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CLONING AND EXPRESSION OF A CYSTEINE-RICH VENOM PROTEIN FROM *TRIMERESURUS MUCROSQUAMATUS* (TAIWAN HABU)

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T.-Y. Chang, S.-H. Mao and Y.-W. Guo. Cloning and expression of a cysteine-rich venom protein from *Trimeresurus mucrosquamatus* (Taiwan habu). *Toxicon* **35**, 879–888, 1997.—A full-length cDNA for cysteine-rich venom protein (CRVP) was constructed by immunoscreening and 5′-rapid amplification of cDNA ends from a cDNA library of venom gland of *Trimeresurus mucrosquamatus*. The predicted CRVP consisted of 183 amino acid residues including a putative signal peptide of 21 residues. Northern blot hybridization suggested the tissue-specific expression in venom gland and its corresponding length of cDNA. The predicted amino acid sequence of CRVP was homologous to a rat epididymal metalloprotein and a lizard helothermine. Amino acid sequence analysis suggested that CRVP may be a venom metalloprotein targeted against ryanodine receptors and Ca^{2+} release. Moreover, CRVP expressed in *Escherichia coli* exhibited the same antigenicity as their native venom forms of *T. mucrosquamatus*. This is the first report in the cloning and expression of a CRVP from the venom gland of *T. mucrosquamatus*. © 1997 Elsevier Science

INTRODUCTION

Comprehensive and intensive studies of rare compounds such as venom proteins are often thwarted by the limited availability of the test materials (Grissmer *et al.*, 1994). For proteins, recombinant DNA technology has helped to solve this problem by providing a means by which large quantities can be synthesized. In order to achieve this, however, the encoding cDNAs first have to be constructed. To date, only a few venom cDNAs, including those for phospholipase A_2 (Tsai *et al.*, 1995), β-fibrinogenase (Hung *et al.*, 1994) and metalloproteinase (Huang *et al.*, 1995; Tsai *et al.*, 1994), have been isolated from

The CRVP cDNA nucleotide sequence has been submitted to GenBank, accession number U59447.

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Abbreviations— AEG, Acidic epididymal glycoprotein; CRISP, cysteine-rich secretory protein; CRVP, cysteine-rich venom protein; HELO, helothermine; PRP, pathogenesis-related protein; SCP, sperm-coating glycoprotein; TPX, testicular protein.

Taiwan habu (*Trimeresurus mucrosquamatus*), a common venomous snake in Taiwan belonging to the family Viperidae (Mao *et al.*, 1986).

In this study, a novel cDNA of *T. mucrosquamatus* was constructed by immunoscreening and 5′-rapid amplification of cDNA ends (RACE). An amino acid sequence derived from the cDNA sequence indicating a cysteine-rich venom protein (CRVP) gene was cloned from the cDNA library of venom gland of Taiwan habu. This CRVP cDNA consists of 1318 bp and may code for 183 amino acids with a calculated mol. wt of 20,000. To further our understanding of the CRVP and its characteristics, sequence comparison of the deduced amino acid showed strong homology with cysteine-rich secretory proteins (CRISP) (Charest *et al.*, 1988; Haendler *et al.*, 1993; Kasahara *et al.*, 1989) and a lizard venom protein, helothermine (Morrissette *et al.*, 1995). This sequence analysis shows that CRVP exhibits a zinc-binding consensus domain of rat epididymal glycoprotein (Charest *et al.*, 1988) and reveals 48.6% homology with the Ca^{2+} channel blocker of lizard helothermine (Morrissette *et al.*, 1995). These results suggest that CRVP may be a venom metalloprotein with potential activity in targeting against ryanodine receptors and Ca^{2+} ion release.

The encoding region of CRVP was subcloned into the pET21a expression vector and delivered into an *E. coli* strain of BL21(DE3)pLysS for protein expression. The recombinant fusion protein of CRVP is actively expressed in *E. coli* and exhibits immunological activity in reacting with the venom antibody. This study represents an attempt to construct and characterize a novel cDNA of *T. mucrosquamatus*.

