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Determination of amino acid sequence responsible for suppression of bone resorption by serum calcium-decreasing factor (caldecrin)

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Abstract We previously reported on the serum calcium-decreasing activity of recombinant protein factor referred to as caldecrin [Tomomura et al. (1995) J. Biol. Chem. 270, 30315–30321]. To address the mechanism of this serum calcium-decreasing activity, we investigated the effect of rat caldecrin on osteoclastic bone-resorbing activity. Wild-type caldecrin suppressed resorption pit formation by osteoclast on a dentine slice in a dose-dependent manner. The suppressive effect on the bone resorption was not affected by treatment of caldecrin with phenylmethyl sulfonyl fluoride or by use of protease-deficient mutant caldecrins. Recombinant procaldecrin (−13–239), and its fragments (−13–125), (1–111), (1–46), (47–111), and (126–239) were expressed as His-tagged thioredoxin fusion proteins and investigated for their ability to suppress bone resorption. The proform (−13–239) and fragment (−13–125) did not affect the suppressive activity, whereas fragments (1–111) and (126–239) did suppress the bone resorption. The bone-resorbing activity was also suppressed by fragment (47–111), not by fragment (1–46). Overlapping fragments (47–62), (47–79), (47–98), (56–111), (71–111), and (85–111) were compared for their suppressive activity. The fragments (47–62) and (85–111) did not affect the activity, but the other fragments suppressed the bone resorption. A synthetic peptide having the (71–79) sequence suppressed the bone resorption. These results suggest that amino acid sequence corresponding to rat caldecrin (aa 71–79) is responsible for the suppression of bone resorption by caldecrin. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Caldecrin; Protease; Bone resorption; Osteoclast

1. Introduction

It is well known that blood calcium is regulated by a complex network of calciotropic hormones, e.g. calcitonin, parathyroid hormone, 1α,25-$(OH)_2$ vitamin D_3, and prostaglandins and cytokines [1–3]. It has been reported that acute pancreatitis causes hypocalcemia, suggesting that the pancreas possesses hypocalcemic factors [4]. Earlier we reported the purification and cloning of a serum calcium-decreasing factor, referred to as caldecrin, from porcine, rat, and human pancreas [5–8]. Caldecrin is a novel serine protease with a structure like that of a member of the elastase family, although caldecrin is a chymotrypsin-type protease in terms of its activity [7–9]. Procaldecrin, which does not possess protease or serum calcium-decreasing activity, lowers blood calcium levels in mice as well as protease activity upon trypsin treatment [6]. However, the protease activity of activated caldecrin is abolished by treatment with the irreversible serine protease inhibitor, phenylmethyl sulfonyl fluoride (PMSF), but the protease retains its serum calcium-decreasing activity [5]. Protease activity-deficient mutants of recombinant caldecrin also lower blood calcium [7].

In this paper, we report that caldecrin suppressed bone resorption, we also examined various caldecrin fragments for their ability to suppress such resorption.

2. Materials and methods

2.1. Animals

10-day-old white male rabbits were purchased from Japan Laboratory Animals Inc. (Tokyo, Japan) and were used for preparation of unfractionated bone cells and for the bone resorption pit formation assay.

2.2. Protein expression

Expression of recombinant wild- and mutant-type caldecrins by the baculovirus expression system and purification were performed as described previously [7]. Culture media of Sf9 cells transfected with virus harboring wild-type or mutant-type caldecrin cDNAs (serine 187 mutated to alanine, or histidine 45 mutated to alanine to destroy protease activity) were collected 72 h after transfection, concentrated, and dialyzed against 10 mM sodium phosphate buffer (pH 6.8). Expressed recombinants were purified by Mono Q ion-exchange chromatography and dialyzed against phosphate-buffered saline (PBS).

To express recombinant caldecrin fragments in *Escherichia coli*, we generated histidine-tagged thioredoxin (HTRX) fusion protein expression vectors by subcloning the *Nde*I–*Bam*HI fragment of pTRXFus vector (Invitrogen) into the *Nde*I–*Bam*HI site of pET16b vector (Novagen). Full-length caldecrin or fragments were amplified by polymerase chain reaction (PCR) using the primers listed in Table 1.

