**Name of Journal: *World Journal of Biological Chemistry***

**ESPS Manuscript NO: 20116**

**Manuscript Type: Minireviews**

**Caldecrin: A pancreas-derived hypocalcemic factor, regulates osteoclast formation and function**

Tomomura M *et al*. Caldecrin is an anti-osteoclastogenic factor

**Mineko Tomomura, Akito Tomomura**

**Mineko Tomomura,** Meikai Pharmaco-Medical Laboratory, MPL, and Division of Biochemistry, Department of Oral Biology and Tissue Engineering, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan

**Akito Tomomura,** Division of Biochemistry, Department of Oral Biology and Tissue Engineering, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan

**Author contributions:** Tomomura M and Tomomura A contributed equally to the writing of the manuscript.

**Conflict-of-interest statement:** The authors declare no conflict of interest associated with this manuscript.

**Open-Access:** This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Correspondence to: Mineko Tomomura, PhD, Associate Professor,** Meikai Pharmaco-Medical Laboratory, MPL, and Division of Biochemistry, Department of Oral Biology and Tissue Engineering, Meikai University School of Dentistry, 1-1 Keyakidai, Sakado, Saitama 350-0283, Japan. mineko-t@dent.meikai.ac.jp

**Telephone:** +81-49-2792766

**Fax:** +81-49-2712503

**Received:** May 29, 2015

**Peer-review started:** May 30, 2015

**First decision:** August 7, 2015

**Revised:** September 1, 2015

**Accepted:** September 29, 2015

**Article in press:**

**Published online:**

**Abstract**

Caldecrin was originally isolated from the pancreas as a factor that reduced serum calcium levels. This secreted serine protease has chymotrypsin-like activity and is also known as chymotrypsin C; it belongs to the elastase family. Although intravenous administration of caldecrin decreases the serum calcium concentration even when its protease activity is blocked, this effect does require cleavage of caldecrin’s pro-peptide by trypsin, converting it to the mature enzyme. Ectopic intramuscular expression of caldecrin prevented bone resorption in ovariectomized mice. Caldecrin inhibited parathyroid hormone-stimulated calcium release from fetal mouse long bone organ cultures. Furthermore, caldecrin suppressed the formation of osteoclasts from bone marrow cells by inhibiting the receptor activator of nuclear factor-kappa B ligand (RANKL)-stimulated phospholipase Cγ-calcium oscillation-calcineurin-nuclear factor of activated T-cells, cytoplasmic 1 pathway. Caldecrin also suppressed the bone resorption activity of mature osteoclasts by preventing RANKL-stimulated Src activation, calcium entry, and actin ring formation. *In vivo* and *in vitro* studies have indicated that caldecrin is a unique multifunctional protease with anti-osteoclastogenic activities that are distinct from its protease activity. Caldecrin might be a potential therapeutic target for the treatment of osteolytic diseases such as osteoporosis and osteoarthritis. This mini-review describes caldecrin’s historical background and its mechanisms of action.

**Key words:** Serine protease; Hypocalcemia; Chymotrypsin; Osteoclasts; Bone resorption; Calcium signaling

**© The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** Caldecrin (also known as chymotrypsin C) reduces serum calcium levels. This activity is distinct from its protease activity but also requires trypsin-mediated cleavage of the pro-peptide, converting caldecrin to its active form. Ectopic intramuscular expression of caldecrin prevented bone resorption in ovariectomized mice. Caldecrin inhibited parathyroid hormone-stimulated calcium release from fetal mouse long bones. Furthermore, caldecrin suppressed receptor activator of nuclear factor-kappa B ligand-induced activation of intracellular calcium signaling, thereby reducing osteoclast formation and bone resorption. Caldecrin is a unique multifunctional protease that possesses anti-osteoclastogenic activity, resulting in reduced serum calcium levels.

Tomomura M, Tomomura A. Caldecrin: A pancreas-derived hypocalcemic factor, regulates osteoclast formation and function. *World J Biol Chem* 2015; In press

**INTRODUCTION**

Calcium homeostasis is controlled by intestinal calcium absorption and calcium resorption in the kidney, as well as by bone formation and resorption. Clinical and experimental observations have also linked the pancreas to calcium homeostasis. Pancreas-derived glucagon[1,2], amylin[3,4], and calcitonin gene-related peptide[5,6] have been shown to regulate calcium homeostasis, while acute and chronic pancreatitis have been shown to associate with hypocalcemia[7].

In the 1960s, the pioneering work of Takaoka *et al*[8,9] demonstrated that a porcine pancreatic extract had hypocalcemic activity. In 1992, we first successfully purified a hypocalcemic factor named caldecrin from a pancreatic extract using chromatographic separation techniques including ion exchange, gel filtration chromatography, and high-performance liquid chromatography[10]. To identify caldecrin, each fraction was intravenously administered to overnight-fasted mice and serum calcium concentrations were measured 4 h post-injection. In addition, the samples were assayed for their inhibition of parathyroid hormone-stimulated calcium release from fetal mouse long bone organ cultures. Caldecrin is an anionic protein (pI: 4.5) with a molecular weight of about 28 kDa; it was found to be a serine protease with chymotryptic activity.

