 H, s), 6.28 (1 H, broad s), 7.38 (5 H, s).

t-Butoxycarbonyl-Leu-NH(CH₂)₄NH-Z (3.38 g) in ethyl acetate (35 ml) was treated with HCl (gas) to give Leu-NH(CH₂)₄NH-Z HCl (2.46 g, 85 % yield) as a white solid.

Leu-NH(CH₂)₄NH-Z HCl and monoethyl *trans*-(*S*,*S*)-2,3-oxiranedicarboxylate were coupled with 1,3-dicyclohexylcarbodi-imide, 1-hydroxybenzotriazole, and *N,N*-diisopropylethylamine by the above procedure. Chromatography (CH₂Cl₂/ether, 50:50) gave the ethyl ester of Ep-460 as an off-white solid (33 % yield); m.p. 139–143 °C; NMR δ (p.p.m.) [(²H)chloroform] 0.94 (6 H, 2d), 1.32 (3 H, t), 1.5–1.8 (8 H, m), 3.45 (4 H, m), 3.46 (1 H, d), 3.69 (1 H, d), 4.27 (2 H, 10 line m), 4.48 (1 H, q), 4.90 (1 H, m), 5.11 (2 H, s), 6.29 (1 H, m), 6.59 (1 H, d), 7.37 (5 H, s).

This ethyl ester (470 mg) was hydrolysed for 2.5 h with KOH (1.45 equiv.) in ethanol. The ethanol was evaporated, and the residue suspended in water was extracted three times with ether. The acidified (pH 1.5) aqueous phase was extracted three times with ethyl acetate to give Ep-460 (400 mg, 90 % yield) as a pale yellow solid; m.p. 100–104 °C; NMR δ (p.p.m.) [(²H)chloroform] 0.92 (6 H, 2d), 1.52 (4 H, m), 1.62 (3 H, m), 3.40 (4 H, m), 3.57 (1 H, d), 3.65 (1 H, d), 4.55 (1 H, q), 5.10 (2 H, s), 5.15 (1 H, m), 7.24 (2 H, m), 7.34 (5 H, s), 8.01 (1 H, d).

Z-Leu-Phe-CONHC₂H₅

Z-Leu-Phe-CONHC₂H₅ was prepared from Z-Leu-Phe-CO₂ ethanol essentially as described [44], then chromatographed (CHCl₃/methanol; 98:2) and precipitated from ether, giving a light yellow solid; m.p. 145–152 °C.

Z-Leu-Phe-CHN₂

Z-Leu-Phe-CHN₂ was prepared from Z-Leu-Phe-OH by treatment with isobutylOCOC₂H₅ followed by CH₂N₂ in ether [45]. The product was chromatographed (ether/hexanes; 75:25) and recrystallized (ethyl acetate) to give a light yellow solid; m.p. 131–134 °C.

Z-Leu-Phe-CH₂N(CH₃)₂ [46]

Treatment of Z-Leu-Phe-CHN₂ with HCl gas (115 mol %) in CH₂Cl₂ for 5 min gave Z-Leu-Phe-CH₂Cl; m.p. 146–149 °C. This (182 mg) was dissolved in CH₂Cl₂ and added to a solution of dimethylamine (590 mg, 32 equiv.) in CH₂Cl₂ at -50 °C. After 25 min at -50 °C, warming to 0 °C over 1 h, and evaporation of the solvent, the residue was suspended in ether and extracted three times with 1 M HCl, then water. The combined aqueous extracts were made basic and extracted with CH₂Cl₂. The residue was chromatographed on silica gel (CH₂Cl₂/methanol; 96:4) to give the product as a wax (91 mg, 49 % yield). NMR δ (p.p.m.) [(²H)chloroform] 0.92 (6 H, m), 1.52 (3 H, m), 1.99 (3 H, m), 2.02 (3 H, m), 3.00 (3 H, m), 3.24 (1 H, t), 4.17 (1 H, broad s), 4.92 (1 H, pentet), 5.08 (2 H, s), 6.80 (1 H, broad s), 7.1–7.4 (10 H, m).

Z-Leu-Phe-CH₂OCO-2,6-dichlorobenzene

Z-Leu-Phe-CH₂OCO-2,6-dichlorobenzene was prepared as described previously [47].