Caldecrin proform (−13–239) and fragments (−13–125), (1–111), (1–46), (47–111), and (126–239) were amplified by using *Pfu* DNA polymerase (Stratagene), rat caldecrin cDNA as a template, and respective primer pairs 1 and 8, 1 and 7, 2 and 6, 2 and 5, 3 and 6, and 4 and 8 for 30 cycles (94°C, 30 s; 50°C, 30 s; 72°C, 1 min). PCR products were cloned into the *Sma*I site of the expression vector. Overlapping caldecrin fragments (47–62), (47–79), (47–98), (56–111), (71–111), and (85–111) were amplified using caldecrin (47–111) cDNA as a template and respective primer pairs, 9 and 10, 9 and 11, 9 and 12, 13 and 16, 14 and 16, and 15 and 16. PCR products (47–62), (47–79) and (47–98) were digested with *Rsr*II and *Bam*HI, and cloned into the *Rsr*II–*Bam*HI site of the expression vector. PCR products (56–111), (71–111) and (85–111) were digested with *Kpn*I and *Bam*HI, and cloned into the *Kpn*I–*Bam*HI site of the expression vector. All DNA constructs were sequenced by using a BigDye Terminal Cycle Se-

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Abbreviations: caldecrin, serum calcium-decreasing factor; PCR, polymerase chain reaction; PMSF, phenylmethyl sulfonyl fluoride; FBS, fetal bovine serum

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quencing kit and an ABI PRISM 310 genetic analyzer (PE Biosystems). The recombinant proteins were expressed in *E. coli* BL21(DE3)pLysS (Novagen) and their synthesis induced for 4 h in M9ZB medium with 1 mM isopropyl-β-D-thiogalactopyranoside at 30°C. Cells were harvested, solubilized in buffer (20 mM Tris–HCl (pH 7.9), 0.5 M NaCl, 1 mM PMSF), sonicated five times (30 s each time) and centrifuged at 10 000×*g* for 30 min at 4°C. The supernatants were applied onto a nickel-NTA-resin column. The resin was washed with buffer, and bound proteins were eluted with buffer (20 mM Tris–HCl (pH 7.9), 0.5 M NaCl, 1 mM imidazole, 1 mM PMSF). Recombinant proteins were dialyzed against PBS prior to use. Purification of recombinant proteins was evaluated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting using anti-caldecrin antibody and anti-TRX antibody (Invitrogen).

2.3. Activation of wild-type and mutant caldecrin

The purified recombinant wild-type and mutant caldecrins produced by baculovirus were activated for 30 min by trypsin at a ratio of 1:50, and then the activation was stopped by 1 mM 4-amidinophenylmethanesulfonyl fluoride. Activated caldecrin was incubated with or without 1 mM PMSF for 10 min to abolish its activated protease activity.

2.4. Preparation of synthetic peptides

Peptide fragments corresponding to rat caldecrin (aa 71–79; VYAEVDTIY) and reverse peptide (YITDVEAYV) were synthesized by Sawady Co. (Tokyo, Japan). The lyophilized peptide was dissolved in 30% dimethylsulfoxide at a concentration of 10 mg/ml. The stock solution was diluted with PBS and stored before use.

2.5. Culture and bone resorption pit assay

Preparation of bone cells and resorption pit formation assay were described elsewhere [10,11]. Briefly, unfractionated bone cells were prepared from tibiae, femora, humeri, ulnae, and radii of 10-day-old male rabbits. After removal of connective tissues, the long bones were minced in 20 ml of α-MEM medium (Gibco) supplemented with 5% fetal bovine serum (FBS) (ICN). After having been separated by vortexing for 30 s and sedimented for 2 min, unfractionated bone cells were centrifuged for 2 min at 500×*g*, and resuspended in the same medium. The cells were seeded onto dentine slices (6 mm in diameter and 0.07 mm in thickness) that had previously been plated with the medium in a 96-well plate. After 2 h of incubation, the culture medium was changed to fresh medium supplemented with 5% FBS and caldecrin or caldecrin fragments; and the cells were cultured at 37°C in humidified 10% CO_2 and 90% air. At 48 h of culture, the cells were scraped off the dentine slices, and the slices were stained with acid hematoxylin (Sigma-Aldrich) for 6 min to visualize the excavated pit formed by osteoclasts. Pit formation activity was expressed as a pit area by counting the number of mesh squares covered by the pits. In some cultures, cells were formaldehyde-fixed and stained for tartrate-resistant acid phosphatase (TRAP) using Sigma Diagnostics Acid Phosphatase, Leukocyte kit (Sigma-Aldrich). Total number of TRAP-positive multinucleated cells (MNCs) per well was determined.