In 1995, we isolated rat caldecrin cDNA from pancreatic cDNA expression library by immunoscreening with an anti-caldecrin antibody[11]. A partial amino acid sequence of caldecrin purified from rat pancreas was completely matched with that encoded by the cDNA. The nucleotide sequence was almost identical (except for three nucleotides) to that of a PCR clone referred as elastase IV (ELA4)[12]. Comparison of the amino acid sequences encoded by these two cDNAs indicated that the central region of caldecrin differed from that of ELA4 due to a frame shift caused by this minor nucleotide change (Figure 1). The amino acid sequences of the purified caldecrin fragments, including the central region, were consistent with the deduced amino acid sequence of caldecrin but not with that of ELA4. Over-expression of the ELA4 PCR clone in Sf9 cells caused a complete loss of secretion, low expression levels, and much lower protease activity[13]. Furthermore, the rat genomic DNA sequence matched that of the caldecrin cDNA, but not that of the ELA4 clone[13]. Therefore, the ELA4 PCR clone may be a cloning artifact or represent a mutant caldecrin gene. In 1995, the crystalline structure of bovine chymotrypsinogen C was reported[14-16] and its amino acid sequence was very close to that of rat caldecrin, thereby suggesting a similarity between caldecrin and chymotrypsin C (CTRC). It is now known that CTRC, caldecrin, and ELA4 are the same protein, which is encoded by the *CTRC* gene and known officially as CTRC (caldecrin), according to the HUGO Gene Nomenclature Committee. Table 1 compares the amino acid sequence of rat caldecrin with that of other members of the rat and human pancreatic chymotrypsin and elastase families. Caldecrin shows a greater similarity with elastase than with chymotrypsin. In addition, expressed recombinant human caldecrin also showed serum calcium-decreasing activity, even following phenylmethylsulfonyl fluoride treatment to abolish its protease activity[17].

In 1996, another research group purified a calcium metabolism-regulating factor from the porcine pancreas by determining its stimulatory effects on proliferation of the osteosarcoma MG-63 cell line and its inhibition of 1, 25 vitamin D3-stimulated calcium release in organ cultures[18]. The terminal sequence of the 28-kDa protein that was isolated corresponded to that of human elastase IIIB. Recombinant elastase IIIB decreased interleukin-1-induced hypercalcemia and this effect was dependent on its protease activity. Although both have been isolated from the pancreas, caldecrin and elastase IIIB were found to be different molecules that exerted their hypocalcemic effects via different mechanisms of action.

**PROTEIN STRUCTURE AND PROTEASE ACTIVITY OF CALDECRIN**

The human *CTRC* gene maps to chromosome 1p36.21. The homologous mouse and rat genes are located on chromosomes 4E1 and 5q36, respectively. The *CTRC* genes consist of 8 exons in these species. Northern blot analysis has indicated that caldecrin is mainly expressed in the pancreas (Figure 2A).

CTRC (caldecrin) is a single protein consisting of 268 amino acids, with a signal peptide (16 amino acids), pro-peptide (13 amino acids), and the mature protein (239 amino acids; Figure 2B). The three-dimensional structure demonstrated that five disulfide bridges were formed at Cys1-Cys125 (according to the chymotrypsin numbering), Cys43-Cys59, Cys139-Cys206, Cys170-Cys186, and Cys196-Cys227 (Figure 2B). CTRC (caldecrin) was shown to have a two-barrel structure, each composed of 6‑7 β-sheets and a C-terminal α-helix long tail[14-16] (Figure 2C). Following tryptic cleavage at Arg13-Val14, the caldecrin pro-peptide remains associated with the mature enzyme via the Cys1-Cys125 disulfide bridge; this generates a structure resembling those of chymotrypsin A and B, as well as elastase IIA, but not those of elastase I, IIIA, and IIIB, where the pro-peptide is removed from the mature enzyme after tryptic activation[11,14-16].

CTRC (caldecrin) is a serine protease with the characteristic charge-relayed catalytic triad (His58, Asp105, and Ser200), located in the active site cleft between the barrel structures[14-16]. After tryptic activation, caldecrin changes its structure to a substrate-accessible catalytic cleft form. Active caldecrin hydrolyzes the leucyl bond (*e.g.*, in the N-Succinyl-Ala-Ala-Pro-Leu-p-nitroanilide substrate) more efficiently than chymotrypsin A and B; Caldecrin also cleaves the phenylalanyl bond (*e.g.*, in the N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide substrate) and the tyrosyl bond (*e.g.*, in the N-Succinyl-Leu-Leu-Val-Tyr-p-nitroanilide substrate)[10,19-21]. The protease activity of caldecrin is inhibited by serine protease inhibitors (phenylmethylsulfonyl fluoride or diisopropyl fluorophosphate), chymotrypsin inhibitor (chymostatin), and the Bowman-Birk trypsin and chymotrypsin inhibitor. The amino acid sequence and protease activity of caldecrin indicate that it is a hybrid of chymotrypsin and elastase.

**CALDECRIN AND BONE METABOLISM**

Caldecrin produces dose-dependent decreases in serum calcium concentrations[10]. The administration of purified porcine and rat caldecrin via the tail vein of mice decreased their serum calcium concentration dose-dependently and the maximum effect was attained 2‑4 h post-injection with 20‑100 µg (about 0.7–3.6 nmol)/kg body weight. The hypocalcemic potency of caldecrin was almost equivalent to that of porcine calcitonin (1 nmol/kg body weight, Tomomura *et al*[10] data not shown). The caldecrin proform (procaldecrin), purified from the porcine pancreas in the presence of diisopropyl fluorophosphate, appeared to show time- and concentration-dependent chymotryptic activity following cleavage by trypsin. Administration of activated caldecrin reduced the serum calcium level in mice, even after treatment with the serine protease inhibitor, phenylmethylsulfonyl fluoride, which abolished the chymotryptic activity. However, administration of procaldecrin did not decrease serum calcium levels[22]. Recombinant rat[11] and human[17] caldecrin also decreased serum calcium levels. In addition, rat protease activity-deficient caldecrin mutants (with His58Ala or Ser200Ala substitutions) decreased the levels of serum calcium. Therefore, the effect of caldecrin on serum calcium levels *in vivo* requires its activation by trypsin cleavage. An intramolecular responsive region required for this calcium decreasing activity may therefore be exposed by trypsin activation.