Calcium sensitivity

The effect of calcium on calpain I enzymic activity was assessed essentially as described by Yoshimura et al. [48]. Enzyme dialysed overnight against 110 mM imidazole/HCl (pH 7.3)/1 mM EGTA/5 mM β -mercapthoethanol was assayed using 1 mM Suc-Leu-Tyr-MNA substrate in the same buffer with varying amounts of CaCl₂. Free calcium concentrations were calculated using a dissociation constant of 5.5×10^{-6} M [49]. The $1/2 V_{max}$.

was determined as the rate of substrate hydrolysis that was 50 % of the maximal rate achieved in the presence of calcium.

RESULTS

Recombinant viruses for the expression of human calpain I

The cDNAs for the coding region of the ~30 kDa and ~80 kDa human calpain I subunits were obtained by a combination of PCR amplification and hybridization screening of a human spleen cDNA library (Clontech, Palo Alto, CA, U.S.A.) using published sequences [2,50]. The origins of the clones for both subunits are diagrammed in Figure 1(a). A spleen library was chosen based on the reported abundant expression of calpain I and II in rat spleen [51]. These cDNA constructs were then used to prepare recombinant viruses expressing either the individual subunits separately (AcNPV-hCANP30 and AcNPV-hCANPI80, expressing the 30 kDa or the 80 kDa calpain I subunits respectively) or both subunits on the same virus (AcNPV-hCANPI-2-5; Figure 1b).

Characterization of proteins expressed by the recombinant viruses

The expression of the recombinant calpain subunits was initially examined by immunoblot analysis using Ab#6 (Figure 2). This antiserum detected two polypeptides, one ~80 kDa and another ~30 kDa, in preparations of purified human erythrocyte calpain I (results not shown). There was no reactivity with this antiserum to proteins in extracts from wild-type-virus-infected cells (Figure 2, lane 1). Cells infected with either AcNPV-hCANP30 (Figure 2, lanes 2 and 3) or AcNPV-hCANPI80 (Figure 2, lanes 4 and 5) contained an immunoreactive polypeptide of ~30 kDa or ~80 kDa respectively. Cells infected simultaneously with both of those viruses (Figure 2, lanes 6–8) or with AcNPV-hCANPI-2-5, the double-subunit virus (Figure 2, lanes 9 and 10), displayed two immunoreactive polypeptides of ~30 kDa and ~80 kDa. These data demonstrate that the expected calpain subunit(s) were being synthesized from the viral constructs. The accumulated amount of the ~80 kDa subunit was significantly increased (~10-fold) by co-expression with the ~30 kDa subunit, either by co-infection of cells with AcNPV-hCANP30 and AcNPV-hCANPI80 (Figure 2, lanes 6–8) or by expression of the double

construct AcNPV-hCANPI-2-5 (Figure 2, lanes 9 and 10), as compared with expression in the absence of the ~30 kDa subunit (Figure 2, lanes 4 and 5). In addition, heterogeneity of the large subunit was observed, with an ~76 kDa polypeptide being present in all extracts containing the ~80 kDa subunit (Figure 2, lanes 4–10) and an additional intermediate ~78 kDa polypeptide detectable when the ~80 kDa subunit was expressed alone (Figure 2, lanes 4 and 5).

Calcium-dependent enzymic activity of the rhcalpain I

Because the appropriate calpain I subunits were being expressed in infected cells, unfractionated extracts were then examined for calcium-dependent enzymic activity *in vitro*. Lysates from cells infected with AcNPV-hCANPI-2-5 or co-infected with AcNPV-hCANP30 and AcNPV-hCANPI80 demonstrated calcium-dependent hydrolysis of the fluorogenic peptide calpain substrate, Suc-Leu-Tyr-MNA, that was linear with time and extract concentration and completely inhibited by 12.5 μ M calpain inhibitor I (results not shown). Extracts from AcNPV-hCANPI80-infected cells, expressing monomeric ~80 kDa protein displayed the same profile, but had substantially reduced activity (results not shown). Extracts from wild-type-virus-infected cells exhibited no endogenous substrate cleavage, either in the presence or absence of calcium. Therefore all activity in the unfractionated extracts results from expression of proteolytically active rhcalpain I protein.

