The calcium-activated neutral cysteine proteinase calpain (CANP) is a heterodimer of a catalytic 80-kDa subunit and a regulatory 30-kDa subunit (1–3). The physiological role of CANP is not fully established, but its participation in events such as cell division, signal transduction, and long-term potentiation has been suggested (4–7). Calpains have also been proposed to play a role in various pathological processes associated with altered protein metabolism and/or altered calcium homeostasis (8, 9). Two major isoforms of the protease are known, μCANP (or calpain I) and mCANP (or calpain II), which require about 50 and 300 μM Ca\(^{2+}\) for half-maximal activity \textit{in vitro}, respectively (10, 11). More recently, a third calpain isoform, termed p94, which is specific for skeletal muscle and appears to be partly located in the cell nucleus, has also been identified (12, 13). The large subunit of CANP is composed of four domains (I–IV) as follows: domain I probably acts as a “repressor” of the catalytic activity and is cleaved at its N-terminal portion during the autolytic activation of the protease (3, 14); domain II contains the essential cysteine and histidine residues of the active site (15); the role of domain III, which has no obvious sequence homology to other proteins, is still obscure; domain IV is a calmodulin-like domain that contains four putative Ca\(^{2+}\)-binding sites corresponding to the helix-loop-helix Ca\(^{2+}\)-binding motif typical of calmodulin-like proteins (15–17). The small subunit consists of an N-terminal glycine-rich hydrophobic domain V and of a calmodulin-like domain IV that is homologous to domain IV of the catalytic subunit. The association of the two subunits to form the heterodimer results from the interaction of domains IV and IV’ (8). The function of the 30-kDa subunit is not established, but Ca\(^{2+}\) appears to dissociate it from the catalytic subunit, activating it (18). Ca\(^{2+}\) also promotes the autoproteolysis of the enzyme, cleaving amino acids from both subunits (11, 19). The autoproteolytic processing is linked to the activation of CANP (two degradation products of about 78 and 76 kDa are produced seconds after the incubation of the enzyme with Ca\(^{2+}\)), but recent work has shown that non-autoproteolyzed μCANP may also be active (20).

Reports have appeared describing the purification of both heterodimeric CANP isoforms from several tissues, e.g. muscle, liver, and kidney (2), but the isolation of large amounts of pure and active protein is laborious. cDNAs for the large and small subunits of calpains I and II have been cloned (21–24), and the expression of the large subunit of calpain II (22) and of an N-terminally truncated 21-kDa variant of the small subunit (24) has been achieved in \textit{Escherichia coli}. Domain IV of the large subunit of calpain I has also been expressed (21). In a
recent publication the expression of active human calpain I using the baculovirus system has been described (25).

The work described in this contribution reports the expression of the catalytic subunit of calpain I (L-μCANP) and of two mutants lacking domains III or IV (L-μCANP3A and L-μCANP4) in E. coli and using the baculovirus expression system. A chimeric form (L-μμCANP), in which domain IV of μCANP was replaced by that of mCANP, was also expressed to study the role of domain IV in the Ca2+ requirement for the activation of the enzyme.

EXPERIMENTAL PROCEDURES

Materials and General Methods—Restriction enzymes were obtained from New England Biolabs (Schwalbach, Germany). For the PCR purification, mid-plasmid purification and gel extraction kits from QIAGEN AG (Basel, Switzerland) were used. The ExpandTM Long Template PCR System kit was from Boehringer Mannheim (Mannheim, Germany). The oligonucleotides were purchased from Microsynth (Balgach, Switzerland).

