Site-directed mutation makes rabbit calcyclin dimer

Yuhko Ando, Masato Watanabe, Hajime Akatsuka, Hiroshi Tokumitsu and Hiroyoshi Hidaka

Department of Pharmacology, Nagoya University School of Medicine, Showa-ku, Nagoya 466, Japan

Unlike human, rat and mouse calcyclin, purified rabbit calcyclin did not form a dimer on Tricine SDS-PAGE under non-reduced conditions. Based on the internal peptide sequence of rabbit calcyclin, we isolated and sequenced a cDNA clone encoding calcyclin. The sequence of this clone (pCaiC) is 629 bp long and codes 90 amino acid residues of a protein with a molecular mass of 10,153 Da. By Northern blot analysis, a major band of 0.9 kbp and a minor band of 2.6 kbp were detected in the lung. The recombinant calcyclin mutated serine at the third position to cysteine was expressed in *E. coli* and made dimer formation under non-reduced conditions on SDS-PAGE. Whether or not this type of mutation which prevents dimer formation of calcyclin plays a physiological role in the rabbit lung is the subject of an ongoing study.

1. INTRODUCTION

Intracellular calcium ions are involved in regulating various biochemical events in excitable cells and these events are mediated by a family of calcium binding proteins which exhibit an EF-hand structure [1]. Many members of this family with the EF-hand structure have been documented.

Calcyclin is a member of the S-100 protein family with two EF-hand structures. Originally, calcyclin was thought to be a cDNA clone whose cognate mRNA was growth regulated [2]. Calcium binding protein from Ehrlich-ascites tumor cells [3] and prolactin receptor-associated protein [4] were identified as calcyclin. cDNA and peptide sequences for mouse calcyclin were reported from two laboratories, independently [5,6]. In 1991, we reported the purification of rabbit calcyclin, using calcium-dependent affinity chromatography [7]. To date, calcyclin has been purified from human, rat, mouse and rabbit. Unlike calcyclin from other species, rabbit calcyclin exists as a monomer, as determined on the basis of gel filtration in either native or denatured conditions and native gel electrophoresis in the presence or absence of 2-mercaptoethanol [7]. We considered the

Correspondence address: H. Hidaka, Department of Pharmacology, Nagoya University School of Medicine, Tsurumai-65, Showa-ku, Nagoya 466, Japan. Fax: (81) (52) 733 4774.

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; bp, base pairs; PCR, polymerase chain reaction; BPB, bromophenol blue, Tricine, Tris(hydroxymethyl)methylglycine; IPTG, isopropyl-β-D-thiogalactoside.

following two possibilities; the first one is that the cysteine residue in the primary structure of rabbit calcyclin is substituted by another amino acid residue, and the second is that the cysteine residues does not function by post translational modification. In an attempt at clarification, we isolated a cDNA clone encoding calcyclin from a rabbit lung cDNA library. Analysis of the cDNA clone revealed that rabbit calcyclin has no cysteine residue and has 90% homology with human calcyclin. Using this clone and *E. coli* we characterized the recombinant protein in which the cysteine residue had been introduced.

2. MATERIALS AND METHODS

2.1. *Materials*

Human calcyclin cDNA, hp2A9, was kindly donated by Dr. R. Baserga of Temple University. [α-^{32}P]dCTP (110 TBq/mmol) was purchased from Amersham International. T$_4$ polynucleotide kinase, T$_4$ DNA ligase, *Taq* polymerase (Ampli *Taq*), restriction endonucleases and other enzymes were obtained from Takara Shuzo. Oligonucleotides were synthesized on a Model 392A DNA synthesizer (Applied Biosystems Inc.). A rabbit lung λgt10 library was purchased from Clontech All other chemicals and reagents were of analytical grade and were obtained from commercial suppliers.