Correct autolytic activation of the 80 kDa subunit expressed alone or co-expressed with the 30 kDa subunit

The autolytic activation of the large subunit was examined in more detail in order to determine the proportion of expressed protein capable of being activated by calcium and to verify the site of cleavage using an antibody that specifically recognizes only autolytically activated calpain I. The antibody was raised against the peptide Leu-Gly-Arg-His-Glu (directionally linked to keyhole limpet hemocyanin by a C-terminal cysteine), corresponding to the N-terminus of the autolysed ~76 kDa subunit of calpain I [37]. Either partially purified native human erythrocyte calpain I or lysates from infected cells were incubated with or without calcium at room temperature, and samples were analysed by immunoblot analysis to examine either all species of the ~80 kDa subunit (Figure 3A) or only the correctly cleaved ~76 kDa form (Figure 3B). All of the untreated partially purified native calpain I was in the ~80 kDa form (Figure 3A, lane 1) and was shifted to the ~76 kDa form by calcium addition (Figure 3A, lane 2). Only the latter form was recognized by an antibody (Ab#34) specific for the autolytically cleaved ~76 kDa subunit (compare lane 2 with lane 1 in Figure 3b). Neither antiserum detected any proteins in the wild-type-virus-infected lysates (Figure 3, lane 3, both panels). In the rhcalpain I samples without added calcium (Figure 3A; lanes 4, 6 and 8), the lowest immunoreactive polypeptide co-migrated with ~76 kDa form of the native enzyme generated by calcium addition (Figure 3A; lane 2). This was verified as being the authentic ~76 kDa species by its immunoreactivity with Ab#34 (Figure 3B; lanes 4, 6 and 8). With calcium incubation, all the immunoreactive protein was converted to a single polypeptide (Figure 3A; lanes 2, 5, 7 and 9) that co-migrated with the ~76 kDa native human calpain I (Figure 3, lane 2) and was reactive with Ab#34. Combining the information from the two panels, this experiment demonstrated that all of the 80 kDa subunit recombinant protein was capable of undergoing autocatalytic cleavage by the addition of calcium, either with or without the presence of the 30 kDa subunit, and

Figure 2 Expression of calpain protein subunits from infected Sf21 cells

Immunoblot analysis using polyclonal anti-(human calpain I) serum of 10 μ g of soluble protein extracts (see the Experimental section) from cells infected with wild-type virus (lane 1), AcNPV-hCANP30 (lanes 2 and 3), AcNPV-hCANPI80 (lanes 4 and 5), AcNPV-hCANP30 plus AcNPV-hCANPI80 (lanes 6–8), and AcNPV-hCANPI-2-5 (lanes 9 and 10). All cells were infected at an MOI of 5 except the co-infections (lanes 6–8), which were done at an MOI of 5 for each virus.

Figure 3 Complete autocatalytic activation of the 80 kDa subunit in unfractionated extracts

Immunoblot analysis of the large subunit of calpain I with (A) Ab#4 and (B) Ab#34 (see the Experimental section for details). Lanes are with or without the addition of 6.7 mM CaCl_2 as designated. Lanes 1 and 2, partially purified native human calpain I from red blood cells (0.9 μg); 15–20 mg of soluble protein each from Sf21 cells infected as in Figure 2 with: lane 3, wild-type virus; lanes 4 and 5, AcNPV-hCANPI80; lanes 6 and 7, AcNPV-hCANP30-5 plus AcNPV-hCANPI80; lanes 8 and 9, AcNPV-hCANPI-2-5.

that the activated catalytic subunit was cleaved at the same site as native calpain I (between residues 27 and 28).

Purification of the rhcalpain I

The double-subunit virus AcNPV-hCANPI-2-5 was chosen for heterodimer production since it ensured synthesis of both subunits in each infected cell. Optimal conditions for the expression and purification of rhcalpain I using this virus were determined based on achieving maximal protein levels with minimal calpain I autolysis. Table 1 outlines the purification scheme. The initial extract of soluble proteins was enriched 1.4-fold for calpain I by ammonium sulphate precipitation and was further fractionated by anion-exchange, hydrophobic-interaction and affinity chromatographies. These four steps gave a 21-fold purification with a 36% recovery of the initial calpain I present, to yield 5–6 mg of purified rhcalpain I per litre of infected cells. The SDS/PAGE profile of total proteins at each step of the purification is in Figure 4. The two calpain subunits were readily

Figure 4 SDS/PAGE analysis of the purification of rhcalpain I from infected Sf21 cells

The indicated amounts of total protein from each step in the purification were separated by SDS/PAGE (12.5% polyacrylamide) and stained with Coomassie Blue. Lanes 1 and 2, 10 μg each of the initial extract and the ammonium sulphate fractionation respectively; lanes 3–5, 4 μg each of the pooled fractions eluted from Q-Sepharose, phenyl-Sepharose and Mimetic Red 2 columns respectively.

apparent in the first chromatographic step (Q-Sepharose; Figure 4, lane 3). The purified rhcalpain I (Figure 4, lane 5) was estimated to be ~95% pure by reversed-phase HPLC analysis of a sample denatured in 6 M guanidine hydrochloride (results not shown). The enzymic activity of the purified rhcalpain I or partially purified preparations from the phenyl-Sepharose column was stable for greater than 12 months at 4 °C (results not shown).

