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Molecular cloning and expression of human caldecrin

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Abstract Earlier we reported the primary structure of serum calcium-decreasing factor (caldecrin) from rat pancreas, a protein which is considered to be a member of the elastase family. In this report, we describe the isolation of the two homologous cDNA clones encoding caldecrin from human pancreas, the structures of which are identical except for one base and the corresponding amino acid residue. These human caldecrin isoforms are composed of a signal peptide of 16 amino acids, a propeptide of 13 amino acids, and a mature form of 239 amino acids. Both recombinant caldecrins showed the same chymotrypsin-type protease activity and hypocalcemic activity. The hypocalcemic activity of both remained intact even after treatment with PMSF to abolish their protease activity. These results suggest that human caldecrin possesses hypocalcemic activity that has no connection with its protease activity.

Key words: Pancreas; Calcium; Elastase; Human

1. Introduction

Acute pancreatitis causes hypocalcemia, which suggests that the pancreas contains hypocalcemic factors [1]. We previously reported the purification of a serum calcium-decreasing factor (caldecrin) from porcine and rat pancreas, and the cloning of rat caldecrin cDNA [2-4]. The primary structure of the rat caldecrin cDNA is, with the exception of that of the central region, almost identical to that of elastase IV cDNA which was identified by cloning with PCR from rat pancreas, as reported by Kang et al. [5]. The amino acid sequence of fragments derived from purified rat caldecrin was identical to the amino acid sequence deduced from rat caldecrin cDNA but different from that of elastase IV, especially in the central region [4].

In this report, we isolated caldecrin cDNAs from human pancreas and purified the recombinant proteins produced in a baculovirus expression system. The recombinant human caldecrin showed serum calcium-decreasing activity even after inactivation of its protease activity.

2. Materials and methods

2.1. Screening for human caldecrin cDNA

In order to obtain the hybridization probe, we amplified human pancreas cDNA, QUICK-Clone cDNA (Clontech), by PCR with *Eco*RI tagged-mixed oligonucleotide primer pairs corresponding to the caldecrin-specific sequence (encoding amino acids 63–69) and the elastase-conserved sequence (encoding amino acids 125–131) of rat caldecrin. The amplified product of about 200 bp was cloned into pUC19, sequenced, and ^{32}P-labeled with a Ready-To-Go DNA labeling kit (Pharmacia Biotech). Recombinant plaques from a human λgt11 pancreas 5′-stretch cDNA library (Clontech) were transferred onto a nylon membrane (Hybond-N, Amersham), fixed with ultraviolet light, and hybridized with the probe in hybridization solution (50% formamide, 5×SSC, 2×Denhardt, 0.1% SDS, and 0.1 mg/ml denatured salmon testis DNA) at 42°C for 16 h. The membranes were subsequently washed twice in 2×SSC, 0.1% SDS at 25°C for 30 min and twice in 0.2×SSC, 0.1% SDS at 50°C for 30 min. After autoradiography, positive plaques were subcloned into pUC19 and sequenced.

2.2. Production of recombinant virus

Production of recombinant virus (pBacHC) was performed according to the manual of the baculovirus expression system (Clontech) and as described previously [4]. The entire coding region of human caldecrin isolated from λgt 11 or Quick-clone cDNA library was amplified by PCR with two *Eco*RI tagged-oligonucleotides (5′-TTGAATTCATGTTGGGCATCACTGTCCTCGCTG-3′) and (5′-TTGAATTCTCACAGCTGCATTTTCTCGTT-3′). The PCR products were subcloned into pBacPAK9 transfer vector. After centrifugation with $CsCl_2$, the purified transfer vectors carrying human caldecrin and BacPAK6 viral DNA were cotransfected into Sf9 cells.

2.3. Expression and purification of human caldecrin

Sf9 cells were transfected with the recombinant virus and cultured for 3 days. The medium containing recombinant peptide was collected, concentrated, dialyzed against 10 mM sodium phosphate buffer (pH 6.8), and applied to a hydroxyapatite column, Bio-Scale CHT-1 (Bio-Rad). After extensive washing, absorbed materials were eluted by stepwise increases in the phosphate buffer concentration up to 0.5 M. Caldecrin in each fraction was assayed for proteolytic activity after trypsin activation, and caldecrin protein was detected by Western blotting using anti-porcine caldecrin antibody.

2.4. Assay for protease activity

Proteolytic activity of caldecrin was measured by hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA (Peptide Institute Inc.), as described previously [6].

2.5. In vivo assays

The serum calcium-decreasing activity (SCDA) was measured as described previously [4]. Recombinant caldecrin was activated at 24°C for 30 min with trypsin at 1/50th of the protein amount of caldecrin, and the activation was terminated with 1 mM APMSF, a specific inhibitor of trypsin. After the activated caldecrin had been treated for an additional 10 min with or without 1 mM PMSF, the buffer was changed to phosphate-buffered saline by use of a PD-10 desalting column (Pharmacia Biotech). The purified recombinant human caldecrin was injected into male Balb/C mice, and blood was taken 4 h after injection. Serum calcium concentration was measured by the *o*-cresolphthalein complexon method [7].