The caldecrin-induced serum calcium decrease occurred concomitantly with a decrease in the serum concentration of hydroxyproline, which is a marker of bone resorption. This observation suggested that this serum calcium decrease may be due to the suppression of bone resorption[10]. The effects of caldecrin on osteoclast function have also been investigated; recombinant wild-type and protease activity-deficient mutant caldecrin produced concentration-dependent suppression of bone resorption in isolated rabbit mature osteoclasts[23].

Osteoclasts execute bone resorption, which is modulated by macrophage colony-stimulating factor and receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL), produced by osteoblasts and osteocytes. An imbalance between bone formation and resorption leads to bone diseases, including osteoporosis. Osteoclast differentiation and maturation involves the following three steps: (1) Osteoclast precursor cells are generated from bone marrow cells in response to macrophage colony-stimulating factor; (2) osteoclasts begin to differentiate from the precursor cells following stimulation by RANKL; and (3) at the later stage of differentiation, osteoclasts fuse to become multinucleated giant cells, leading to the cytoskeletal actin ring formation required for bone resorption. These processes are tightly regulated to maintain bone homeostasis, and many molecules are involved in osteoclast differentiation[24-26]. The key molecule involved in osteoclastogenesis is RANKL, which is a member of the tumor necrosis factor superfamily that is expressed by osteoblasts and osteocytes in membrane-bound and secreted forms[27-33]. RANKL induces osteoclast differentiation by activating two signaling pathways: the mitogen-activated protein kinase (MAPK), NF-κB, and c-Fos activation axis and the phospholipase C γ (PLCγ)-mediated calcium oscillation-calcineurin-nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) axis. Caldecrin did not inhibit macrophage colony-stimulating factor-induced osteoclast progenitor formation from bone marrow cells but did inhibit RANKL-induced osteoclast differentiation, even in the absence of protease activity[34]. Caldecrin inhibited the RANKL-stimulated spleen tyrosine kinase- and PLCγ-induced calcium oscillation, leading to an inhibition of calcineurin and NFATc1 activity (Figure 3). Caldecrin also inhibited the RANKL-mediated actin ring formation in mature osteoclasts, which is associated with RANKL-evoked calcium entry via the transient receptor potential vanilloid channel 4[35]. Caldecrin significantly inhibited RANKL-stimulated phosphorylation of c-Src in association with spleen tyrosine kinase, which is upstream of transient receptor potential vanilloid channel 4 and actin ring formation. On the other hand, caldecrin did not inhibit RANKL-mediated stimulation of MAPK, NF-κB, and c-Fos activation in osteoclast precursors or mature osteoclasts[34,35]. Therefore, caldecrin antagonized the RANKL-stimulated calcium signaling pathway involved in both osteoclast differentiation and activation.

Caldecrin is a therapeutic target in osteoporosis. The ovariectomized (OVX) mouse provides a model of postmenopausal osteoporosis and exhibits an increased serum calcium level due to elevated bone resorption. This is evidenced by an increase in the bone surface to bone volume ratio, increased trabecular separation, decreased bone volume density, and decreased trabecular thickness and number. Expression of the caldecrin plasmid vector, which harbors the wild-type rat caldecrin cDNA, in the femoral muscle of this mouse model reversed this increase in serum calcium levels and restored bone resorption parameters to normal levels[36].

An important, but unaddressed, question relates to how the caldecrin released from the pancreas targets the bone. Recently, osteocalcin, which is osteoblast-derived, stored in the bone matrix, and then released by osteoclastic bone resorption, was shown to increase insulin secretion from pancreatic islets[37,38]. This activity appears to provide a physiological link between bone and pancreas, in relation to the regulation of energy metabolism. It is possible that some of the caldecrin derived from the pancreas enters the circulation and then inhibits osteoclasts, in order to regulate calcium homeostasis. The physiological activation and functions of caldecrin are not defined; however, considering its obvious effects on serum calcium levels and osteogenesis, caldecrin might be an intrinsic calcium regulating factor. The expression and distribution of caldecrin, peptide fragments of caldecrin, and its binding proteins should be explored in order to determine their physiological roles in bone metabolism.

**OTHER BIOLOGICAL ASPECTS**

The *CTRC* gene modulates risk for pancreatitis. Rosendahl *et al*[39] reported that *CTRC* gene mutations were significantly associated with hereditary chronic pancreatitis. Masson *et al*[40] also identified a *CTRC* mutation in patients with idiopathic chronic pancreatitis. CTRC hydrolyzes the pro-peptide and calcium-binding loop of the trypsinogens, enhancing their activation and degradation, respectively[41-43]. Loss-of-function *CTRC* variants increase the risk for chronic pancreatitis. *CTRC* is also a susceptibility gene for tropical calcific pancreatitis, which is a juvenile form of chronic nonalcoholic pancreatitis that occurs in Asians and Africans and is associated with nearly 90% pancreatic calcium deposition[44].