3. Results and discussion

To determine whether caldecrin suppresses bone resorption as well as lowers the serum calcium level, we performed rabbit osteoclastic bone resorption pit formation assay. Recombinant activated wild-type caldecrin suppressed the bone resorption in a dose-dependent manner, of which suppression reached a maximum of about 50% at a 30 nM concentration of the protease (Fig. 1A). Treatment of the activated caldecrin with PMSF or mutation of histidine 45 or serine 187 to alanine (H45A and S187A) destroyed the protease activity but not the serum calcium-lowering activity, suggesting that the hypocalcemic activity of caldecrin has no connection with its protease activity [5]. Thus, caldecrins without protease activity were compared in the bone resorption pit assay. As shown in Fig. 1B, wild-type caldecrin inactivated by PMSF treatment suppressed the bone resorption equally well as the activated wild-type caldecrin. Again, activated protease activity-deficient mutant caldecrins, H45A and S187A, suppressed the bone resorption. The number of TRAP-positive osteoclastic MNCs was not significant between the culture with or without caldecrin (Table 2). These results suggest that caldecrin suppressed bone resorption independent of its protease activity and cytotoxicity, and that the suppression of the bone resorption may cause the serum calcium decrease.

To determine the domain(s) of caldecrin responsible for the suppression of osteoclastic bone-resorbing activity, we constructed several caldecrin fragments. The amino acid sequence of cattle chymotrypsin C (EC 3.4.21.2) is close to that of rat and human caldecrins. Chymotrypsin C is composed of two β-barrel-like structures, each of which contains six to seven β-sheets. The C-terminal barrel structure contains a short intermediate helical segment and an extended C-terminal helix [12]. The positions of cysteine residues of chymotrypsin C are conserved at the same positions in caldecrin, and the amino acids that differ between chymotrypsin C and caldecrins are in the same category of amino acids, suggesting that the three-dimensional structure of caldecrin may resemble that of chymotrypsin C. The X-ray structure of cattle chymotrypsin C dis-

Table 1
Primer sequence used for PCR

Primer no.	Primer sequence (5′–3′)	Starting or ending at amino acid position
1	S: 5′-TGCGGGAACCCCGCCTTCCCA-3′	starting at −13
2	S: 5′-GTGGTAGGAGGAGAGGATGCT-3′	starting at 1
3	S: 5′-ATCAACAAAGACTTCACTTAC-3′	starting at 47
4	S: 5′-TGCTATGTCACGGGCTGGGGT-3′	starting at 126
5	A: 5′-**TCA**GCAGTGGGCGGCAGTGAGGAC-3′	ending at 46
6	A: 5′-**TCA**GGCCACCTGGATGGTGTTGCT-3′	ending at 111
7	A: 5′-**TCA**GGGATAGTCCTGAGGCAGCAG-3′	ending at 125
8	A: 5′-**TCACAGTTG**TATTTTCTCGAA-3′	ending at 239
9	S: 5′-TGCGGTCCGTGCAAAATGAT-3′	starting at 33 of TRX
10	A: 5′-AAGGATCC**TCA**CAGATTATACTTCCCCA-3′	ending at 62
11	A: 5′-TTGGATCC**TCA**GTAGATGGTGTCCACCTC-3′	ending at 79
12	A: 5′-TTGGATCC**TCA**CAACTTAATGATAGCGAT-3′	ending at 98
13	S: 5′-TTGGTACCCGGCCTGGGGAAGTATAATCT-3′	starting at 56
14	S: 5′-AAGGTACCCGTGTACGCTGAGGTGGACA-3′	starting at 71
15	S: 5′-AAGGTACCCAACCGACTCTTCCTGTGGAA-3′	starting at 85
16	A: 5′-TTGGATCC**TCA**GGCCACCTGGATGGTGTTGCT-3′	ending at 111