Baculovirus-expressed monomeric 80 kDa subunit has substantial, but lower, activity than the heterodimer

The specific activity of baculovirus-expressed monomeric ~80 kDa calpain I was estimated in unfractionated extracts, with calpain concentrations determined by active site titration using Ep-460 at 0.2 mM Suc-Leu-Tyr-MNA substrate. The average of two determinations gave a value of 2.5 nmol of MNA product/min per nmol of enzyme in comparison with a value of 6.2 nmol of MNA/min per nmol of enzyme for the purified heterodimer (~40% that of the heterodimer). The activity of the baculovirus-expressed monomer was confirmed to be lower than that of the heterodimer, because its catalytic rate was increased by preincubation with an extract containing the ~30 kDa subunit

Table 1 Purification of rhcalpain I from infected Sf21 cells

Purification	Protein (mg)	Units ($\mu\text{mol}/\text{min}$)	Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)	Fold increase
Initial extract ^a	511.5	5.13	0.010	1.0
Ammonium sulphate	269.9	3.97	0.014	1.4
Q-Sepharose	43.1	2.99	0.070	7.0
Phenyl-Sepharose	14.7	2.39	0.163	16.3
Mimetic Red 2	8.7	1.85	0.213	21.3

^a Starting material was 2.5×10^9 infected serum-free-adapted Sf21 cells (1.5 litre infection).

Table 2 Comparative analysis of native and rhcalpain I

Purified erythrocyte and rhcalpain I were prepared and assayed as described in the Experimental section. A concentration of 1 mM Suc-Leu-Tyr-MNA substrate was used for specific activity and calcium activation determinations, whereas inhibitor analyses used 0.2 mM. Values are expressed $\pm$ S.E.M. except for inactivation rates, which are expressed $\pm$ S.D.

	Erythrocyte human calpain I	Rhcalpain I
Enzyme properties		
Specific activity (mmol/min per mg)	0.12 $\pm$ 0.02	0.37 $\pm$ 0.06
k_{cat}/K_m for Suc-Leu-Tyr-MNA ($\text{M}^{-1} \cdot \text{s}^{-1}$)	890	870
$[\text{Ca}^{2+}]$ for activation [$1/2 V_{\text{max}}$ (mM)]	15.3	15.4 $\pm$ 3.8
Class-specific inhibitors [K_i (nM)]		
Pepstatin A	> 67000	> 67000
PMSF	> 670000	> 670000
Reversible calpain inhibitors [K_i (nM)]		
Calpain inhibitor I	9.5 $\pm$ 3.8	7.0 $\pm$ 1.9
Z-Leu-Phe-CONHC ₂ H ₅	11.3 $\pm$ 2.1	11.2 $\pm$ 3.7
Human calpastatin domain I (14 kDa)	3.1 $\pm$ 0.3	6.6 $\pm$ 0.1
Irreversible calpain inhibitors [$k_{\text{obs}}/[I]$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)]		
Z-Leu-Phe-CHN ₂	1470 $\pm$ 10	1700 $\pm$ 100
Z-Leu-Phe-CH ₂ N(CH ₃) ₂	1700 $\pm$ 50	1260 $\pm$ 160
Ep-460	26100 $\pm$ 830	28000 $\pm$ 4300
Z-Leu-Phe-CH ₂ OCO-2,6-dichlorobenzene	18700 $\pm$ 2200	13300 $\pm$ 200

(1.6-fold increase after 2 h preincubation at 0 °C). Active-site titration of the $\sim$ 80 kDa plus $\sim$ 30 kDa mixture gave essentially the same extract enzyme concentration (0.31 μM) as in the absence of the $\sim$ 30 kDa extract (0.30 μM), even though approximately twice as much extract was present during the former measurement. The similarity of these values suggests that the increment in the measurement due to Ep-460 binding to other cysteine proteases in the unfractionated extract is negligible. This also demonstrates that the increase in activity with the $\sim$ 30 kDa subunit was not simply due to recruitment of previously inactive $\sim$ 80 kDa protein.