It is of clinical interest that five decades ago, Takaoka *et al*[8,9,45] administered pancreatic extract to patients diagnosed with myasthenia gravis and muscular dystrophy. The symptoms of the patients treated with the extract improved progressively, suggesting that the hypocalcemic effect of the extract could have contributed to protecting them against the development of muscular dystrophy. The effect of caldecrin was also investigated in the dy/dy muscular dystrophic mouse model[46]. These mice genetically lack M-laminin and exhibit defective muscle basement membranes. Peritoneal administration of caldecrin protein or intramuscular expression of a caldecrin vector inhibited muscular destruction in the dy/dy mice. This indicated that caldecrin was responsible for the effects of the pancreatic extract on muscular dystrophy.

In 2011, Lacruz *et al*[47] found that CTRC (caldecrin) was expressed by ameloblasts and was up-regulated during enamel maturation, suggesting that caldecrin might be involved in tooth development.

CTRC (caldecrin) has been reported to be associated with pancreatic cancer, where its expression is drastically reduced. Individuals with chronic pancreatitis who show low or no activity of caldecrin show an increased risk for pancreatic cancer[48]. Furthermore, Wang *et al*[49] demonstrated that overexpression of CTRC (caldecrin) downregulated the migration of human pancreatic adenocarcinoma Aspc-1 cells, whereas the knockdown of CTRC (caldecrin) increased cell migration. It would be interesting to explore the potential use of caldecrin in pancreatic cancer diagnosis and treatment. In addition, breast cancer is highly associated with osteolytic metastatic disease. RANKL is important in mammary gland development and also in the progression of metastatic breast cancer cells[50,51]. RANKL may partly contribute to the activation of metastatic breast cancer via the calcineurin/NFAT pathway[52], which is modulated by caldecrin. It would therefore be interesting to investigate whether caldecrin suppresses RANKL-dependent tumor metastases.

**CONCLUSION**

The serum calcium-decreasing factor, caldecrin, was discovered in the pancreas. Caldecrin inhibits osteoclast differentiation and bone resorption in mature osteoclasts via inhibition of RANKL-induced intracellular calcium signaling. This effect occurs independently of its inherent protease activity. Therefore, caldecrin might be a potential therapeutic target for the treatment of osteolytic diseases such as osteoporosis and osteoarthritis.

**REFERENCES**

1 **Williams GA**, Bowser EN, Henderson WJ. Mode of hypocalcemic action of glucagon in the rat. *Endocrinology* 1969; **85**: 538-541 [PMID: 5793033 DOI: 10.1210/endo-85-3-537]

2 **Stern PH**, Bell NH. Effects of glucagon on serum calcium in the rat and on bone resorption in tissue culture. *Endocrinology* 1970; **87**: 111-117 [PMID: 4315590 DOI: 10.1210/endo-87-1-111]

3 **Zaidi M**, Datta HK, Bevis PJ, Wimalawansa SJ, MacIntyre I. Amylin-amide: a new bone-conserving peptide from the pancreas. *Exp Physiol* 1990; **75**: 529-536 [PMID: 2223054 DOI: 10.1113/expphysiol.1990.sp003429]

4 **Alam AS**, Moonga BS, Bevis PJ, Huang CL, Zaidi M. Amylin inhibits bone resorption by a direct effect on the motility of rat osteoclasts. *Exp Physiol* 1993; **78**: 183-196 [PMID: 8385961 DOI: 10.1113/expphysiol.1993.sp003679]

5 **D'Souza SM**, MacIntyre I, Girgis SI, Mundy GR. Human synthetic calcitonin gene-related peptide inhibits bone resorption in vitro. *Endocrinology* 1986; **119**: 58-61 [PMID: 3487444 DOI: 10.1210/endo-119-1-58]

6 **Zaidi M**, Chambers TJ, Gaines Das RE, Morris HR, MacIntyre I. A direct action of human calcitonin gene-related peptide on isolated osteoclasts. *J Endocrinol* 1987; **115**: 511-518 [PMID: 3502132 DOI: 10.1677/joe.0.1150511]

7 **D'Souza A**, Floch MH. Calcium metabolism in pancreatic disease. *Am J Clin Nutr* 1973; **26**: 352-361 [PMID: 4347666]

8 **Takaoka Y**, Hiwaki C, Ozawa H, Ichinose M, Otsubo Y, Shikaya T. A pancreatic protein anabolic extract. Proposal of a protein anabolic extract from pancreas. I. Preliminary report. *Acta Med Nagasaki* 1966; **10**: 51-57 [PMID: 5961056]

9 **Takaoka Y**, Takamori M, Ichinose M, Shikaya T, Igawa N. Hypocalcemic action of a pancreatic factor and its clinical significance on the myasthenic patients. *Acta Med Nagasaki* 1969; **14**: 28-35 [PMID: 5393331]

10 **Tomomura A**, Fukushige T, Noda T, Noikura T, Saheki T. Serum calcium-decreasing factor (caldecrin) from porcine pancreas has proteolytic activity which has no clear connection with the calcium decrease. *FEBS Lett* 1992; **301**: 277-281 [PMID: 1577166 DOI: 10.1016/0014-5793(92)80256-G]

11 **Tomomura A**, Tomomura M, Fukushige T, Akiyama M, Kubota N, Kumaki K, Nishii Y, Noikura T, Saheki T. Molecular cloning and expression of serum calcium-decreasing factor (caldecrin). *J Biol Chem* 1995; **270**: 30315-30321 [PMID: 8530454 DOI: 10.1074/jbc.270.51.30315]