MATERIALS AND METHODS

Preparation of polyclonal antibodies of Taiwan habu venom

Crude venom (35 μl) was extracted from *T. mucrosquamatus* and neutralized with an equal volume of 10% formalin at room temperature for 1 hr. Neutralized venom protein was increased to a volume of 0.5 ml with PBS buffer (137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) and then mixed thoroughly with 0.5 ml of Freund's complete adjuvant. The resulting immunization mixture (1.0 ml) was injected into the restrained rabbit by multiple intradermal applications. Further immunizations were administered by boosting three times at 2–3-week intervals.

cDNA library construction and screening

Poly(A) RNA (10 μg) isolated from adult *T. mucrosquamatus* venom gland was used to prepare double-stranded cDNA by the method of Maniatis *et al.* (1989). cDNA clones were initially screened by immunoblotting plate lysate on *E. coli* Y 1090 as described by Young and Davis (1983). CRVP fusion protein was identified by binding polyclonal antisera and labelling with [^{125}I]protein A, followed by autoradiography.

RNA preparation and analysis

Organs from *T. mucrosquamatus* were surgically removed and immediately frozen in liquid nitrogen before being ground into a powder. Total RNA was extracted from venom gland, brain, lung, testis, liver and heart of *T. mucrosquamatus* by the guanidine isothiocyante method (Chirgwin *et al.*, 1979). cDNA probes were labelled with [α-^{32}P]dCTP (Amersham, IL, U.S.A.) using the rediprimer DNA labelling system (Amersham, IL, U.S.A.) (Feinberg and Vogelstein, 1983). Northern blot hybridization was performed with labelled CRVP cDNA and exposed to X-ray film (Kodak, AR or XRP) at −70°C with intensifying screens.

5′-Rapid amplification of cDNA ends

Two specific antisense primers for the 3′-end coding regions of CRVP cDNA were synthesized: the GSP1 primer 5′-GCA GAA GCA AGA AGC AGG GC-3′ and a nested GSP2 primer 5′-CCA AAC TAT TGC AGT

TCG AG-3′ for 5′-RACE (Fig. 1). Synthesis of 5′-end cDNA of CRVP was performed by 5′-RACE System kit according to the manufacturer's protocols (Life Technologies, NY, U.S.A.) (Frohman *et al.*, 1988; Loh *et al.*, 1989). 5′-End cDNA was then amplified by polymerase chain reaction (PCR) by the GSP2 and anchor primer. The reaction was subjected to 39 cycles of heat denaturation at 94 C for 30 sec, primer annealing at 51 C for 25 sec and primer extension at 72 C for 4 min with U1Tma polymerase (Perkin Elmer, CA, U.S.A.) using a 1605 Air Thermo-Cycler (Idaho Technology, ID, U.S.A.).

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cacgattttctgaaagcaacaaagaagttatttttacgtttattcaaaacaatagaaatg   60
attgccttcattgttttgccaattcttgctgcagtgctgcatcagtcttctggaaatgtt  120
gattttgattctgaatcacccaggaaaccagaaatacaaaacgagattattgacttgcac  180
aattccttaaggagatctgtgaatccaactgctagcaacatgctaaaaATGGAATGGTAT  240
                                              M  E  W  Y       4

CCTGAAGCTGCTGCTAATGCAGAACGTTGGGCGTACAGATGTATTGAGAGTCACAGTTCA  300
P  E  A  A  A  N  A  E  R  W  A  Y  R  C  I  E  S  H  S  S    24

   GSP-3 →
CGCGATTCAAGAGTTATTGGAGGAATAAAATGTGGTGAAAATATATATATGTCACCTTAT  360
R  D  S  R  V  I  G  G  I  K  C  G  E  N  I  Y  M  S  P  Y    44

CCTGCGAAATGGACTGACATTATTCACGCTTGGCATGGTGAATACAAAGATTTCAAGTAT  420
P  A  K  W  T  D  I  I  H  A  W  H  G  E  Y  K  D  F  K  Y    64