3. Results and discussion

We first investigated unique sequences of caldecrin for the

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Abbreviations: caldecrin, serum calcium-decreasing factor; SCDA, serum calcium-decreasing activity; PCR, polymerase chain reaction; APMSF, 4-amidinophenylmethanesulphonyl fluoride; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PX, pancreatic extract

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Table 1
Sequence homology of human caldecrins with porcine (Por.), rat and human elastases

	Caldecrin	Elastase							
	Rat	Por.	Rat	Por.	Rat	Human			
		I	I	II	II	IIA	IIB	IIIA	IIIB
Human caldecrin	(% of homology)								
HC1	78.0	50.8	51.5	61.1	61.3	63.4	59.6	52.2	53.0
HC2	78.0	50.8	51.5	60.8	60.9	63.0	59.2	52.2	52.6

The amino acid sequences deduced from human caldecrins HC1 and HC2 were compared with those of rat caldecrin [4], porcine elastase I [8], rat elastase I [9], porcine elastase II [10], rat elastase II [9], human elastase IIA and IIB [10], and human elastase IIIA and IIIB [11].

screening for caldecrin cDNA from human pancreas. When the primary structure of the deduced amino acid sequence of rat caldecrin cDNA was compared with that of human elastase family members, IIA, IIB [10], IIIA and IIIB [11], the amino acid sequence at positions 63–69 of rat caldecrin appeared to be unique for caldecrin. We obtained a PCR product carrying the unique sequence of caldecrin with the human pancreas Quick-Clone cDNA library, and used it as a probe for the screening for caldecrin cDNA from the λgt11 human pancreas cDNA library.

Approx. 2×10^5 transformants from the latter library were screened with the caldecrin-specific probe. After 3 rounds of screening, 6 positive clones (pHC) were obtained. Among these positive clones, pHC70 had a length of 851 bp and contained a poly(A) tail but no ATG start codon. pHC66 was 953 bp in length and contained a full coding region from the 5′-noncoding sequence to the TGA stop codon, but had no poly(A) tail. The other clones appeared to lack regions corresponding to the 5′- or 3′-side of non-coding regions. The open reading frame of all clones was identical except for thymine (C) or cytosine (T) at nucleotide position 238 (nucleotide numbering commencing at the start codon) corresponding to Arg or Trp, respectively, at amino acid sequence position 51 (amino acid sequence numbering of the mature form). Arg and Trp types of human caldecrin were denoted HC1 and HC2, respectively; 5 out of 6 clones were found to be HC2, the remaining one being HC1. Human caldecrin cDNA was also amplified from the QUICK-Clone human pancreas cDNA library by PCR with oligonucleotide primers involving the start and stop codons of the open reading frame of the human caldecrin cDNA. The sequence of PCR product, pHCQC2/9, was found to be the HC1 type. These results suggest that there is polymorphism of caldecrin expression in the human pancreas. The nucleotide sequence and the primary structure of human caldecrin are shown in Fig. 1. The human caldecrin cDNAs, pHC1 and pHC2, showed an open reading frame of 807 bp, corresponding to 239 amino acid residues with a calculated molecular mass of

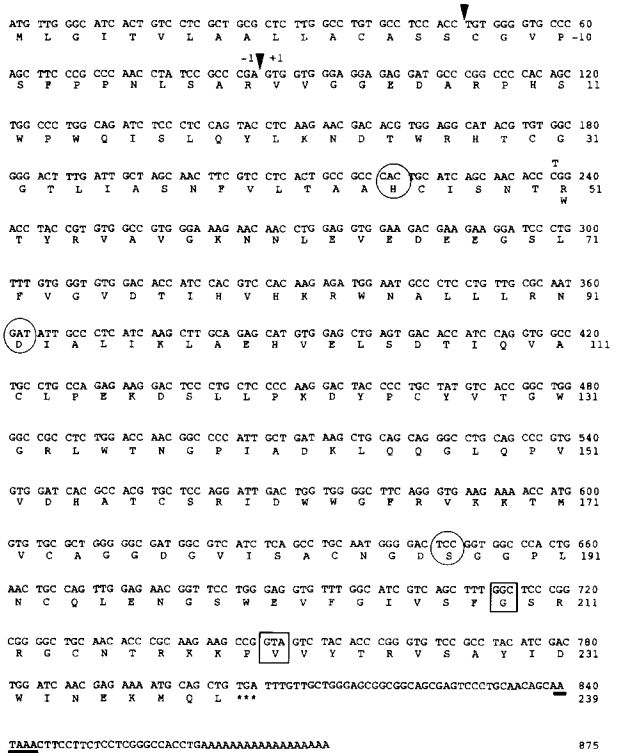

Fig. 1. The nucleotide and deduced amino acid sequences of human caldecrin. The nucleotide and deduced sequences of HC2, which differ from those of HC1, are shown above and below the HC1 sequence, respectively. The polyadenylation signal is underlined. The protein sequence is indicated using the single-letter code and is sequentially numbered from the amino-terminus of the mature form. The arrowheads indicate the proteolytic cleavage sites. The amino acid residues of the charge-relay system are circled, and those for the substrate specificity are boxed.