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Expression of Calpain I Mutants

**RESULTS**

**Preparation of the Recombinants of L-µCANP in E. coli**—The cDNA fragments for the wild-type L-µCANP and the mutants L-µCANP∆3 and L-µCANP∆4 were prepared as illustrated in Fig. 1A, by subcloning PCR products first in the pGEM-T vector and then in the expression vector pET-3d. All three recombinant proteins accumulated as inactive insoluble aggregates. The inclusion bodies containing the proteins expressed at 30 °C were purified through a combination of centrifugation steps. At the end of the washing steps, highly enriched (about 95–97%) preparations of the recombinant proteins were obtained (Fig. 2). To achieve solubilization, in the final step of the purification procedure (30) the pH was initially shifted to 12.0 and after some minutes brought back rapidly to 8.0. However, pH 12.0 irreversibly inactivated calpain, as established in control experiments on the purified erythrocyte calpain (EC CANP). Therefore, the solubilization step at pH 12.0 was omitted, and the isolated inclusion bodies were instead solubilized with guanidine hydrochloride (GdnHCl).

**Denaturation and Refolding of the Bacterially Expressed Proteins**—During the treatment with 6 M GdnHCl and prior to the refolding attempts by dialysis, the recombinant proteins were diluted to 10–50 µg/ml (37, 38). As already reported for other proteins (39), a non-denaturing concentration of GdnHCl (0.05

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(50 mM Tris acetate, pH 7.5, 0.02% (w/v) NaN₃, 100 mM NaCl, 5 µM PEG 4,000, 0.1% (v/v) Triton X-100) containing 1 mM EGTA.

**Purification of the Recombinant Proteins Expressed in Insect Cells**—When Sf9 cells were co-infected with viruses for calpastatin and calpain recombinants, the expressed inhibitor (HA1-CALST) was removed using a modification of the procedure of Wadzinski et al. (35). Briefly, 5 µg of anti-HA (12CA5) antibodies (Boehringer Mannheim, Rotkreuz, Switzerland) were added to these preparations, and the mixtures were rotated end-over-end overnight at 4 °C. About 100 µl of protein A-Sepharose (Pharmacia, Uppsala, Sweden) were added, and the incubation at 4 °C was continued for 2 h. The beads were washed with a low salt buffer (50 mM Tris-HCl, pH 7.5, 1 mM EGTA) to elute the calpain recombinants, and HA1-CALST remained bound to the matrix. To the released proteins, final concentrations of 100 mM NaCl, 5 µM PEG 4,000, and 0.1% (v/v) Triton X-100 were added. The mixtures were then dialyzed overnight against buffer B containing 1 mM EGTA. These samples and the crude enzyme preparations, obtained as described above, were incubated with 100 µM final concentration of calpeptin for 30 min at 4 °C. Then an equal volume of buffer B containing CaCl₂ was added dropwise with stirring to obtain a total free calcium concentration of 0.8 mM, and the incubation was continued for 1 additional h. The inhibitor-modified enzyme preparations were loaded onto a B27-Sepharose calpain affinity column (28) pre-equilibrated with buffer B containing 0.8 mM, and the incubation was continued for 1 additional h. The beads were washed with a low salt buffer (50 mM Tris acetate, pH 7.5, 0.02% (w/v) NaN₃, 100 mM NaCl, 5 mM mercaptoethanol, 5 mM dithiothreitol) in Ultrafree filters (Millipore, Bedford, MA) prior to the determination of protein concentrations and the assay of calpain activity.

**In Vitro Association of the Subunits to Form Heterodimeric Calpain**—The truncated 21-kDa small subunit was added to the refolding mixtures for L-µCANP or L-µCANP∆3 purified from the inclusion bodies as described above, or to L-µCANP, L-µCANP∆3, or L-µµmCANP produced with the baculovirus expression system. The small subunit was in 10–20-fold molar excess of the recombinant catalytic subunit. The protein mixtures were kept at room temperature, and their activities were measured.

**Dissociation of the Subunits**—The dissociation of subunits by Ca²⁺ was studied using a chromatographic procedure. Heterodimeric calpain (L-µµmCANP with the 21-kDa protein) was incubated with calpeptin, loaded onto the B27-Sepharose calpain affinity column (28) in the presence of Ca²⁺, and eluted with EGTA as described above.