S, sense primer; A, antisense primer. The stop codon is marked in bold. The restriction endonuclease site is underlined. *Rsr*II, CGGTCCG; *Bam*HI, GGATCC; *Kpn*I, GGTACC.

plays disulfide bonds formed between Cys (−13)–Cys 112, Cys 30–Cys 46, Cys 126–Cys 193, Cys 157–Cys 173, and Cys 183–Cys 214. Therefore, the following HTRX fused procaldecrin and its fragments were constructed: full-length rat caldecrin (aa −13–239), which is the proform of caldecrin; fragment (aa −13–125), which is an N-terminal barrel-like structure of the proform; fragment (aa 1–111), which is an N-terminal part of activated caldecrin; and fragment (126–239), which is a C-terminal barrel-like structure (Fig. 2A).

The recombinant proteins were compared by the bone-resorbing activity. As shown in Fig. 2B, HTRX did not affect the bone resorption when used as a control. HTRX caldecrin (−13–239) and HTRX caldecrin (−13–125) did not affect the bone-resorbing activity. The bone resorption was suppressed by HTRX caldecrin (1–111). The HTRX caldecrin (126–239) also suppressed the bone resorption. Moreover, with respect to the part of the active fragment (1–111), HTRX caldecrin (1–46) and (47–111) were constructed and examined for their bone-resorbing activity. The bone resorption was suppressed by HTRX caldecrin (47–111) but not by HTRX caldecrin (1–46). These results and the previous finding that activation of procaldecrin was required for the serum calcium-decreasing activity [4] suggest that suppressive domain for the bone resorption located in residues 47–111 requires a conformational change caused by trypsin activation to become exposed on the outer part of the molecule. Recently, it was reported that human elastase IIIB displayed hypocalcemic activity and suppression of bone resorption, both of which depended on its protease activity [13–15]. Homology of the amino acid sequence between rat caldecrin and human elastase IIIB is rather high (64.3%) in their C-terminal structure, compared with (47.3%) the N-terminal structure. Thus, the suppressive activity for the bone resorption of HTRX caldecrin (126–239) may be similar to that of elastase IIIB. However, caldecrin decreased serum calcium and suppressed the bone resorption without protease activity, suggesting that the mechanism of suppression of bone resorption by caldecrin without protease activity is different from that for the protease activity-dependent human elastase IIIB.

To address what part of the sequence was involved in suppressing the bone resorption, we constructed overlapping caldecrin fragments HTRX caldecrin (47–62), (47–79), (47–98), (56–111), (71–111), and (85–111) and compared their suppressive activities toward bone resorption (Fig. 2C). As shown in Fig. 2D, HTRX caldecrin (47–62) and (85–111) did not affect the suppressive activity, but the other fragments, HTRX caldecrin (47–79), (47–98), (56–111) and (71–111), were active in the suppression. These results suggest that the suppressive sequence for bone resorption may be caldecrin residues 71–79. The region is different from that of human elastase IIIB.

Finally, we synthesized a peptide corresponding to caldecrin 71–79 and measured its suppressive activity toward bone resorption. As shown in Fig. 3, this peptide suppressed the bone resorption in a dose-dependent manner whereas the reverse peptide had no effect, suggesting caldecrin 71–79 to be the suppressive sequence. The region may locate in a β-sheet structure deduced from the crystallographic results on chymotrypsin C [12].

The mechanism of the suppression of the bone resorption by caldecrin 71–79 is presently unclear. This amino acid sequence has no homology with that of other calciotropic hormones or factors. Osteoclasts are the primary cells responsible for bone resorption and are controlled by stromal cells through the balanced regulatory mechanism of osteoclast differentiation factor (ODF) and osteoprotegrin, and by binding

Table 2
TRAP-positive MNCs in culture

Treatment	TRAP-positive MNCs/well
Control	138 ± 22
Wild-type caldecrin	131 ± 13
+PMSF	134 ± 20
Mutant caldecrin	
H45A	135 ± 31
S187A	132 ± 23

Data are mean ± S.D.