12 **Kang J**, Wiegand U, Müller-Hill B. Identification of cDNAs encoding two novel rat pancreatic serine proteases. *Gene* 1992; **110**: 181-187 [PMID: 1537555 DOI: 10.1016/0378-1119(92)90646-7]

13 **Yoshino-Yasuda I**, Kobayashi K, Akiyama M, Itoh H, Tomomura A, Saheki T. Caldecrin is a novel-type serine protease expressed in pancreas, but its homologue, elastase IV, is an artifact during cloning derived from caldecrin gene. *J Biochem* 1998; **123**: 546-554 [PMID: 9538241 DOI: 10.1093/oxfordjournals.jbchem.a021971]

14 **Gomis-Rüth FX**, Gómez M, Bode W, Huber R, Avilés FX. The three-dimensional structure of the native ternary complex of bovine pancreatic procarboxypeptidase A with proproteinase E and chymotrypsinogen C. *EMBO J* 1995; **14**: 4387-4394 [PMID: 7556081]

15 **Gomis-Rüth FX**, Gómez-Ortiz M, Vendrell J, Ventura S, Bode W, Huber R, Avilés FX. Crystal structure of an oligomer of proteolytic zymogens: detailed conformational analysis of the bovine ternary complex and implications for their activation. *J Mol Biol* 1997; **269**: 861-880 [PMID: 9223647 DOI: 10.1006/jmbi.1997.1040]

16 **Batra J**, Szabó A, Caulfield TR, Soares AS, Sahin-Tóth M, Radisky ES. Long-range electrostatic complementarity governs substrate recognition by human chymotrypsin C, a key regulator of digestive enzyme activation. *J Biol Chem* 2013; **288**: 9848-9859 [PMID: 23430245 DOI: 10.1074/jbc.M113.457382]

17 **Tomomura A**, Akiyama M, Itoh H, Yoshino I, Tomomura M, Nishii Y, Noikura T, Saheki T. Molecular cloning and expression of human caldecrin. *FEBS Lett* 1996; **386**: 26-28 [PMID: 8635596 DOI: 10.1016/0014-5793(96)00377-8]

18 **Izbicka E**, Yoneda T, Takaoka Y, Horn D, Williams P, Mundy GR. Identification of a novel bone/calcium metabolism-regulating factor in porcine pancreas. *J Biol Chem* 1996; **271**: 23230-23234 [PMID: 8798519 DOI: 10.1074/jbc.271.38.23230]

19 **Folk JE**, Schirmer EW. Chymotrypsin C. I. Isolation of the zymogen and the active enzyme: preliminary structure and specificities. *J Biol Chem* 1965; **240**: 181-192 [PMID: 14253410]

20 **Folk JE**, Cole PW. Chymotrypsin C. II. Enzymatic specificity toward several polypeptides. *J Biol Chem* 1965; **240**: 193-197 [PMID: 14253411]

21 **Keil-Dlouha V**, Puigserver A, Marie A, Keil B. On subunit II of bovine procarboxypeptidase A. Enzymatic specificity after tryptic activation. *Biochim Biophys Acta* 1972; **276**: 531-535 [PMID: 4672120 DOI: 10.1016/0005-2744(72)91013-3]

22 **Tomomura A**, Fukushige T, Tomomura M, Noikura T, Nishii Y, Saheki T. Caldecrin proform requires trypsin activation for the acquisition of serum calcium-decreasing activity. *FEBS Lett* 1993; **335**: 213-216 [PMID: 8253199 DOI: 10.1016/0014-5793(93)80732-A]

23 **Tomomura A**, Yamada H, Fujimoto K, Inaba A, Katoh S. Determination of amino acid sequence responsible for suppression of bone resorption by serum calcium-decreasing factor (caldecrin). *FEBS Lett* 2001; **508**: 454-458 [PMID: 11728471 DOI: 10.1016/S0014-5793(01)03107-6]

24 **Suda T**, Takahashi N, Martin TJ. Modulation of osteoclast differentiation. *Endocr Rev* 1992; **13**: 66-80 [PMID: 1555533]

25 **Teitelbaum SL**. Bone resorption by osteoclasts. *Science* 2000; **289**: 1504-1508 [PMID: 10968780 DOI: 10.1126/science.289.5484.1504]

26 **Asagiri M**, Takayanagi H. The molecular understanding of osteoclast differentiation. *Bone* 2007; **40**: 251-264 [PMID: 17098490 DOI: 10.1016/j.bone.2006.09.023]

27 **Anderson DM**, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* 1997; **390**: 175-179 [PMID: 9367155 DOI: 10.1038/36593]

28 **Wong BR**, Rho J, Arron J, Robinson E, Orlinick J, Chao M, Kalachikov S, Cayani E, Bartlett FS, Frankel WN, Lee SY, Choi Y. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J Biol Chem* 1997; **272**: 25190-25194 [PMID: 9312132 DOI: 10.1074/jbc.272.40.25190]

29 **Lacey DL**, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998; **93**: 165-176 [PMID: 9568710 DOI: 10.1016/S0092-8674(00)81569-X]

30 **Yasuda H**, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A* 1998; **95**: 3597-3602 [PMID: 9520411 DOI: 10.1073/pnas.95.7.3597]

31 **Fuller K**, Wong B, Fox S, Choi Y, Chambers TJ. TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *J Exp Med* 1998; **188**: 997-1001 [PMID: 9730902 DOI: 10.1084/jem.188.5.997]