Fig. 2. Comparison of amino acid sequences among mammalian caldecrins. The amino acid sequence of human caldecrin HC2, which differs from that of HC1, is shown above the HC1 sequence. The data for the deduced full-length rat caldecrin and the partial sequence of purified porcine caldecrin were reported previously [4]. The numbering starts from the amino-terminus of the predicted active form of rat caldecrin. An asterisk indicates the conserved amino acid residue among all sequences compared.

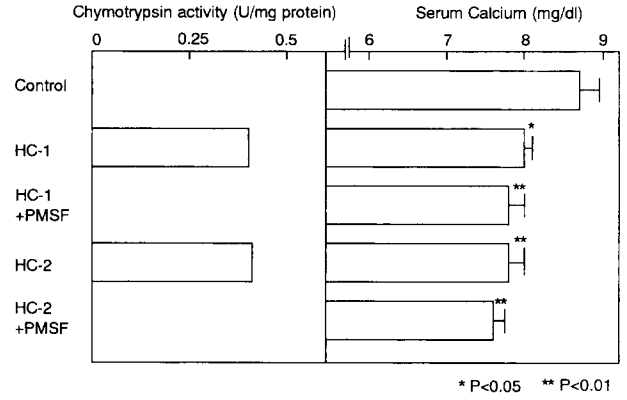

Fig. 3. Comparison of the protease activities and hypocalcemic activities of purified recombinant human caldecrin HC1 and HC2. Recombinant human caldecrins HC1 and HC2 were treated or not with PMSF and assayed for chymotrypsin activity and hypocalcemic activity.

29 327 Da. Both amino acid sequences deduced from HC1 and HC2 showed higher homology (78%) with the rat full-length and porcine partial sequences than with other elastase family members (Fig. 2 and Table 1). The deduced amino acid sequence of human caldecrin cDNA consisted of 16 amino acid residues of the pre-sequence, 13 amino acid residues of the pro-sequence, and 239 amino acid residues of the mature form just as that of rat caldecrin. All cysteine residues in human caldecrin were conserved at the same position as those of rat caldecrin. The cysteine residue at position −13 of the pro-sequence may form a disulfide bond with the cysteine residue at position 112, which is known to be important for the chymotrypsin activity of rat caldecrin [4]. The amino acid residues His-45, Asp-92 and Ser-187 in human caldecrin, which are active-site residues of serine proteases, are conserved at the same positions as in rat caldecrin. Also, the amino acid residues Gly-209 and Val-221 in the human caldecrin, thought to contribute to the substrate specificity, are identical to those of rat caldecrin [4]. These results indicate that the protease moiety of human caldecrin isoforms is identical to that of the rat protein.

To investigate the biological function of human caldecrin, we expressed recombinant human caldecrins HC1 and HC2 in a previously described baculovirus expression system [4]. The recombinant human caldecrins HC1 and HC2 were collected from the medium of Sf9 cells transfected with the baculovirus carrying pBacHC1 and pBacHC2, respectively, and were purified to homogeneity by hydroxyapatite column chromatography.

We previously reported that not only purified porcine and rat caldecrins but also recombinant rat caldecrin showed SCDA [2-4]. The SCDA of these caldecrins remained unaltered even after inhibition of their protease activity by treatment with PMSF [2]. Additionally, recombinant mutant caldecrins, in which the protease activity had been abolished by the replacement of catalytic amino acids histidine or serine with alanine, showed SCDA, again indicating that the protease activity of caldecrin is not connected with SCDA [4]. Therefore, the purified recombinant human caldecrins HC1 and HC2 were assayed for SCDA. As shown in Fig. 3, both HC1 and HC2 showed not only chymotrypsin activity but also SCDA. The protease activity and SCDA of HC1 and HC2 were at almost the same level as those of recombinant rat caldecrin. Furthermore, treatment of HC1 and HC2 with PMSF abolished their chymotrypsin activity, but did not affect their SCDA. Thus, like the other caldecrins, human caldecrin possesses hypocalcemic activity which had no connection with its protease activity.

Hypocalcemia is associated with acute pancreatitis, and hypocalcemic factors are postulated to exist in the pancreas [1]. Recently, Yoneda et al. purified a hypocalcemic factor, PX, from porcine pancreas, and reported that it resembled human elastase IIIB [12-15]. Additionally, recombinant human elastase IIIB showed hypocalcemic activity that was connected with its protease activity [14,15]. The primary structure of human and rat caldecrins is different from that of elastase IIIB (Table 1), the difference undoubtedly being responsible for the difference in protease requirement for SCDA between caldecrins and elastase IIIB (presumably PX). Caldecrin suppressed osteoclastic function as did calcitonin and this suppressive effect was protease activity-independent (manuscript in preparation). Recombinant elastase IIIB suppressed bone resorption in a protease activity-dependent manner [14,15]. The reason for this difference in requirement for protease activity for the effects of caldecrin and elastase IIIB (and presumably PX) on bone metabolism is unclear. Thus, it is of great importance to define the mechanism of hypocalcemia induction by elastase family members.

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