2.2. *Isolation of the calcyclin cDNA clone*

Two degenerate primers corresponding to the partial sequence of rabbit lung calcyclin reported by Tokumitsu et al. [7] were synthesized: sense primer; 5'-AA(TC) AA(AG) GA(TC) CA(AG) GT-3' based on 'NKDQE', and antisense primer: 5'-A(AG)(GATC) GC(TC) TC(AG) TT(AG) TA(AGT) AT-3' based on 'IYNEA'. Twenty-five cycles of PCR amplification were carried out as described [8]. The PCR product was cloned into pUC8 and used as the authentic cDNA probe. About 2.4 × 10^5 plaques from a rabbit lung λgt10 cDNA library were screened with the authentic cDNA probe labeled by the multi-priming method [9] Hybridization was performed overnight at 60°C, and the final wash was carried out with 2 × SSC (20 × SSC = 3 M NaCl, 0.3 M sodium citrate) containing 0.1% SDS at 60°C.

2.3. Northern blotting

Total RNA was extracted from rabbit lung by the guanidinium isothiocyanate/cesium chloride method [10]. Poly(A)$^+$ RNA was prepared on an oligo(dT) cellulose column. Fifteen μg of total RNA and five hundred ng of poly(A)$^+$ RNA were denatured and electrophoresed on a formaldehyde-containing agarose gel. The RNA was transferred onto a Hybond-N (Amersham) and hybridized in solution containing 90 mM Tris-HCl, pH 7.5, 0.9 M NaCl, 6 mM EDTA, 50% formamide, 1 × Denhardt's solution (5 × Denhardt's solution = 1% bovine serum albumin, 1% polyvinylpyrrolidone, 1% Ficoll 400), 0.1% SDS and 200 μg/ml heat-denatured salmon testis DNA at 42°C with (α-^{32}P)dCTP-labeled cDNA fragments. Finally, the filter was washed in 0 2 × SSC containing 0.1% SDS at 55°C and exposed to an imaging plate. The image was analyzed and visualized using a Bioimage analyzer (Model Fujix BAS2000, Fuji Film Corp).

2.4. Expression of the recombinant calcyclin gene

The recombinant protein of calcyclin was expressed in E. coli as a maltose binding protein (MBP) fusion protein, using pMAL-c vector (New England Biolabs.). Oligonucleotides based on boundaries of the coding region of cDNA were used to amplify the desired stretch of cDNA by PCR. The amplified DNA was ligated into the StuI and EcoRI site of pMAL-c and the recombinant plasmid was introduced into E. coli. Expression of the MBP fusion protein was induced with 0.5 mM IPTG and the fusion protein was isolated by bacterial lysate utilizing affinity chromatography with amylose resin, followed by elution with 10 mM maltose The pMAL vector contains the sequence coding for the recognition site of the specific protease factor Xa [11], located just 5' to the polylinker insertion sites. Factor Xa cleavage was carried out at a w/w ratio of 1:100 at 37°C for 24 h Recombinant calcyclin was purified, as described [7].

2.4. Other methods

DNA sequencing was achieved using the dideoxy chain termination method [12,13]. Tricine SDS-PAGE was performed by the method of Schagger and Jagow [14].

3. RESULTS AND DISCUSSION

3.1. Purification of calcyclin

We reported the purification of calcyclin from rabbit lung using calcium dependent affinity chromatography [7]. Purified rabbit calcyclin did not take on a dimer form, unlike human and mouse calcyclin. Fig. 1 shows SDS-PAGE of calcyclin purified from rabbit lung and human placenta, under reduced or non-reduced conditions. There are at least two possibilities for this event; one is that the cysteine residue present at the third position from the N-terminal of human calcyclin molecule might be lacking in rabbit calcyclin, and the other is that the cysteine residue does not function by post-translational modification. For clarification, we isolated a cDNA clone encoding rabbit calcyclin.