GGCGTTGGAGCAGTCCCATCAAATGCTGCTACTGGCCATTACACTCAGATAGTTTGGTAC  480
G  V  G  A  V  P  S  N  A  A  T  G  H  Y  T  Q  I  V  W  Y    84

AAAAGTTACCGTGGTGGTTGTGCTGCTGCCTATTGTCCTTCATCAAAATACAGATACTTC  540
K  S  Y  R  G  G  C  A  A  A  Y  C  P  S  S  K  Y  R  Y  F   104

TATGTTTGTCAGTACTGCCCAGCAGGAAACATGATAGGTAAAACTGCTACTCCATATACA  600
Y  V  C  Q  Y  C  P  A  G  N  M  I  G  K  T  A  T  P  Y  T   124

TCAGGGCCACCTTGTGGGGATTGTCCTTCGGATTGTGACAATGGACTATGCACAAATCCT  660
S  G  P  P  C  G  D  C  P  S  D  C  D  N  G  L  C  T  N  P   144

TGCACACAAGAAAATACGTACTCGAACTGCAATAGTTTGGTCCAACAAAGTAGCTGCCAG  720
C  T  Q  E  N  T  Y  S  N  C  N  S  L  V  Q  Q  S  S  C  Q   164
                     ← GSP-2

GATAATAATATGAAGACAAAATGCCCTGCTTCTTGCTTCTGCCAAAATAAAATAATATAG  780
D  N  N  M  K  T  K  C  P  A  S  C  F  C  Q  N  K  I  I      183
                       ← GSP-1
                                    ← GSP-4

tgggcttcccattcaatatttttatttttgccggaaatatcttaaaatcatggcatctt  840
ttagtatcagcaaattcttacttgacatttgatttcatatactttgcatgaatgtcctat  900
aaatgcctaagggaaacatcagcaggagtagaggctagggataaaaactgtaagttcaaa  960
gggtcacaggaaaaatcacaagcctatgtagcatcaaaaaaagttgattaaaagaaaata 1020
ctgaaggcctgaaaataacatcaccaaaattctttgcactccaactataccacaaggatt 1080
tcttaaatgcttatattaaaacgaatctagaactgatctgatgccattcatgcaaaatat 1140
atgccaggatttggtgtttttaatgttttaattgggtatggttcataatttgatatgatt 1200
ttatatgattttaatttttgattgggagccacccagagttgattgaaagatgggcagct 1260
ttataaatgtaataaataattaaataagcaaacaaataaataaattctgtagaagagc   1318
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Fig. 1. DNA and deduced amino acid sequence of CRVP cDNA.
Top line: Nucleotide sequence of the 1318 bp cDNA; bottom line: predicted amino acid sequence of cDNA clones. Nucleotides and amino acids are numbered on the right. Amino acids are denoted by one-letter symbols. The putative signal peptide are indicated by a dashed line. The Kozak sequence located in the boxed region, AAAATGG, and the polyadenylation signal, AATAAA, are underlined. Arrows indicate GSP-1, -2, -3 and -4 primers.

DNA sequencing

CRVP cDNA was subcloned into M13mp18/19 bacteriophages and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977).

Homology search and sequence comparison

Sequence comparisons of the deduced amino acid sequences were conducted using two sequence analysis software packages. DNASTAR software (DNASTAR, WI, U.S.A.) and GCG sequence analysis software (Genetics Computer Group, WI, U.S.A.) were used for the optimal alignment of various related sequences and estimation of sequence homology.