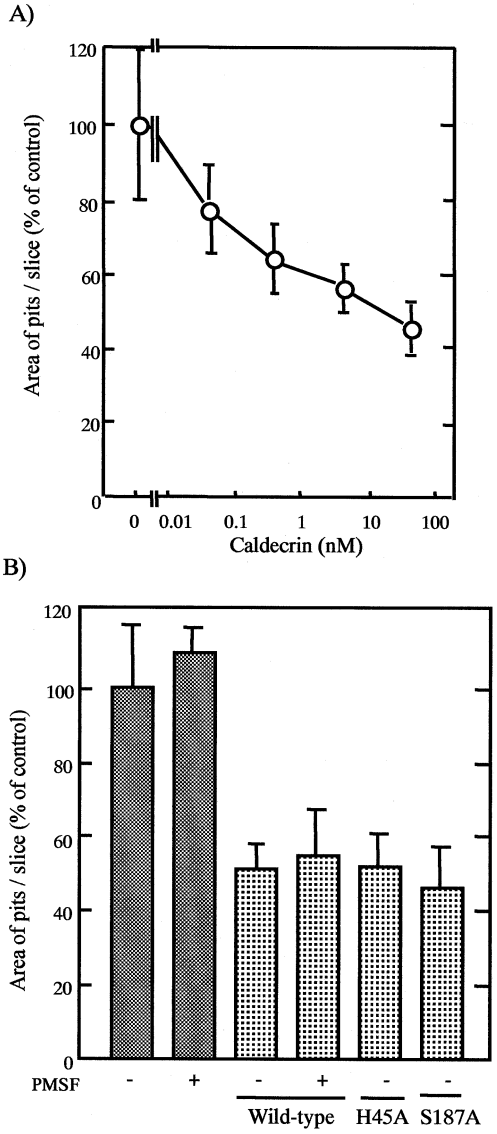

Fig. 1. Suppression of bone resorption by recombinant rat caldecrin. A: Dose-dependent suppression of the bone resorption by wild-type rat caldecrin. B: Wild-type caldecrin with or without protease activity, and protease activity-deficient mutant caldecrins suppressed the bone resorption. Purified recombinant rat caldecrin with or without PMSF treatment or protease activity-deficient mutants (S187A, H45A) (30 nM) were used for the bone resorption pit formation assay. Data are expressed as means ± S.D. values obtained from quadruplicates of two experiments.