3.2. Cloning of rabbit calcyclin

We screened a λgt10 cDNA library with the human calcyclin cDNA as a probe. We hybridized the filter at 65°C and the final wash was performed with 0.1 × SSC at 65°C. We did not isolate a cDNA clone of rabbit calcyclin. We then attempted to obtain the authentic cDNA probe of rabbit calcyclin, using PCR techniques. PCR amplification was performed using two oligonucleotides, sense and antisense (see section 2), as primers.
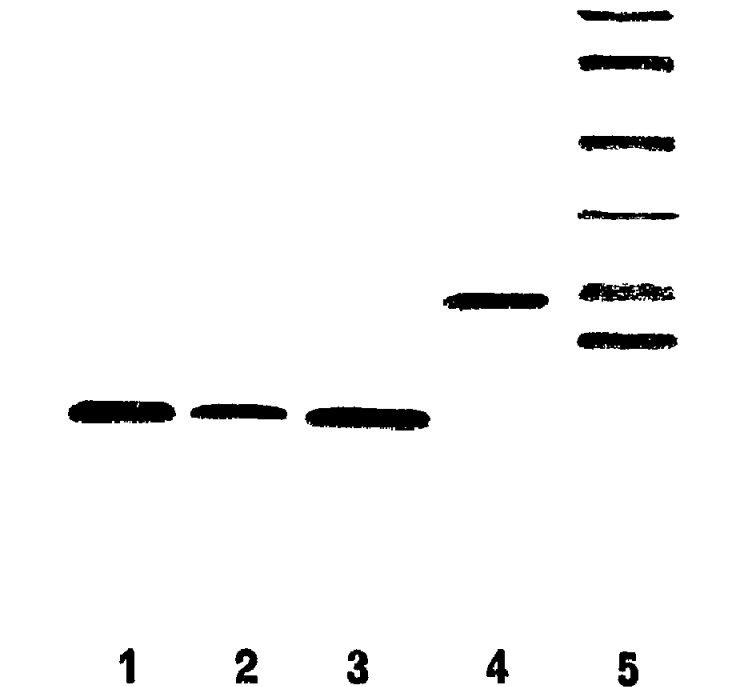

Fig. 1. SDS-PAGE of purified calcyclin from rabbit lung and human placenta. One μg of rabbit calcyclin was applied to lane 1 (reduced conditions) and to lane 2 (non-reduced conditions). One μg of human calcyclin was applied to lane 3 (reduced conditions) and to lane 4 (non-reduced conditions). Low-range molecular weight standards (Bio-Rad) were applied to lane 5 (rabbit muscle phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; hen egg white ovalbumin, 43 kDa, bovine carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21 kDa; hen egg white lysozyme, 14 kDa).

The PCR product was electrophoresed and separated on a 4% Nu-Sieve (FMC) agarose gel. A 77 bp band of the expected size was isolated from the agarose gel and cloned into pUC8. The 77 bp insert identified by sequencing was used. About 2.4 × 10^5 plaques from a rabbit lung λgt10 cDNA library were screened with the authentic probe. Hybridization and the final wash were carried out according to conditions described in section 2. Two clones gave a positive signal throughout two rounds of screening procedure. Both clones digested with EcoRI yielded about 600 base pairs. Partial restriction maps of both clones were identical. Thus, we selected one of two clones (pCalC) for further analysis. The primary nucleotide and the deduced amino acid sequences of the entire cDNA insert are shown in Fig. 2A. pCalC is 629 bp long and contains 268 bp of the 5' leader region, 273 bp coding region including termination codon TGA and 88 bp of the 3' nontranslated region. The apparent polyadenylation signal (AATAAA) was not seen, but a similar sequence (ATAAA) was noted at nucleotide positions 614–618. All sequences derived from Edman degradation (Fig. 2A, underlined) were present. The calculated molecular weight of pCalC was 10,153, a value which was in good

```
                              gggcaggcctgggtgctggtcccaagtc      28
tgccctcactggaccctgggttcctggatgagacacatggccctaccctgtcctcaccct    88
gttgagccgcaaagcgggagccaggcctgtagtctcccccctgccagctgcccacgcgcg   148
ccctcctcgcgaccttcttcaaaaggaccttgcactggtggatgcaagacccagtgtg     208
tagccacagccctgcctccacctcgtctgtcccagccgtaatcacagcccaactgaaacc   268
ATGGCAAGCCCCCTGGACCAGGCCATTGGCCTCCTGATCGGCATCTTCCACAAGTACTCT   328
 M   A   S   P   L   D   Q   A   I   G   L   L   I   G   I   F   H   K   Y   S
 1                                                              20
GGCAAGGAGGGTGACAAGCACACCCTGAGCAAGAAGGAACTGAAGGAACTAATCCAAAAG   388
 G   K   E   G   D   K   H   T   L   S   K   K   E   L   K   E   L   I   Q   K
 21                                                             40
GAGCTCACCATCGGCTCGAAGCTGCAGGATGCTGAGATTGTCAAGCTGATGGACGACCTG   448
 E   L   T   I   G   S   K   L   Q   D   A   E   I   V   K   L   M   D   D   L
 41                                                             60
GACCGCAATAAGGACCAGGAGGTGAACTTCCAGGAATACATCACCTTCCTGGGGGCCTTG   508
 D   R   N   K   D   Q   E   V   N   F   Q   E   Y   I   T   F   L   G   A   L
 61                                                             80
GCCATGATCTACAATGAAGCTCTCAAGGGCTGAagacagactggggaaggtggaggcacc   568
 A   M   I   Y   N   E   A   L   K   G   *
 81                            90
cctcaggggcctgtctgggtcacacccagtgtgggtgattgtaagataaatatatatttt   628
t                                                             629
```