Reverse transcriptase polymerase chain reaction

The sequences of reverse transcriptase polymerase chain reaction (RT-PCR) primer were 5′-GTG GAT CCG TTA TTG GAG GAA TAA AAT G-3′ of GSP3 sense primer and 5′-CGA ATT CGC TAT TAT TTT ATT TTG GCA GAA GC-3′ of GSP4 antisense primer for RT-PCR (Fig. 1). In the reverse transcription, the single-stranded cDNA was first synthesized with GSP4 primer with SuperScript™ II reverse transcriptase (Life Technologies, NY, U.S.A.). PCR amplification was then performed both with GSP3 sense primer and GSP4 antisense primer. PCR reactions were subjected to 39 cycles of heat denaturation at 94 C for 30 sec, primer annealing at 53 C for 10 sec and primer extension at 72 C for 1 min with UITma polymerase using a 1605 Air Thermo-Cycler. The restriction enzyme sites of *Bam*HI in GSP3 primer and *Eco*RI in GSP4 primer were used in plasmid construction.

Cloning for a CRVP expression plasmid

The expression plasmids were constructed by ligating the CRVP cDNA insert into a pET21a vector (Novagen, WI, U.S.A.) at *Bam*HI and *Eco*RI restriction endonuclease sites. The resulting recombinant plasmid, designated pCRVP, was characterized by the presence of a 0.47 kb DNA insert and then transformed into competent *E. coli* BL21(DE3)pLysS cells for protein expression.

Expression and purification of fusion protein

BL21(DE3)pLysS cells containing the pCRVP were grown to a late log phase (A_{600} = 0.3–0.4) in Luria broth and induced with 1 mM isopropyl-1-3-β-thiogalactoside (IPTG) for 2 hr. The cells were centrifuged and lysed in buffer B (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris Cl, pH 8.0). The recombinant expressed CRVP was purified by a Ni-NTA-affinity resin according to the manufacturer's protocols (Qiagen, Hilden, Germany).

Western blotting analysis

Western blotting was carried out following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) according to Burnette (1981). The membrane was first treated with rabbit antivenom of *T. mucrosquamatus* venom antiserum (1:50), reacted with peroxidase-conjugated secondary antibody of goat anti-rabbit IgG, and finally developed with 3,3′-diaminobenzidine (DAB) and hydrogen peroxide.

RESULTS

Identification of CRVP cDNA

The *Trimeresurus mucrosquamatus* cDNA library was constructed in the expression vector, lambda gt11. A CRVP cDNA was isolated by screening 10^5 recombinant clones with polyclonal antisera. Analysis of DNA from 20 immunopositive clones revealed inserts ranging in size from 300 to 670 base pairs (bp). The largest immunopositive clone, with a insert of 670 bp, was used for further investigation. To obtain a full-length clone, the 5′-end cDNA of a single sequence of 0.75 kb was isolated by the method of 5′-rapid amplification of cDNA ends (RACE) (data not shown).

Nucleotide sequence of CRVP cDNA

The nucleotide sequence and derived amino acid sequence are shown in Fig. 1. The 1318 bp cDNA had an open reading frame beginning at nucleotide 229 and ending with

the termination codon, TAG, at position 778. According to the sequence analysis method (Kozak, 1991), the first in-frame methionine codon was located at position 229 and was contained within the translation initiation consensus sequence, AAAATGG. The coding region had 550 bases and could code for 183 amino acids with a calculated mol. wt of 20,000. The 5′-untranslated region started from nucleotide 1 to 228. The 3′-untranslated region contained a long stretch of 538 nucleotides (781–1318) and was followed by three contiguous polyadenylation signals, AATAAA, located in the upstream of the poly(A) tail at position 1271, 1294 and 1299, respectively (Fig. 1).