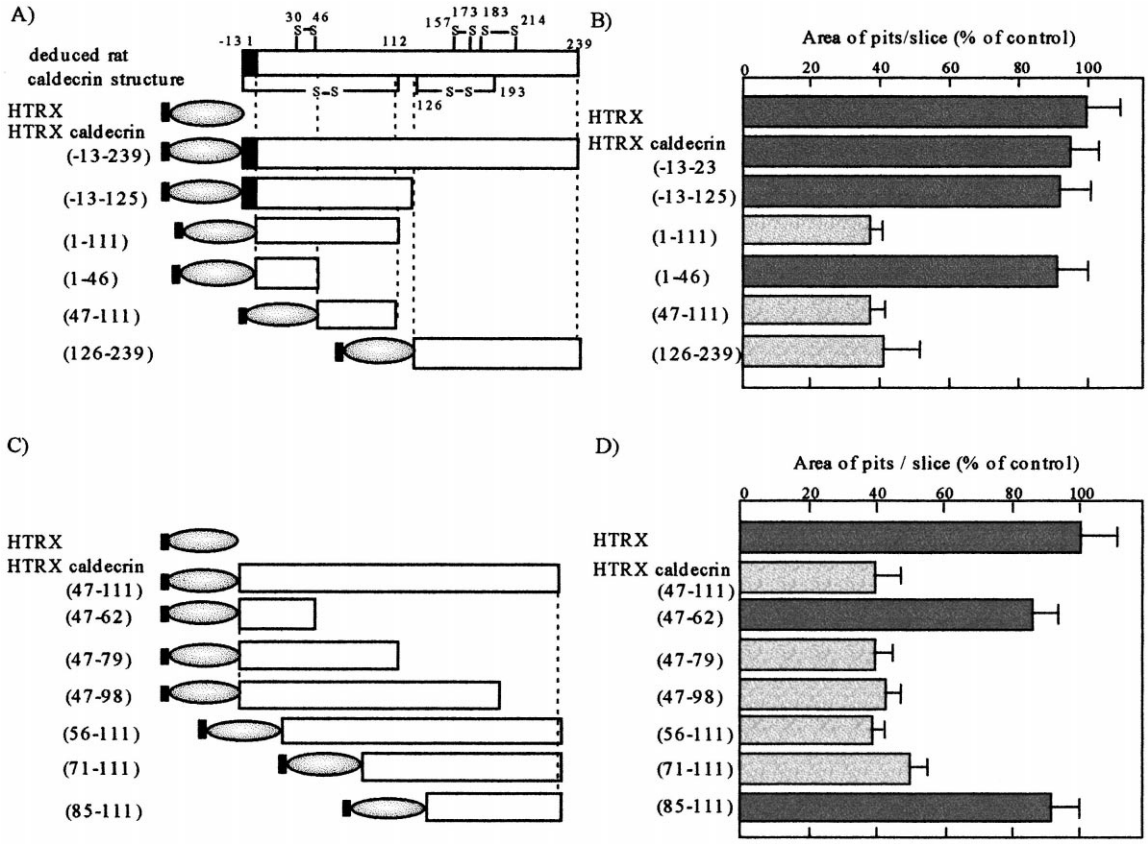

Fig. 2. Caldecrin fragments and their bone-resorbing activities. A: Structure of caldecrin and construction of caldecrin fragments. Structure of rat caldecrin is deduced from that of cattle chymotrypsin C (upper low of A). S–S means disulfide bridge and number represents the position of cysteine residue. Dotted box represents activation peptide (aa −13) and filled box and gray-colored oval represent histidine tag and TRX, respectively. B: The bone-resorbing activities of caldecrin or caldecrin fragments. C: Construction of overlapping caldecrin fragments. D: The bone-resorbing activities of the overlapping caldecrin fragments. Data are expressed as means ± S.D. values obtained from quadruplicates of three experiments.

of ODF to its receptor activator of nuclear factor-κB (RANK) on the osteoclasts [16,17]. ODF is a member of the tumor necrosis factor-related ligand family, which ligand is composed of β-strand [16]. The β-strand in the receptor-binding domain of vascular endothelial growth factor forms a complex with a 20-mer receptor-blocking peptide containing a β-strand [18]. The possibility of caldecrin interaction with RANK or some unknown receptor or with ODF may cause the suppression of the bone resorption. The inhibiting activity of the peptide 71–79 to the bone resorption was less than that of the recombinant protein (Figs. 1A and 3). It may be considered that steric factor might enhance the affinity of interaction of caldecrin with target proteins. In any event, the segment comprising residues 71–79 of rat caldecrin contains the major determinant suppressing bone resorption.

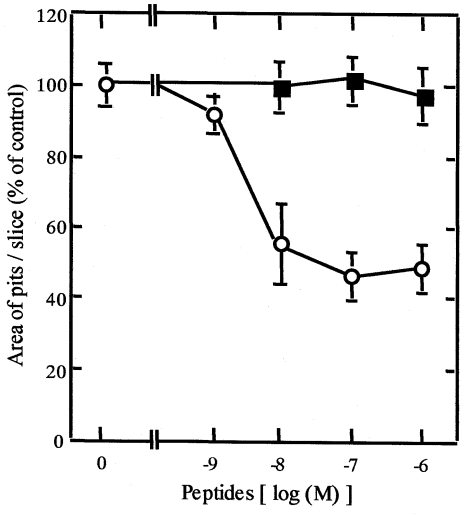

Fig. 3. Effect of synthetic peptide residues 71–79 on the bone resorption. Caldecrin 71–79 peptide (VYAEVDTIY) (○) and reverse peptide (YITDVEAYV) (■) were used for the bone resorption assay. Data are expressed as means ± S.D. values obtained from quadruplicates of three experiments.

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