Comparison of baculovirus rhcalpain I with native human erythrocyte calpain I

In order to verify the suitability of using rhcalpain I to study native human calpain I, the latter was purified from human erythrocytes and a detailed comparison of enzymic properties performed (Table 2). Although the specific activities of the native and recombinant calpains I differed, virtually all other enzymic properties of the enzymes were identical. For instance, the kinetic constant k_{cat}/K_m for the synthetic fluorogenic peptide substrate Suc-Leu-Tyr-MNA was the same for both enzymes. In addition, the concentration of calcium required to give $1/2 V_{\text{max}}$ activity was identical for both and within the low micromolar range expected for calpain I [48,52–54], with a value consistent with having a somewhat higher Ca^{2+} requirement with synthetic dipeptide substrates [55]. The two forms were also characterized for their sensitivity to a structurally diverse set of known calpain inhibitors (Table 2). The enzymes were inhibited with equal potency by two classes of active-site-directed reversible calpain inhibitors, as well as by the 14 kDa domain I of the endogenous inhibitor calpastatin, which probes a different site on the enzyme [56,57]. The four irreversible calpain inhibitors examined were chosen both for their structural diversity and to include a fairly broad range of inactivation rates (varying $\sim$ 17-fold). These displayed equivalent inhibition rates for the native and recombinant enzymes, and are of the same order of magnitude as those published for chicken gizzard calpain II [58,59]. As expected, neither enzyme was inhibited by pepstatin A or PMSF, inhibitors

of aspartic acid and serine proteases respectively. Lastly, mass spectrometry was used to demonstrate that two peptide substrates, angiotensin I and dynorphin A, were cleaved at identical sites by both enzymes (results not shown).

DISCUSSION

Although both subunits for human calpain I were initially cloned in 1986 [2,50], until recently the only characterized recombinant expression of an entire subunit of calpain has been the production of an inactive large subunit of rat calpain II in *Escherichia coli* [60]. Co-expression in *E. coli* of the large subunit with a portion of the small subunit has been shown to result in proteolytically active recombinant calpain II [61], but direct comparisons were not described between the recombinant and native rat calpains II. We demonstrate in this report that simultaneous expression of both subunits of human calpain I in baculovirus produces the fully enzymically active heterodimer. Upon purification to near homogeneity, the rhcalpain I is indistinguishable from purified human erythrocyte calpain I in its autocatalysis, calcium sensitivity, and susceptibility to inhibition by a structurally and mechanistically diverse array of inhibitors that probed two sites on the enzyme (Table 2). The authenticity of the cleavage-site specificity of the enzyme is evidenced by recognition of the autolytically cleaved catalytic subunit by antiserum generated against the N-terminal peptide of the autolytically cleaved native enzyme [37] (Figure 3B), showing proper cleavage of a protein substrate. In addition, the generation of comparable peptides after cleavage of the endogenous substrates, dynorphin A and angiotensin I, further underscores that the extended active sites of the enzymes are very similar, if not identical. The specific activity of the recombinant enzyme using a fluorogenic peptide substrate was higher than that of the native protease. This is most likely due to the fewer manipulations required to purify recombinant calpain to homogeneity, reducing the potential for protein oxidation.

The $\sim$ 80 kDa subunit, in the absence of the $\sim$ 30 kDa subunit, has substantial calcium-dependent proteolytic activity and can generate both an $\sim$ 78 kDa and $\sim$ 76 kDa form by autolytic cleavage (Figures 2 and 3). Based on its antibody reactivity, the

~76 kDa form is produced by autolysis of the same bond cleaved during activation of native and recombinant heterodimer (Figure 3B). The presence of the ~78 kDa polypeptide is probably also due to autolysis, since the large subunit of the calpain I heterodimer has been shown to undergo a two-step activation process, with formation of an ~78 kDa polypeptide preceding the appearance of the ~76 kDa form [25].