Predicted amino acid sequence of CRVP

Using the method of von Heijne (1983), a putative signal peptide of 21 amino acids in length was predicted from the N-terminal residues of CRVP (Fig. 1). A search of the Sequence Analysis Software Package (GCG) using the computer program BLAST revealed that the CRVP protein exhibited sequence similarities with testicular proteins from human/mouse testis (TPX1-human/TPX1-mouse) (Kasahara *et al.*, 1989), mouse/rat epididymis (SCP1-mouse/AEG-rat) (Charest *et al.*, 1988; Haendler *et al.*, 1993), the helothermine protein (HELO) from lizards (Morrissette *et al.*, 1995), the allergic venom antigen (Ag 5) from vespids (Lu *et al.*, 1993) and the pathogenesis-related proteins from plants (Cornelissen *et al.*, 1986; Lucas *et al.*, 1985; Muradov *et al.*, 1993), as can be seen in Fig. 2. The highest identities were found with a family of CRISPs containing TPX1-human (50.3%), TPX1-mouse (53.0%), SCP1-mouse (46.2%), AEG-rat (46.7%) and HELO (48.6%). There were 16 cysteine residues which were highly conserved between CRVP and CRISP (Fig. 2). Moreover, a weaker, but none the less significant, similarity existed with insect venom protein of vespid Ag 5 from hornet (Dol m VA and VB; 29.8 and 30.5%), yellow jacket (Ves v V; 28.7%), wasp (Pol a V; 33.6%) and plant pathogenesis-related proteins from tobacco (PRP-tobacco; 34.8%), tomato (PRP-tomato; 15.7%) and barley (PRP-barley; 38.0%).

Tissue-specific transcription of CRVP mRNA

Northern blot analysis with radiolabelled CRVP cDNA in six major anatomic organs including the venom gland, brain, lung, testis, liver and heart from *T. mucrosquamatus* indicated that only the venom gland specifically hybridized with the mRNA species in approximately 1300 bases (Fig. 3).

Expression and immunological analysis of CRVP

The encoding region of CRVP was excised from the M13/mp18 vector by *Bam*HI/*Eco*RI and subsequently cloned into the pET21a expression vector with an in-frame histidine tag following the *Eco*RI cloning site. After induction by IPTG, the expressed fusion protein was analysed by 12% SDS-PAGE [Fig. 4(A)] and Western blot analysis [Fig. 4(B)]. The CRVP, purified through a Ni-NTA-resin, was recognizable by a polyclonal antibody to the venom of *T. mucrosquamatus* and had a mol. wt of 20,000.

DISCUSSION

CRVP cDNA was isolated from Taiwan habu and appeared to be encoded by one mRNA species corresponding to the full-length cDNAs. The result of Northern blot

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                                     *                *
CONSENSUS CRISP   L-MEW------NAQ--A--C---HS----R------CGENL-MS-----W----S--IQ-WY-E--------GPK-------VGHYTQ-VW-S---
CRVP              --MEWYPEAAANAERWAYRCIESHSSRDSRVIGGIKCGENIYMSPYPAKW----TDIIHAWHGEYKDFKYGVGAVPSN--AATGHYTQIVWYKSYR
CRVP-HELO           M.W  . A.NA:R A: C. .H:S::.R.I:G:.CGEN::.S: P .W    :  I:.W :E K F::. G:.: N  . .GHYTQ:VWY:SY
HELO              LKMTWSNKIAQNAQRSANQCTLEHTSKEERTIDGVECGENLFFSSAPYTW----SYAIQNWFDERKYFRFNYGPTAQN--VMIGHYTQVVWYRSYE
AEG-rat           LRVEWDHDAYVNAQKWANRCIYNHSPLQHRTT-TLKCGENLFMANYPASW----SSVIQDWYDESLDFVFGFGPKKVG--VKVGHYTQVVWNSTFL
SCP1-mouse        LKMEWNYDAQVNAQQWADKCTFSHSPIELRTT-NLRCGENLFMSSYLASW----SSAIQGWYNEYKDLTYDVGPKQPD--SVVGHYTQVVWNSTFQ
TPX1-human        LKMEWSREVTTNAQRWANKCTLQHSDPEDRKT-STRCGENLYMSSDPTSW----SSAIQSWYDEILDFVYGVGPKSPN--AVVGHYTQLVWYSTYQ
TPX1-mouse        LKMEWSIQATTNAQKWANKCILEHSSKDDRKI-NIRCGENLYMSTDPTLW----STVIQSWYNENEDFVYGVGAK-PN--SAVGHYTQLVWYSSFK