A

	1	11	21	31	41
Rabbit	MASPLDQAIG	LLIGIFHKYS	GKEGDKHTLS	KKELKELIQK	ELTIGSKLQD
Human	**C*******	**VA******	*R********	**********	**********
Rat	**C*******	**VA******	**********	**********	*****A****
Mouse	**C*******	**VA******	**********	**********	**********

	51	61	71	81
Rabbit	AEIVKLMDDL	DRNKDQEVNF	QEYITFLGAL	AMIYNEALKG
Human	***AR**E**	**********	***V******	*L********
Rat	***AR*****	**********	***VA*****	*L********
Mouse	***AR*****	**********	***V?*****	*L********

B

Fig. 2. (A) Nucleotide sequence and deduced amino acid sequence of the cDNA encoding rabbit calcyclin. The nucleotide sequence is numbered starting at initiation of this clone (right side). The translated region is shown in capital letters and the 5'-, 3'-noncoding regions are shown in lower case letters. The predicted amino acid sequence is shown below the nucleotide sequence by one-letter code. Underlining indicates the peptide sequence derived from Edman degradation. *Termination codon (B) Comparison of the amino acid sequence of rabbit calcyclin with that of other species. Human [2], rat [4] and mouse [5,6] calcyclin were aligned with the corresponding sequence of rabbit calcyclin. Identical amino acids with rabbit calcyclin are indicated by asterisks (*) Underlines indicate the putative EF hand structures.

accord with that of the purified rabbit calcyclin assessed by Tricine-SDS-PAGE [7].

Rabbit calcyclin bound to 2 mol of calcium [7]. As predicted from the primary structure, amino acid residues at positions 21–33 and 61–72 (indicated in Fig. 2B) were homologous to the EF-hand motif of another S-100 family proteins and functioned as a calcium binding structure. Amino acids of rabbit calcyclin have a 90% homology to human calcyclin and the remaining were