32 **Nakashima T**, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, Bonewald LF, Kodama T, Wutz A, Wagner EF, Penninger JM, Takayanagi H. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med* 2011; **17**: 1231-1234 [PMID: 21909105 DOI: 10.1038/nm.2452]

33 **Xiong J**, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. *Nat Med* 2011; **17**: 1235-1241 [PMID: 21909103 DOI: 10.1038/nm.2448]

34 **Hasegawa H**, Kido S, Tomomura M, Fujimoto K, Ohi M, Kiyomura M, Kanegae H, Inaba A, Sakagami H, Tomomura A. Serum calcium-decreasing factor, caldecrin, inhibits osteoclast differentiation by suppression of NFATc1 activity. *J Biol Chem* 2010; **285**: 25448-25457 [PMID: 20547767 DOI: 10.1074/jbc.M109.068742]

35 **Tomomura M**, Hasegawa H, Suda N, Sakagami H, Tomomura A. Serum calcium-decreasing factor, caldecrin, inhibits receptor activator of NF-κB ligand (RANKL)-mediated Ca2+ signaling and actin ring formation in mature osteoclasts via suppression of Src signaling pathway. *J Biol Chem* 2012; **287**: 17963-17974 [PMID: 22461633 DOI: 10.1074/jbc.M112.358796]

36 **Oi M**, Kido S, Hasegawa H, Fujimoto K, Tomomura M, Kanegae H, Suda N, Tomomura A. Inhibitory effects on bone resorption in postmenopausal osteoporosis model mice by delivery of serum calcium decreasing factor (Caldecrin) gene. *J Meikai Dent Med* 2011; **40**: 146−154

37 **Lee NK**, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C, Dacquin R, Mee PJ, McKee MD, Jung DY, Zhang Z, Kim JK, Mauvais-Jarvis F, Ducy P, Karsenty G. Endocrine regulation of energy metabolism by the skeleton. *Cell* 2007; **130**: 456-469 [PMID: 17693256 DOI: 10.1016/j.cell.2007.05.047]

38 **Confavreux CB**, Levine RL, Karsenty G. A paradigm of integrative physiology, the crosstalk between bone and energy metabolisms. *Mol Cell Endocrinol* 2009; **310**: 21-29 [PMID: 19376193 DOI: 10.1016/j.mce.2009.04.004]

39 **Rosendahl J**, Witt H, Szmola R, Bhatia E, Ozsvári B, Landt O, Schulz HU, Gress TM, Pfützer R, Löhr M, Kovacs P, Blüher M, Stumvoll M, Choudhuri G, Hegyi P, te Morsche RH, Drenth JP, Truninger K, Macek M, Puhl G, Witt U, Schmidt H, Büning C, Ockenga J, Kage A, Groneberg DA, Nickel R, Berg T, Wiedenmann B, Bödeker H, Keim V, Mössner J, Teich N, Sahin-Tóth M. Chymotrypsin C (CTRC) variants that diminish activity or secretion are associated with chronic pancreatitis. *Nat Genet* 2008; **40**: 78-82 [PMID: 18059268 DOI: 10.1038/ng.2007.44]

40 **Masson E**, Chen JM, Scotet V, Le Maréchal C, Férec C. Association of rare chymotrypsinogen C (CTRC) gene variations in patients with idiopathic chronic pancreatitis. *Hum Genet* 2008; **123**: 83-91 [PMID: 18172691 DOI: 10.1007/s00439-007-0459-3]

41 **Nemoda Z**, Sahin-Tóth M. Chymotrypsin C (caldecrin) stimulates autoactivation of human cationic trypsinogen. *J Biol Chem* 2006; **281**: 11879-11886 [PMID: 16505482 DOI: 10.1074/jbc.M600124200]

42 **Szmola R**, Sahin-Tóth M. Chymotrypsin C (caldecrin) promotes degradation of human cationic trypsin: identity with Rinderknecht's enzyme Y. *Proc Natl Acad Sci U S A* 2007; **104**: 11227-11232 [PMID: 17592142 DOI: 10.1073/pnas.0703714104]

43 **Zhou J**, Sahin-Tóth M. Chymotrypsin C mutations in chronic pancreatitis. *J Gastroenterol Hepatol* 2011; **26**: 1238-1246 [PMID: 21631589 DOI: 10.1111/j.1440-1746.2011.06791.x]

44 **Paliwal S**, Bhaskar S, Mani KR, Reddy DN, Rao GV, Singh SP, Thomas V, Chandak GR. Comprehensive screening of chymotrypsin C (CTRC) gene in tropical calcific pancreatitis identifies novel variants. *Gut* 2013; **62**: 1602-1606 [PMID: 22580415 DOI: 10.1136/gutjnl-2012-302448]

45 **Takaoka Y**, Takamori M, Ichinose M, Tsujihata M, Mori M, Eguchi K. A protein anabolic factor from porcine pancreas and its affects on mice and patients with progressive muscular dystrophy. *IRCS J Med Sci* 1977; **5**: 103-105

46 **Tomomura M**, Fujii T, Sakagami H, Tomomura A. Serum calcium-decreasing factor, caldecrin, ameliorates muscular dystrophy in dy/dy mice. *In Vivo* 2011; **25**: 157-163 [PMID: 21471529]

47 **Lacruz RS**, Smith CE, Smith SM, Hu P, Bringas P, Sahin-Tóth M, Moradian-Oldak J, Paine ML. Chymotrypsin C (caldecrin) is associated with enamel development. *J Dent Res* 2011; **90**: 1228-1233 [PMID: 21828354 DOI: 10.1177/0022034511418231]