**Calpain Assay**—Calpain activity was assayed fluorimetrically according to a modified method of Sasaki et al. (36), with a Spex Fluorolog 1680 (Spex Industries, Inc., Edison, NJ). In this assay, 40-µl portions of the samples (300–400 nm) were added to 500 µl of total volume of assay buffer (50 mM Tris acetate, pH 7.5, 0.25 mM Suc-Leu-Tyr-AMC, 5 mM cysteine, 0.02% (v/v) 2-mercaptoethanol). Ca²⁺ was added in a 10-µl volume to initiate the hydrolysis of the fluorogenic substrate at 22 °C. Excitation was at 370 nm, and the emission of the released AMC was measured at 460 nm. In the case of L-µCANP∆4 (produced in E. coli), it was necessary to reduce the amount of calpeptin in the 40-µl portions; the reversible inhibitor was removed by ultrafiltration with several buffer changes in Ultrafree filters. The volume of the retained samples was adjusted to 40 µl.
Expression of Calpain I Mutants

Characterization of the Proteins Expressed Using the Baculovirus System—The expression of the recombinant catalytic subunit was examined in immunoblots of proteins of infected Sf9 cells (Fig. 4A). An antiserum against platelet mCANP detected L-μCANP, L-μCANPΔ3, and L-μCANPΔ4 but failed to detect L-μCANPΔ4. The latter evidently was not expressed, as expected from the low level of baculovirus production. Antibodies against calpastatin detected HA1-CALST (Fig. 4B). As reported by Meyer et al. (25), heterogeneity of the large subunit was observed; in addition to the 80-kDa subunit, two polypeptides of about 78 and 76 kDa were also present, the latter in smaller amounts (Fig. 4A, lane 1). When L-μCANP was co-

**TABLE I**

| Constituent                  | Maximal specific activity | Relative maximal activity | Ca²⁺ requirement for half-maximal activity |
|------------------------------|---------------------------|--------------------------|------------------------------------------|
| Native EC CANP               | 70.1                      | 100                      | 53                                       |
| Control EC CANP              | 42.1                      | 60                       | 104                                      |
| PEG-EC CANP                  | 56.7                      | 81                       | 94                                       |
| μ(E. coli)/                  | 47.2                      | 67                       | 215                                      |
| μ(E. coli)/+ 21 kDa          | 45.8                      | 65                       | 199                                      |
| μ(SIB)/                      | 64.3                      | 92                       | 68                                       |
| μ(SIB)/+ 21 kDa              | 66.9                      | 95                       | 65                                       |
| Δ3(E. coli)/                 | 38.0                      | 54                       | 528                                      |
| Δ3(E. coli)/+ 21 kDa         | 39.4                      | 56                       | 511                                      |
| Δ3(SIB)/                     | 61.3                      | 87                       | 399                                      |
| Δ3(SIB)/+ 21 kDa             | 61.9                      | 88                       | 340                                      |
| μ(SIB)/                      | 65.9                      | 94                       | 51                                       |
| μ(SIB)/+ 21 kDa              | 72.7                      | 104                      | 44                                       |

*The maximal specific activity is defined as the pmol of Suc-Leu-Tyr-AMC hydrolyzed by 12.5 pmol of the protease within 1 min at room temperature.

**FIG. 2.** SDS-PAGE analysis of the recombinant proteins produced in E. coli. Proteins extracted from IPTG-induced E. coli cells (30 °C) were subjected to purification as described under “Experimental Procedures” and analyzed by SDS-PAGE on 10% slab gels stained with Coomassie Brilliant Blue. Lanes 1, 6, and 11, total cell lysates; lanes 2, 7, and 12, soluble fractions; lanes 3, 8, and 13, supernatants after washing with urea; lanes 4, 9, and 14, supernatants after washing with Zwittergent 3-14; lanes 5, 10, and 15, solubilized recombinants; lane 16, L-μCANPΔ4 solubilized in the presence of calpeptin.