Dol m VA          NVLVWNDELAKIAQTWANQCSF--GHDQCRNTEKYQVGQNVAIASTTGNSYATMSKLIEMWENEVKDFNPKKGTMGDNNFSKVGHYTQMVWGKTKE
Dol m VB          NVLVWNDELAKIAQTWANQCDF--NHDDCRNTAKYQVGQNIAISSTTATQFDRPSKLIKQWEDEVTEFNYKVG-LQNSNFRKVGHYTQMVWGKTKE
Ves v V           KNLVWNDELAYVAQVWANQCQY--GHDTCRDVAKYQVGQNVALTGSTAAKYDDPVKLVKMWEDEVKDYNPKK-KFSGNDFLKTGHYTQMVWANTKE
Pol a V           NDLVWNDELAHIAQVWASQCQF-LVHDKCRNTAKYPVGQNIAYAG--GSNLPDVVSLIKLWENEVKDFNYNTG-ITKQNFAKIGHYTQMVWGKTKE
PRP-Tobacco       -PLTWDNGVAAYAQNYVSQ----LAADCNLVHSHGQYGENL--AQG-SGDFMTAAKAVEMWVDEKQYYDHDSNTCAQGQ--VCGHYTQVVWRNSVR
PRP-Tomato        -PMSWDANLASRAQNYANS----RAGDCNLIHSGA--GENL--AKG-GGDF-TGRAAVQLWVSERPSYNYATNQCVGGK--KCRHYTQV-----VR
PRP-Barley        -AVSWSTKLQAFAQNYANQ----RINDCKLQHSGGPYGENIFWGSA-GADW-KASDAVNSWVSEKKDYDYGSNTCAAGK--VCGHYTQVVWRASTS

                    *    *           *  *                  *  *   *    *   *        *        *        *   * *
CONSENSUS CRISP   --CG-A-CP----L-Y-YVC-YCP-GN---R---PY--G-PC--CP--C--GLCTNSC---D---NC--LK----C-H--LK--C-ATC-C---I-
CRVP              GGCAAAYCPS-SKYRYFYVCQYCPAGNMIGKTATPYTSGPPCGDCPSDCDNGLCTNPCTQENTYSNCNSLVQQSSCQDNNMKTKCPASCFCQNKII
CRVP-HELO          GCA AYCP. :.Y:Y: VCQYCP:GN: ::. TPY: GPPCGDCP..CDNGLCTNPC.Q::.Y:NC .L :Q :C :  MK. C A:C C ..I
HELO              LGCAIAYCPDQPTYKYYQVCQYCPGGNIRSRKYTPYSIGPPCGDCPDACDNGLCTNPCKQNDVYNNCPDLKKQVGCGHPIMKD-CMATCKCLTEIK
AEG-rat           VACGVAECPDQ-PLKYFYVCHYCPGGNYVGRLYSPYTEGEPCDSCPGNCEDGLCTNSCEYEDNYSNCGDLKKMVSCDDPLLKEGCRASCFCEDKIH
SCP1-mouse        VACGVAECPKN-PLRYYYVCHYCPVGNYQGRLYTPYTAGEPCASCPDHCEDGLCTNSCGHEDKYTNCKYLKKMLSCEHELLKKGCKATCLCEGKIH
TPX1-human        VGCGIAYCPNQDSLKYYYVCQYCPAGNNMNRKNTPYQQGTPCAGCPDDCDKGLCTNSCQYQDLLSNCDSLKNTAGCEHELLKEKCKATCLCENKIY
TPX1-mouse        IGCGIAYCPNQDNLKYFYVCHYCPMGNNVMKKSTPYQQGTPCASCPNNCENGLCTNSCDFEDLLSNCESLKTSAGCKHELLKTKCQATCLCEDKIH