48 **Lowenfels AB**, Maisonneuve P, DiMagno EP, Elitsur Y, Gates LK, Perrault J, Whitcomb DC. Hereditary pancreatitis and the risk of pancreatic cancer. International Hereditary Pancreatitis Study Group. *J Natl Cancer Inst* 1997; **89**: 442-446 [PMID: 9091646 DOI: 10.1093/jnci/89.6.442]

49 **Wang H**, Sha W, Liu Z, Chi CW. Effect of chymotrypsin C and related proteins on pancreatic cancer cell migration. *Acta Biochim Biophys Sin (Shanghai)* 2011; **43**: 362-371 [PMID: 21460362 DOI: 10.1093/abbs/gmr022]

50 **Schramek D**, Leibbrandt A, Sigl V, Kenner L, Pospisilik JA, Lee HJ, Hanada R, Joshi PA, Aliprantis A, Glimcher L, Pasparakis M, Khokha R, Ormandy CJ, Widschwendter M, Schett G, Penninger JM. Osteoclast differentiation factor RANKL controls development of progestin-driven mammary cancer. *Nature* 2010; **468**: 98-102 [PMID: 20881962 DOI: 10.1038/nature09387]

51 **Gonzalez-Suarez E**, Jacob AP, Jones J, Miller R, Roudier-Meyer MP, Erwert R, Pinkas J, Branstetter D, Dougall WC. RANK ligand mediates progestin-induced mammary epithelial proliferation and carcinogenesis. *Nature* 2010; **468**: 103-107 [PMID: 20881963 DOI: 10.1038/nature09495]

52 **Quang CT**, Leboucher S, Passaro D, Fuhrmann L, Nourieh M, Vincent-Salomon A, Ghysdael J. The calcineurin/NFAT pathway is activated in diagnostic breast cancer cases and is essential to survival and metastasis of mammary cancer cells. *Cell Death Dis* 2015; **6**: e1658 [PMID: 25719243 DOI: 10.1038/cddis.2015.14]

**P-Reviewer:** Vlachostergios PJ, Hegardt FG **S-Editor:** Qiu S **L-Editor: E-Editor:**

**Table 1 Amino acid sequence similarity with rat caldecrin**

|  |  |  |  |
| --- | --- | --- | --- |
| **Species** | **Pancreatic protease** | **Identity (%)** | **Similarity (%)** |
| Rat | Caldecrin | 100 | 100 |
| Chymotrypsin B | 41 | 55 |
| Elastase I | 51 | 67 |
| Elastase IIA | 59 | 72 |
| Elastase IIIB | 57 | 71 |
| Human | Caldecrin | 78 | 88 |
| Chymotrypsin B | 41 | 56 |
| Elastase IIA | 61 | 74 |
| Elastase IIB | 56 | 70 |
| Elastase IIIA | 57 | 70 |
| Elastase IIIB | 55 | 69 |
| Cow | Chymotrypsin A | 39 | 57 |

Sequence identity: Percent of same amino residues in a sequence alignment between 2 sequences; Sequence similarity: Percent amino acid sequence identity and percent positive substitutions between 2 sequences.

rCal: **ATG TTG GGA ATT ACG GTC CTC GCT GCC ATC CTG GCC TGC GCC TCT TGC TGC GGG AAC CCC 60**

**M L G I T V L A A I L A C A S C C G N P 4**

Ela4: **··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· 60**

**\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* 4**

rCal: **GCC TTC CCA CCT AAC CTG TCA ACC AGA GTG GTA GGA GGA GAG GAT GCT GTC CCC AAC AGC 120**

**A F P P N L S T R V V G G E D A V P N S 24**

Ela4: **··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· 120**

**\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* 24**

rCal: **TGG CCT TGG CAG GTC TCT CTC CAG TAC CTC AAG GAC GAC ACA TGG AGG CAC ACC TGT GGG 180**

**W P W Q V S L Q Y L K D D T W R H T C G 44**

Ela4: **··· G·· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· 180**

**\* A \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* 44**

rCal: **GGA AGT CTC ATC ACC ACC AGC CAC GTC CTC ACT GCC GCC CAC TGC ATC AAC AAA GAC TTC 240**

**G S L I T T S H V L T A A H C I N K D F 64**

Ela4: **··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· 240**

**\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* 64**

rCal: **ACT TAC CGT GTG GGC CTG GGG AAG TAT AAT CTG ACA GTG GAG GAT GAG GAA GGC TCC GTG 300**

**T Y R V G L G K Y N L T V E D E E G S V 84**

Ela4: **··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ·C· G AG GCT CCG TGT 300**