**FIG. 3.** Co-expression of L-μCANP and the 21-kDa truncated small subunit in E. coli. Soluble and insoluble fractions from IPTG-induced E. coli cells (30 °C) expressing both L-μCANP and the 21-kDa protein as described under “Experimental Procedures” were subjected to 12% SDS-PAGE analysis. Lane 1, total cell lysate; lane 2, soluble fractions; lane 3, insoluble fraction.
Expression of Calpain I Mutants

**Fig. 4. Expression of recombinant proteins in Sf9 cells.** Immunoblot analysis using antibodies against calpain (A) or against calpastatin (B) of 10 μg of soluble protein extracts from Sf9 cells infected with an m.o.i. of 5.0 for each virus is shown. Lane 1 (A and B), L-μCANP; lane 2 (A), L-μCANP and the 21-kDa protein; lane 3 (A), L-μmCANP; lane 4, L-μmCANP and the 21-kDa protein; lane 5, L-μmCANPΔ3; lane 6, L-μmCANPΔ3 and the 21-kDa protein; lanes 7 and 2 (B), L-μCANP plus HA1-CALST; lanes 8 and 3 (B), L-μmCANP plus HA1-CALST.

expressed with the 21-kDa subunit, the 78-kDa protein was not detected, and the 76-kDa product became the most evident (Fig. 4A, lane 2). The expressed chimeric form L-μmCANP had obvious bands at 80, 78, and 76 kDa (Fig. 4A, lane 3). Also in this case co-expression with the 21-kDa subunit induced the disappearance of the 78-kDa polypeptide (Fig. 4A, lane 4). The intensity of the two remaining bands was weak, indicating autolysis of the chimeric form. At variance with this, the expressed L-μCANPΔ3 only showed one immunoreactive polypeptide of 55 kDa, also when co-expressed with the small subunit (Fig. 4A, lanes 5 and 6). The 78- and 76-kDa polypeptides failed to form when L-μCANP and L-μmCANP were co-expressed with CALST (Fig. 4A, lanes 7 and 8). Thus, co-expression with calpastatin apparently prevented autolysis of recombinant calpains in the Sf9 cells. The expression of CALST was monitored with antibodies against bovine calpastatin (Fig. 4B, lanes 2 and 3).

**Purification of Recombinant Proteins from Sf9**—The recombinant proteins were produced by 1.4 × 10^7 infected Sf9 cells grown in monolayer, and purification of L-μCANP, L-μmCANP, and L-μmCANPΔ3, or of co-expressed L-μCANP–CALST, L-μmCANP–CALST, and L-μmCANPΔ3–21-kDa was performed as described under “Experimental Procedures.” The final purified products are shown in Fig. 5. The extracts containing co-expressed CALST were treated to remove the CALST (see the “Experimental Procedures”) prior to the purification step on the B27 affinity column. The purification yielded 25–35 μg of 90–95% pure recombinant enzymes from 1.4 × 10^7 cells.

**Ca^{2+}-dependent Activity**—The Ca^{2+} dependence of the recombinant calpains was studied fluorimetrically with the synthetic substrate Suc-Leu-Tyr-AMC using samples of purified erythrocyte CANP as controls (Table 1). The recombinant proteins used were produced under conditions that yielded no autoproteolysis products. Thus, as shown in Fig. 4A for the experiment on calpains produced in Sf9 cells, L-μCANP, L-μmCANP, L-μmCANPΔ3, and L-μmCANP were co-expressed with CALST (except for the case of L-μmCANPΔ3) and were subjected to activity measurements after removal of CALST. As mentioned above, the level of activity for the bacterially expressed proteins peaked 2 days after dialysis against PEG, NaCl, and Triton X-100. Half-maximal activity of the native erythrocyte CANP and of the same enzyme submitted to the denaturation/renaturation treatment required 60–65 and 100–105 μM Ca^{2+}, respectively (Fig. 6). The V_max of the purified erythrocyte CANP reached about 80% that of the untreated native enzyme when denatured and refolded in the presence of PEG, NaCl, and Triton X-100 and decreased to about 60% if the GdnHCl treatment followed by refolding was performed in the absence of PEG. The V_max of L-μCANP expressed in E. coli and renatured under optimal conditions reached 65–70% that of native erythrocyte CANP and 90–93% when expressed using the baculovirus system. The apparent K_m(Ca^{2+}) was 65–70 μM in the case of the virally produced protein and 205–220 μM for the protein expressed in bacteria. Higher Ca^{2+} concentrations dramatically decreased the activity, as also observed for erythrocyte CANP. The V_max of mutant L-μmCANPΔ3 produced in E. coli was 52–56% that of the native erythrocyte enzyme (K_m(Ca^{2+}) of 510–540 μM) and increased to 85–90% for L-μmCANPΔ3 from Sf9 cells (K_m(Ca^{2+}) of 380–420 μM). At variance with erythrocyte CANP and the expressed L-μCANP, the activity of mutant L-μmCANPΔ3 did not decline at high Ca^{2+} concentrations. In vitro association of L-μCANP and L-μmCANPΔ3 with the purified recombinant small subunit.
sightly increased the activity and calcium dependence of the recombinant catalytic subunits produced in insect cells (Table I). The maximal activity of mutant L-μCANPΔ4 expressed in E. coli and renatured in the PEG-containing buffer corresponded to only 42–45% that of the native erythrocyte calpain. The mutant was Ca2+ -insensitive (range tested, 0–20 mM Ca2+).