a conservative change. Seven of nine substitutions are unique in the rabbit. The cysteine residue in the human sequence is included in these seven changes (Fig. 2B). Rabbit calcyclin has a serine residue at the third position from the N-terminus of the molecule, as a result of single nucleotide substitution TGC at positions of 110–112 of human calcyclin cDNA [4] to AGC at positions 275–277 of pCalC. This may explain the lack of formation of a dimer.
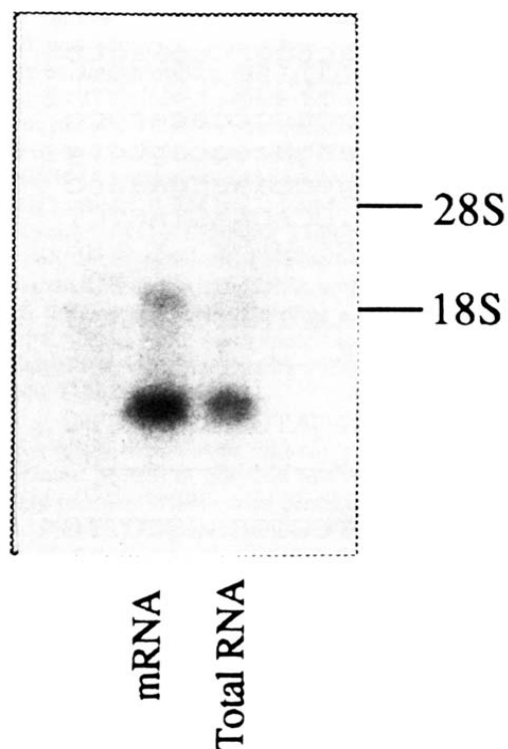
Fig. 3. Northern blot analysis of calcyclin in rabbit tissues. Fifteen μg of total RNA and 0.5 μg of mRNA were electrophoresed and transferred to Hybond-N. Hybridization with the entire insert of pCalC was carried out under the condition described in section 2. 18S and 28S ribosomal RNA were used as size markers, as shown on the right side.

3.3. Northern blotting

Northern blot analysis was performed using the entire inset of pCalC as a probe. Probing of total RNA from rabbit lung revealed a major band at 0.9 kbp. When probing with mRNA with the entire insert, a minor band at 2.6 kbp was detected in addition to the 0.9 kbp band (Fig. 3). It was not clear whether the larger band is a homologue of calcyclin or an alternative spliced gene. Because of the weak polyadenylation signal and the lack of poly(A) tail in the cDNA, the 2.6 kbp band may be an alternative spliced gene. mRNA of calcyclin was abundantly expressed in lung, kidney, testis and moderately in brain but rarely in liver and heart (data not shown). These findings are in accord with the reported data [4,15].

3.4. Expression of recombinant calcyclin

Oligonucleotides based on boundaries of the coding region of cDNA were used to amplify the desired stretch of cDNA by PCR. Two amplified DNAs were ligated into the StuI and EcoRI site of pMAL-c, one (w.t.) is a wild type and the other (S3C) is a mutant in which the serine residue was substituted for cysteine. Two MBP-fusion proteins were expressed and purified from bacterial lysate. After cleavage with Factor Xa, recombinant calcyclins were purified on an affinity column. Fig. 4 shows SDS-PAGE, under non-reduced conditions. The
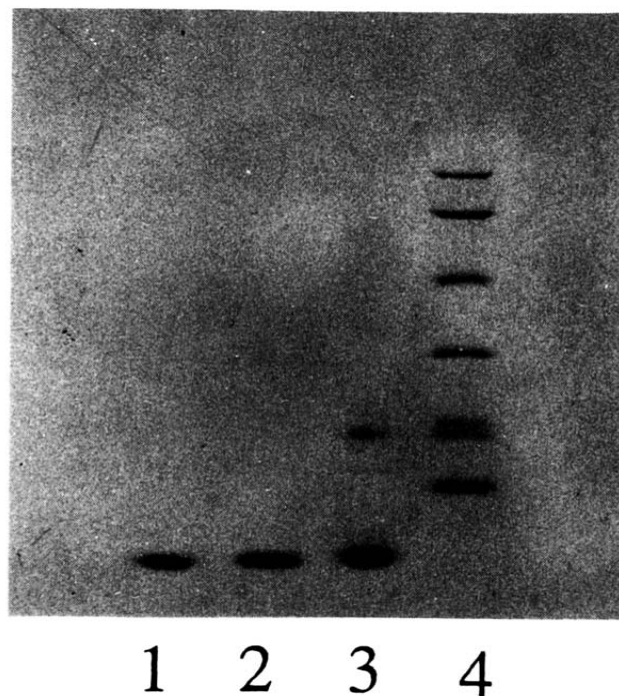

Fig. 4. SDS-PAGE of recombinant calcyclins. One μg of protein was applied to each lane, under non-reduced conditions. Lane 1: native calcyclin, lane 2: wild type calcyclin (w.t.), lane 3: point mutated calcyclin (Ser[3] to Cys) (S3C), lane 4: low range-molecular weight standards (Bio-Rad).

native protein plus the w.t. calcyclin did not allow it to form a dimer, whereas 30% of the S3C formed a dimer, as seen in human calcyclin. This result suggests that the disulfide bridge through the cysteine residues is important for formation of a homodimer, and that other factor(s) may also be involved.