Dol m VA          IGCGSVKYIENNWHTHYLVCNYGPAGNYMDQPIYERK------------------------------------------------------------
Dol m VB          IGCGSIKYIEDNWYTHYLVCNYGPGGNDFNQPIYERK------------------------------------------------------------
Ves v V           VGCGSIKYIQEKWHKHYLVCNYGPSGNFMNEELYQTK------------------------------------------------------------
Pol a V           IGCGSLKYMENNMQNHYLICNYGPAGNYLGQLPYTKK------------------------------------------------------------
PRP-Tobacco       VGCARVKC--NNGG-YVVSCNYDPPGNVIGQSPY---------------------------------------------------------------
PRP-Tomato        LGCGRARC--NNGW-WFISCNYDPVGNWIGQRPY---------------------------------------------------------------
PRP-Barley        IGCARVVC--NNNRGVFITCNYEPRGNIIGQKPY---------------------------------------------------------------
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Fig. 2. Multiple alignment and sequence comparison of the CRVP, mammalian testicular proteins, helothermine, vespid Ag 5 and plant pathogenesis-related proteins.

Sequence alignment was carried out in the GCG program and gaps were introduced for optimal alignment and maximum homology for the sequences. The sequences are proteins from CRVP of *T. mucrosquamatus*, helothermine (HELO) of Mexican beaded lizard, rat androgen epididymal protein (AEG-rat), human testis-specific protein (TPX1-human), mouse testis-specific protein/sperm-coating glycoprotein (TPX1-mouse/SCP1-mouse), vespid Ag 5 from hornet (Dol m VA and VB), yellow jacket (Ves v V), wasp (Pol a V) and plant pathogenesis-related proteins from tobacco (PRP-tobacco), tomato (PRP-tomato) and barley (PRP-barley). The 16 cysteine residues of the protein are marked with asterisks. The upper consensus line shows residues conserved in at least four of the five CRISPs. Amino acid residues are denoted by one-letter symbols. CRVP-HELO is indicated as the consensus sequences between helothermine and CRVP. Identical residues appear between the sequences. Positively related amino acids are indicated by (:) whereas neutral relationships are indicated by (.).

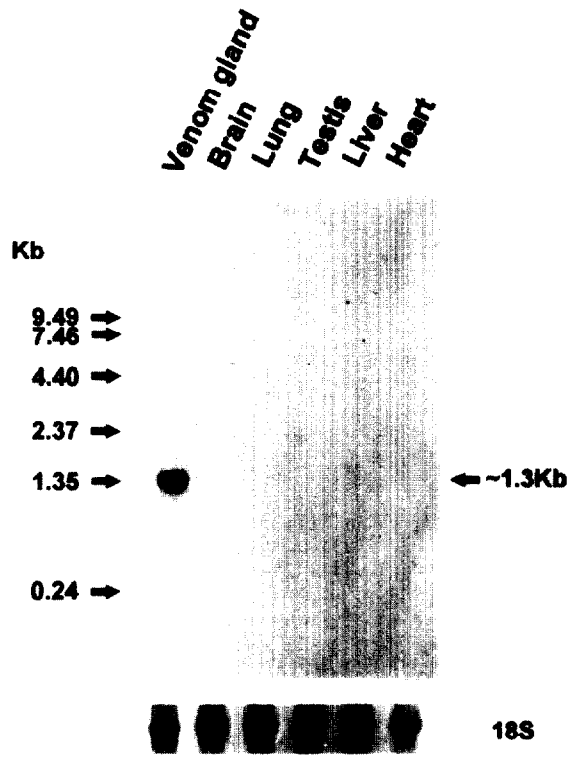

Fig. 3. Tissue-specific transcription of CRVP mRNA.
Northern blots were hybridized with ^{32}P-labelled CRVP cDNA as described in Materials and Methods. Sizes of denatured DNA markers electrophoresed on the same 1.2% agarose gels are indicated. Northern blot analysis of 10 μg total RNA isolated from various tissues of the adults of *T. mucrosquamatus*. Lane 1, Venom gland; lane 2, brain; lane 3, lung; lane 4, testis; lane 5, liver; lane 6, heart.

analysis suggested that a specific message encoded the CRVP protein. The allele encoding the 1.3 kb mRNA was found only in the venom gland, indicating a tissue-specific expression of CRVP in the venom gland of *T. mucrosquamatus*.