Calpeptin had to be present in the dialysis buffer when purifying L-μCANPΔ4. Removal of the inhibitor by dialysis prior to performing the activity assay led to the rapid autoproteolysis of this mutant and to its disappearance from the dialysis tube (not shown). The activity was thus measured after diluting the reversible inhibitor in the assay buffer by ultrafiltration. Possibly, then, the lower activity of this mutant could have been due to its partial degradation during the ultrafiltration step.

The V_max of the chimera L-μCANP expressed using the baculovirus was 92–95% that of native erythrocyte CANP, and in this case the addition of the 21-kDa subunit brought the activity to 100–105%. Unexpectedly, the Ca2+ requirement for half-maximal activity of L-μCANP was even lower than that of erythrocyte CANP, i.e. 49–53 μM, and was decreased to 42–45 μM by in vitro association with the small subunit.

Dissociation of the Subunits upon Ca2+ Activation—The B27 peptide (the product of exon 1B of the CALST gene) is known to bind only the catalytic subunit. As already observed (28), the small subunit failed to bind to the B27 peptide column. Heterodimeric calpain in buffer B containing Ca2+ and 100 μM calpeptin was loaded onto the B27 column (Fig. 7, lane 1) which was then washed thoroughly with a Ca2+-containing buffer. The large subunit was retained, whereas the small subunit was lost in the flow-through (Fig. 7, lane 2). The large subunit was eluted with an EGTA-containing buffer (Fig. 7, lane 3). The isolated catalytic subunit showed full enzyme activity (not shown).

Autoproteolytic Processing of the Recombinant Calpains—The four recombinant monomers (L-μCANP, L-μmCANP, and L-μCANPΔ3 expressed in Sf9 cells and L-μCANPΔ4 produced in E. coli) underwent autoproteolysis when incubated for 2 min with 1 mM Ca2+, L-μCANPΔ4 was rapidly converted to species with lower molecular weight also in its absence. The degradation of the four proteins was completely inhibited by Cbz-Leu-Leu-Tyr-CHN2 (Fig. 8). In all cases, the typical three-band pattern of limited autoproteolysis of the catalytic subunit, resulting from cleavage at two N-terminal sites (3, 14), was observed.

**DISCUSSION**

This study describes the expression and properties of the recombinant catalytic subunit (L-μCANP) of μ-calpain, of two mutants lacking domain III (L-μCANPΔ3) or domain IV (L-μCANPΔ4), and of a chimeric form of the μ- and m-calpain (L-μmCANP). When expressed in E. coli, the proteins were always produced as inactive aggregates in the inclusion bodies, possibly a useful device to prevent cell damage, especially in the case of the Ca2+-independent L-μCANPΔ4. Co-expression of the large CANP subunit with the regulatory subunit was also attempted in E. coli to improve the production of soluble and active catalytic subunit (24, 25). The attempt was unsuccessful, i.e. the small subunit was expressed in soluble form but failed to prevent the aggregation of L-μCANP and did not improve its expression.