**\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* A \* A P C 84**

rCal: **TAC GCT GAG GTG GAC ACC ATC TAC GTC CAT GAG AAG TGG AAC CGA CTC TTC CTG TGG AAC 360**

**Y A E V D T I Y V H E K W N R L F L W N 104**

Ela4: **ACA CTG AGG TGG ACA CCA TCT ACG TCC ATG AGA AGT GGA ACC GAC TCT TCC TGT GGA ACC 360**

**T L R W T P S T S M R S G T D S S C G T 104**

rCal: **GAC ATC GCT ATC ATT AAG TTG GCT GAG CCT GTG GAA CTG AGC AAC ACC ATC CAG GTG GCC 420**

**D I A I I K L A E P V E L S N T I Q V A 124**

Ela4: **··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· 420**

**\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* 124**

rCal: **TGC ATC CCA GAG GAA GGT TCC CTG CTG CCT CAG GAC TAT CCC TGC TAT GTC ACG GGC TGG 480**

**C I P E E G S L L P Q D Y P C Y V T G W 144**

Ela4: **··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· 480**

**\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* 144**

rCal: **GGT CGC CTC TGG ACC AAT GGT CCC ATC GCT GAA GTG CTC CAG CAG GGC CTG CAG CCC ATC 540**

**G R L W T N G P I A E V L Q Q G L Q P I 164**

Ela4: **··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· 540**

**\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* 164**

rCal: **GTG AGC CAT GCC ACG TGC TCC AGG TTG GAC TGG TGG TTC ATC AAG GTC CGG AAG ACG ATG 600**

**V S H A T C S R L D W W F I K V R K T M 184**

Ela4: **··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· 600**

**\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* 184**

rCal: **GTG TGC GCT GGG GGT GAT GGC GTC ATC TCT GCC TGT AAC GGA GAT TCT GGC GGC CCA CTG 660**

**V C A G G D G V I S A C N G D S G G P L 204**

Ela4: **··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· 660**

**\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* 204**

rCal: **AAC TGC CAA GCA GAA GAC GGC TCA TGG CAG GTG CAC GGC ATC GTG AGC TTC GGT TCC AGT 720**

**N C Q A E D G S W Q V H G I V S F G S S 224**

Ela4: **··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· 720**

**\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* 224**

rCal: **AGC GGC TGC AAC GTA CAC AAG AAA CCG GTA GTC TTC ACC CGA GTG TCT GCC TAC AAT GAC 780**

**S G C N V H K K P V V F T R V S A Y N D 244**

Ela4: **··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· 780**

**\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* 244**

rCal: **TGG ATC AAC GAG AAA ATA CAA CTG 804**

**W I N E K I Q L 252**

Ela4: **··· ··· ··· ··· ··· ··· ··· ··· 804**

**\* \* \* \* \* \* \* \* 252**

**Figure 1 Nucleotide and deduced amino acid sequences of rat caldecrin (rCal) and elastase IV (Ela4).** The nucleotide (upper row) and amino acid (lower row) sequences of the indicated molecules are shown. The dots and asterisks indicate nucleotides and amino acid residues, respectively, that are conserved between rCal and Ela4. Circle: Charge-relay system; Vertical arrowhead: Proteolytic cleavage site.

**Figure 2**

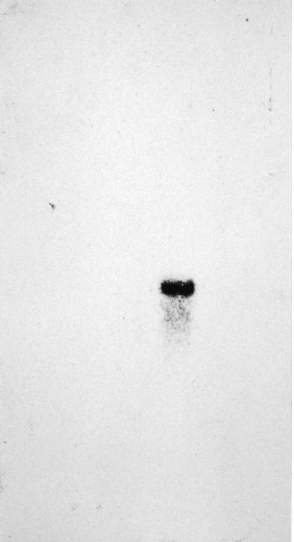
-16 1 13 43 59 125 139 170 186 196 206 227 252

A)

B)

C)

H58 D105 S200



**28S**

**18S**

**Brain**

**Lung**

**Liver**

**Pancreas**

**Kidney**

**Blood**

**Figure 2 Caldecrin expression and protein structure.** A) Caldecrin expression was analyzed by northern blot. 18S, 28S: 18S, 28S ribosomal RNA; B) Domain structures of caldecrin. Black box: signal peptide; orange box: pro-peptide; blue box: mature protein; red line: disulfide bridges with cysteine number; the H (histidine), D (aspartic acid), S (serine) catalytic triad; C) Ribbon diagram of the crystal structure of human caldecrin (adapted from PDB ID: 4H4F, prepared from[16]. Red line: Disulfide bridge; Yellow line: Pro-peptide; Arrow: β-sheet structure; Cylinder: α-helix structure.

RANK

RANK

Bone matrix

NFκB

MAPK

Src/Syk

Pyk2

Actin ring

Ca2+ signal

TRPV4

Ca2+signal

NFATc1

NFκB c-Fos

MAPK

Syk/PLCγ

RANKL

RANKL

differentiation

Mature osteoclasts

Osteoclast precursors

Survive

Caldecrin

Caldecrin

**Figure 3 Caldecrin suppresses receptor activator of RANKL-induced osteoclast differentiation and bone resorption.** RANKL binds to its receptor (RANK) on the osteoclast precursor, leading to simultaneous activation of two pathways: the NF-κB/ MAPK/c-Fos axis and the Syk/ PLCγ-calcium oscillation- NFATc1 axis. In mature osteoclasts, RANKL also activates the NF-κB/MAPK and Src/Syk/Pyk2–TRPV4 channel–calcium entry–actin ring formation axes. Caldecrin inhibits the latter pathways (but not the NF-κB/MAPK pathway) in the precursor and mature osteoclasts. TRPV4: Transient receptor potential vanilloid channel 4; MAPK: Mitogen-activated protein kinase; RANKL: RANK ligand; OVX: Ovariectomy; PLCγ: Phospholipase Cγ; PMSF: Phenylmethylsulfonyl fluoride; Pyk2: Proline-rich tyrosine kinase 2; CTRC: Chymotrypsin C; ELA4: Elastase IV; M-CSF: Macrophage colony-stimulating factor; NFATc1: Nuclear factor of activated T-cells cytoplasmic 1; NF-κB: Nuclear factor-kappa B; RANKL: RANK ligand; Syk: Spleen tyrosine kinase.