In mammalian systems, the ~30 kDa subunit is always co-expressed with the ~80 kDa subunit. Our report is the first to show expression of proteolytically active ~80 kDa calpain I without any prior association with the ~30 kDa subunit. Studies based on the specific activity of the monomeric enzyme estimated through active-site titration, as well as the observed increase in activity of the monomer in the presence of ~30 kDa subunit, suggest the baculoviral-expressed monomeric enzyme to have substantial, but lower activity than the heterodimer. Early reports suggested that the ~30 kDa subunit was needed for full enzymic activity [62–64], but recently it has been reported that the monomeric ~80 kDa subunit can have the same relative activity as the native heterodimer, either when refolded under optimal conditions [53] or when isolated after dissociation from the ~30 kDa subunit during calcium activation [65]. Our data suggest that in a eukaryotic cell the monomeric ~80 kDa subunit may have a different conformation than material refolded after *E. coli* expression. The increase in activity after preincubation with an extract containing the small subunit suggests an association of the two subunits during catalysis, which is difficult to reconcile with the hypothesis that the heterodimer dissociates in the presence of calcium [65]. One possible explanation for the increase in activity would be that association with the ~30 kDa could confer a conformational change that is retained upon dissociation with calcium. Our results confirming enzymic activity of eukaryote-expressed monomer suggest that studies are needed to verify whether monomeric ~80 kDa subunit ever exists in mammalian cells and, if so, how its activity and function may differ from those of the heterodimer.

One striking finding from the initial expression experiments is the increased levels of the large subunit when co-expressed with the ~30 kDa subunit, either by double infection with the two independent viruses or by infection with the double subunit virus (Figure 2). The increased accumulation of the ~80 kDa protein from ~30 kDa co-expression could be due to either a stabilization and increased half-life of the protein, increased solubility of the protein or a combination of the two. Most of the expressed monomeric ~80 kDa subunit protein is present in the pellet after centrifugation to produce the initial extract, whereas the majority of both the subunits are in the supernatant when expressed as the heterodimeric protein (results not shown), suggesting that co-expression of the ~30 kDa subunit primarily increases solubility/proper folding of the catalytic subunit. This idea is supported by expression studies of recombinant calpain II in *E. coli*, where co-expression of a portion of the small subunit is needed to produce soluble enzyme [61]. Some investigators have included monomeric ~80 kDa calpain as a species involved in the intracellular calpain cascade [66], but this is not a universal view [67]. Because the baculovirus-expressed monomeric enzyme is active, the system can be used to examine monomeric ~80 kDa calpain I in more detail, as well as to define better its interaction with the ~30 kDa subunit. Our studies have already provided substantial evidence for the primary role of the ~30 kDa subunit being to promote the proper folding of the catalytic subunit, as opposed to having an important role in catalysis.

Having performed a rigorous comparison of the baculovirus-expressed rhcalpain I with native human enzyme, one can now confidently use this recombinant as a model of the native enzyme.

The combined data presented strongly suggest that the baculovirus expression of rhcalpain I results in a facile method for the production of the enzyme for use in studies of the native enzyme, including X-ray crystallization and site-directed mutagenesis. It has the advantage over *E. coli* expression of producing active monomeric protease. Furthermore, it is anticipated that other calpain variants will be amenable to recombinant expression in proteolytically active forms by the approach employed here.