The recently reported successful expression of biologically active and stable monomeric and heterodimeric human μ-calpains in eukaryotic cells (25) prompted the extension of the work to the baculovirus Sf9 cell expression system. However, the truncated mutant L-μCANPΔ4 could not be expressed in Sf9 cells, and baculovirus particles for this mutant were not produced. The mRNA for this mutant was present after transfection of the insect cells, but its amount was much lower than that for L-μCANP or L-μCANPΔ3. The reasons for the very low level of transcription of L-μCANPΔ4 is not understood at the moment, except that one could speculate that the production of constitutively active L-μCANPΔ4 could be lethal to eukaryotic cells. Possibly, insect cells activate a down-regulating system to prevent the formation of dangerous viral DNA.

The autoproteolytic process leading to the production of the 78- and 76-kDa forms of expressed L-μCANP was enhanced in the baculovirus system by co-expressing the 21-kDa subunit. At variance with Meyer et al. (25), who observed increased accumulation of the 80-kDa protein upon co-expression with the small subunit, in the present work the 21-kDa subunit was found to improve the solubility of the catalytic subunit but not its stability. In fact, in co-expressing cells, L-μCANP underwent more pronounced autolysis with the appearance of signif-

![Fig. 7. Subunit dissociation on the B27 peptide affinity column.](Image)

![Fig. 8. Autoproteolytic processing of the recombinant proteins.](Image)
sensitivity of the protein to Ca\textsuperscript{2+} did not induce significant loss of calpain activity but altered the structure of domain III could increase the accessibility of the activating signal to calpain, i.e. upon binding to produce the active monomer. The suggestion would be compatible with previously published studies (18, 28). A recent report, however, argues for the continued association of calpain subunits in the presence of Ca\textsuperscript{2+} and during the proteolysis of substrates (42).

Despite the large literature on the structure and function of calpains, little is known about the roles of domains III and IV of their native 80-kDa subunits. The experiments presented in this contribution have shown that the removal of domain III did not induce significant loss of calpain activity but altered the sensitivity of the protein to Ca\textsuperscript{2+}, i.e. it increased very substantially the apparent K\textsubscript{m}(Ca\textsuperscript{2+}) of the mutant. It would thus be attractive to speculate that domain III mediates the Ca\textsuperscript{2+}-activating signal to calpain, i.e. upon binding of Ca\textsuperscript{2+} to domain IV, domain III would amplify the activating message to the active site in domain II. Possibly a change in the tertiary structure of domain III could increase the accessibility of the active site.

As for domain IV, its removal has fortified the proposal that this domain is the Ca\textsuperscript{2+} receptor in the molecule. The finding that the replacement of domain IV of mCANP with that of recombinant mCANP improved the affinity of the protein for Ca\textsuperscript{2+} was unexpected, given the considerably lower Ca\textsuperscript{2+} affinity of mCANP. This suggests that domain IV may play an important role in the interaction of the molecule with Ca\textsuperscript{2+} but not as the only domain in the large subunit that mediates the Ca\textsuperscript{2+} response. As for the finding that L-\textmu-mCANP, L-\textmu-mCANP, and L-\textmu-mCANP\textDelta 3 were all active and Ca\textsuperscript{2+}-dependent in the absence of the small subunit, it clearly indicates that Ca\textsuperscript{2+}, in addition to its function in dissociating the two calpain subunits, also has other roles; it could, for example, induce conformational changes of the large subunit that may be essential for its activation.

Acknowledgments—We are grateful to our colleagues Dr. A. Kraev for help in the subcloning of the recombinant mutants and Dr. D. Guerini for numerous discussions on the experiments involving the baculovirus expression system. We thank Dr. J. S. Elce (Kingston, Ontario, Canada) for kindly donating the CDNs of the catalytic subunit of mCANP and of the truncated regulatory subunit.

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