It was reported that S100-β stimulated the extension of neurites and enhanced cell maintenance of embryonic chick cerebral cortex neurons [16,17]. This neurotropic activity of S100-β was abolished in the presence of a reducing agent [16]. Moreover, when either of the two cysteines in S100-β were altered by site-directed mutagenesis, the resultant proteins lost both neurite extension and neuronal survival activities [17]. These findings suggested that neurotropic activity is related to the dimer form of S100-β, through the disulfide bonds. We purified and identified calcyclin-associated protein interacting in a calcium-dependent manner [18,19]. As calcyclins from human and rabbit can specifically bind to calcyclin-associated protein (data not shown), the biological activity of calcyclin is probably independent of dimer–monomer change.

Both w.t. and S3C recombinants were seen to bind calcium ions, using gel overlay methods, and were cross-reacted to antibody against native calcyclin by Western blotting (data not shown). Analyses of interactions and binding sites of calcyclin to calcyclin-associated protein are ongoing using this expression system and purified mutants.

REFERENCES

[1] Kretsinger, R.H. (1980) CRC Crit Rev. Biochem. 8, 119–174.

[2] Calabretta, B., Battini, R., Kaczmarek, L., de Riel, J.K. and Baserga, R. (1986) J. Biol. Chem. 261, 12628–12632.

[3] Kuznicki, J. and Filipek, A. (1987) Biochem J. 247, 663–667.

[4] Murphy, L.C., Murphy, L.J., Tsuyuki, D., Duckworth, M.L. and Shiu, R.P.C. (1988) J. Biol. Chem. 263, 2397–2401.

[5] Filipek, A., Gerke, V., Weber, K. and Kuznicki, J. (1991) Eur. J. Biochem. 195, 795–800.

[6] Wood, L., Carter, D., Mills, M., Hatzenbuhler, N. and Vogeli, G. (1991) J. Invest. Dermatol. 96, 383–387.

[7] Tokumitsu, H, Kobayashi, R. and Hidaka, H. (1991) Arch. Biochem. Biophys. 288, 202–207.

[8] Watanabe, M., Ando, Y, Todoroki, H., Minami, H. and Hidaka, H. (1991) Biochem. Biophys. Res. Commun. 181, 644–649.

[9] Feinberg, A P and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.

[10] Sambrook, J., Fritsch, E F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

[11] Nagia, K. and Thogersin, H C. (1984) Nature 309, 810–812.

[12] Sanger, F., Coulson, A.R. and Barrell, B.G. (1980) J. Mol. Biol. 143, 161–178.

[13] Hattori, M. and Sakaki, Y. (1986) Anal. Biochem. 152, 232–238.

[14] Schagger, H and Jagow, G. (1987) Anal Biochem. 166, 368–379.

[15] Kuznicki, J., Filipek, A., Heimann, P., Kaczmarek, L. and Kaminska, B. (1989) FEBS Lett. 254, 141–144.

[16] Kligman, D. and Marshak, D.R. (1985) Proc. Natl. Acad. Sci. USA 82, 7136–7139.

[17] Winningham-Major, F., Staecker, J.L., Barger, S.W., Coats, S. and Van Eldik, L J. (1989) J. Cell Biol. 109, 3063–3071.

[18] Tokumitsu, H., Mizutani, A., Minami, H., Kobayashi, R. and Hidaka, H (1992) J. Biol. Chem. 267, 8919–8924.

[19] Minami, H., Tokumitsu, H., Mizutani, A., Watanabe, Y., Watanabe, M. and Hidaka, H (1992) FEBS Lett. 305, 217–219.