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REFERENCES

- Mellgren, R. L. and Murachi, T. (1990) Intracellular Calcium-dependent Proteolysis, CRC Press, Boca Raton
- Aoki, K., Imajoh, S., Ohno, S., Emori, Y., Koike, M., Kosaki, G. and Suzuki, K. (1986) FEBS Lett. **205**, 313–317
- Emori, Y., Kawasaki, H., Sugihara, H., Imajoh, S., Kawashima, S. and Suzuki, K. (1986) J. Biol. Chem. **261**, 9465–9471
- Sakihama, T., Kakidani, H., Zenita, K., Yumoto, N., Kikuchi, T., Sasaki, T., Kannagi, R., Nakanishi, S., Ohmori, M., Takio, K., Titani, K. and Murachi, T. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 6075–6079
- Kawasaki, H., Imajoh, S., Kawashima, S., Hayashi, H. and Suzuki, K. (1986) J. Biochem. (Tokyo) **99**, 1525–1532
- Coolican, S. A. and Hathaway, D. R. (1984) J. Biol. Chem. **259**, 11627–11630
- Gopalakrishna, R. and Barsky, S. H. (1986) J. Biol. Chem. **261**, 13936–13942
- Saigo, T. C., Shibata, M., Takenawa, T., Murofushi, H. and Suzuki, K. (1992) J. Biol. Chem. **267**, 24585–24590
- Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F. and Horecker, B. L. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 6435–6439
- Suzuki, K., Imajoh, S., Emori, Y., Kawasaki, H., Minami, Y. and Ohno, S. (1987) FEBS Lett. **220**, 271–277
- Sorimachi, H., Toyama-Sorimachi, N., Saigo, T. C., Kawasaki, H., Sugita, H., Miyasaka, M., Arahata, K., Ishiura, S. and Suzuki, K. (1993) J. Biol. Chem. **268**, 10593–10605
- Sorimachi, H., Ishiura, S. and Suzuki, K. (1993) J. Biol. Chem. **268**, 19476–19482
- Wang, K. K., Villalobo, A. and Roufogalis, B. D. (1989) Biochem. J. **262**, 693–706
- Mellgren, R. L. (1991) J. Biol. Chem. **266**, 13920–13924
- Siman, R. (1990) in Neurotoxicity of Excitatory Amino Acids (Guidotti, A., ed.), pp. 145–161, Raven Press, New York
- Seubert, P. and Lynch, G. (1990) in Intracellular Calcium-dependent Proteolysis (Mellgren, R. L. and Murachi, T., eds.), pp. 251–263, CRC Press, Boca Raton
- Lee, K. S., Frank, S., Vanderklish, P., Arai, A. and Lynch, G. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 7233–7237
- Saigo, T. C., Yokota, M., Nagao, S., Yamaura, I., Tani, E., Tsuchiya, T., Suzuki, K. and Kawashima, S. (1993) J. Biol. Chem. **268**, 25239–25243
- Roberts-Lewis, J. M., Savage, M. J., Marcy, V. R., Pinsker, L. R. and Siman, R. (1994) J. Neurosci. **14**, 3934–3944
- Imajoh, S., Kawasaki, H. and Suzuki, K. (1986) J. Biochem. (Tokyo) **99**, 1281–1284
- Imajoh, S., Kawasaki, H. and Suzuki, K. (1986) J. Biochem. (Tokyo) **100**, 633–642
- Pontremoli, S. and Melloni, E. (1986) in Calcium and Cell Function, vol. 6 (Cheung, W. Y., ed.), pp. 159–183, Academic Press, San Diego
- Cong, J., Goll, D. E., Peterson, A. M. and Kapprell, H.-P. (1989) J. Biol. Chem. **264**, 10096–10103
- Croall, D. E. and DeMartino, G. N. (1990) in Intracellular Calcium-dependent Proteolysis (Mellgren, R. L., and Murachi, T., eds.), pp. 103–114, CRC Press, Boca Raton
- Zimmerman, U.-J. P. and Schlaepfer, W. W. (1991) Biochim. Biophys. Acta **1078**, 192–198
- Suzuki, K., Tsuji, S. and Ishiura, S. (1981) FEBS Lett. **136**, 119–122
- Suzuki, K. and Ishiura, S. (1983) J. Biochem. (Tokyo) **93**, 1463–1471
- Takahashi-Nakamura, M., Tsuji, S., Suzuki, K. and Imahori, K. (1981) J. Biochem. (Tokyo) **90**, 1583–1589
- Takano, E. and Murachi, T. (1982) J. Biochem. (Tokyo) **92**, 2021–2028
- Emori, Y., Kawasaki, H., Imajoh, S., Imahori, K. and Suzuki, K. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 3590–3594
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor

- 32 O'Reilly, D. R., Miller, L. K. and Luckow, V. A. (1992) Baculovirus Expression Vectors: A Laboratory Manual, W. H. Freeman and Company, New York
- 33 Luckow, V. A. and Summers, M. D. (1989) *Virology* **170**, 31–39
- 34 Vaughn, J. L., Goodwin, R. H., Thompson, G. J. and McCawley, P. (1977) *In Vitro* **13**, 213–217
- 35 Meyer, S. L., Lang, D. M., Forbes, M. E., Knight, E., Jr., Hirsch, J. D., Trusko, S. P. and Scott, R. W. (1994) *J. Neurochem.* **62**, 825–833
- 36 Siman, R. and Noszek, J. C. (1988) *Neuron* **1**, 279–287
- 37 Saido, T. C., Nagao, S., Shiramine, M., Tsukaguchi, M., Sorimachi, H., Murofushi, H., Tsuchiya, T., Ito, H. and Suzuki, K. (1992) *J. Biochem. (Tokyo)* **111**, 81–86
- 38 Lee, W. J., Adachi, Y., Maki, M., Hatanaka, M. and Murachi, T. (1990) *Biochem. Int.* **22**, 163–171
- 39 Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M. and Hanada, K. (1982) *Biochem. J.* **201**, 189–198
- 40 Cheng, Y.-C. and Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22**, 3099–3108
- 41 Tian, W.-X. and Tsou, C.-L. (1982) *Biochemistry* **21**, 1028–1032
- 42 Krantz, A., Copp, L. J., Coles, P. J., Smith, R. A. and Heard, S. B. (1991) *Biochemistry* **30**, 4678–4687
- 43 Hanada, K., Tamai, M., Ohmura, S., Sawada, J., Seki, T. and Tanaka, I. (1978) *Agric. Biol. Chem.* **42**, 529–536
- 44 Li, Z., Patil, G. S., Golubski, Z. G., Hori, H., Terani, K., Foreman, J. E., Eveleth, D. D., Bartus, R. T. and Powers, J. C. (1993) *J. Med. Chem.* **36**, 3472–3480
- 45 Harbison, S. L. and Rich, D. H. (1989) *J. Med. Chem.* **32**, 1378–1392
- 46 Ando, R., Ando, N., Masuda, H., Morinaka, Y., Takahashi, C., Tamao, Y. and Tobe, A. (1993) *Eur. Pat. Appl.* 525,420
- 47 Dolle, R. E., Singh, J., Whipple, D., Osifo, I. K., Speier, G., Graybill, T. L., Gregory, J. S., Harris, A. L., Helaszek, C. T., Miller, R. E. and Ator, M. A. (1995) *J. Med. Chem.* **38**, 220–222
- 48 Yoshimura, N., Kikuchi, T., Sasaki, T., Kitahara, A., Hatanaka, M. and Murachi, T. (1983) *J. Biol. Chem.* **258**, 8883–8889
- 49 Harafuji, H. and Ogawa, Y. (1980) *J. Biochem. (Tokyo)* **87**, 1305–1312
- 50 Ohno, S., Emori, Y. and Suzuki, K. (1986) *Nucleic Acids Res.* **14**, 5559
- 51 Murachi, T. (1983) *Trends Biochem. Sci.* **8**, 167–169
- 52 Kitahara, A., Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., Hatanaka, M. and Murachi, T. (1984) *J. Biochem. (Tokyo)* **95**, 1759–1766
- 53 Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S. and Suzuki, K. (1995) *FEBS Lett.* **358**, 101–103
- 54 Murakami, T., Hatanaka, M. and Murachi, T. (1981) *J. Biochem. (Tokyo)* **90**, 1809–1816
- 55 Sasaki, T., Kikuchi, T., Noboru, Y., Yoshimura, N. and Murachi, T. (1984) *J. Biol. Chem.* **259**, 12489–12494
- 56 Kawasaki, H., Emori, Y. and Suzuki, K. (1990) in *Protease Inhibitors* (Takada, A., Samama, M. M. and Collen, D., eds.), pp. 189–199, Elsevier Science Publishing, New York
- 57 Croall, D. and McGrody, K. S. (1994) *Biochemistry* **33**, 13223–13230
- 58 Parkes, C., Kembhavi, A. A. and Barrett, A. J. (1985) *Biochem. J.* **230**, 509–516
- 59 Crawford, C., Mason, R. W., Wikstrom, P. and Shaw, E. (1988) *Biochem. J.* **253**, 751–758
- 60 DeLuca, C. I., Davies, P. L., Samis, J. A. and Elce, J. S. (1993) *Biochim. Biophys. Acta* **1216**, 81–93
- 61 Graham-Siegenthaler, K., Gauthier, S., Davies, P. L. and Elce, J. S. (1994) *J. Biol. Chem.* **269**, 30457–30460
- 62 Kikuchi, T., Yumoto, N., Sasaki, T. and Murachi, T. (1984) *Arch. Biochem. Biophys.* **234**, 639–645
- 63 Crawford, C., Brown, N. R. and Willis, A. C. (1993) *Biochem. J.* **296**, 135–142
- 64 Tsuji, S. and Imahori, K. (1981) *J. Biochem. (Tokyo)* **90**, 233–240
- 65 Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S. and Suzuki, K. (1995) *Biochem. Biophys. Res. Commun.* **208**, 376–383
- 66 Melloni, E. and Pontremoli, S. (1989) *Trends Neurol. Sci.* **12**, 438–444
- 67 Suzuki, K., Saido, T. C. and Hirai, S. (1992) *Ann. N. Y. Acad. Sci.* **674**, 218–227