The zinc-binding domain found in both CRVP and AEG metalloprotein strongly suggested that CRVP might be a zinc-binding protein in venom (see below). In AEG, it contains two zinc-binding consensus sequences (Charest *et al.*, 1988). The zinc ion in AEG is tetrahedrally coordinated with the four cysteine residues in two consecutive stretches of

Consensus:	1	2	3	4	5	5a	5b	6	7	8	9	10	11
Sequence	Xh-	X-	Cys-	X-	X-	(X-	X)-	Cys-	X-	X-	X-	X-	Xa-
CRVP	Gly-	Gly-	Cys-	Ala-	Ala-	Ala-	Tyr-	Cys-	Pro-	Ser-	Ser-	Lys-	Tyr
	Tyr-	Val-	Cys-	Gln-	Tyr-			Cys-	Pro-	Ala-	Gly-	Asn-	Met
AEG	Val-	Ala-	Cys-	Gly-	Val-	Ala-	Glu-	Cys-	Pro-	Asp-	Gln-	Pro-	Leu
	Tyr-	Val-	Cys-	His-	Tyr-			Cys-	Pro-	Gly-	Gly-	Asn-	Tyr

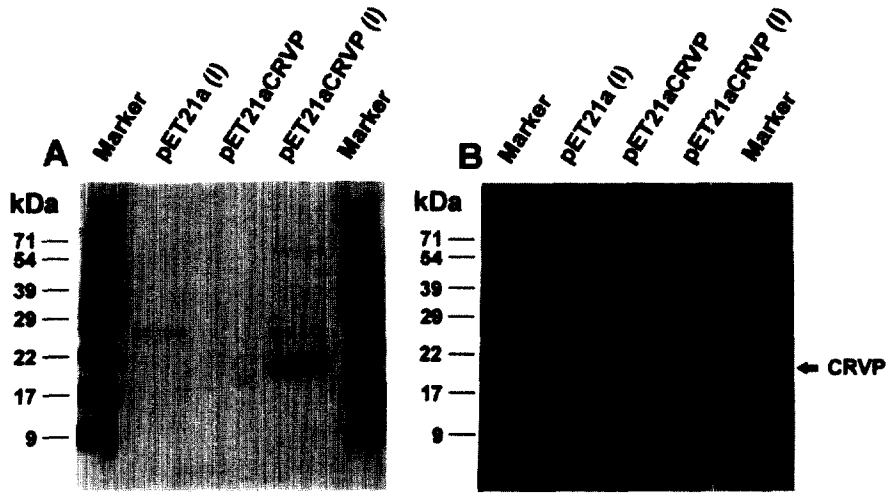

Fig. 4. SDS–PAGE and immunological analysis of the recombinant CRVP generated from CRVP cDNA clone.

Protein profiles of expressed CRVP analysed in 12% SDS–PAGE with (A) Coomassie blue staining and Western blotting analysis of the recombinant CRVP purified from Ni-NTA agarose. (B) Purified recombinant CRVP were reacted with a constant amount (× 50 dilution) of *T. mucrosquamatus* venom antiserum. The arrow indicates the expressed CRVP. The lane marker is prestained protein ladder (6–60 kDa). Lane pET21a(I) is Ni-NTA agarose purified cell extract of *E. coli* BL21(DE3)pLysS with pET21a vector and induced with IPTG. Lanes pET21aCRVP and pET21aCRVP(I) are Ni-NTA agarose-purified recombinant CRVP protein without and with IPTG induction, respectively.

11 amino acid consensus sequences separated by two or four amino acids at positions 3 and 6. In addition to the highly conserved cysteine residues, position 1 is occupied by a hydrophobic residue (Xh) and, 50% of the time, position 11 is occupied by an aromatic amino acid (Xa). In CRVP, not only the two cysteines at position 3 and 6 but also the hydrophobic residues at position 1 and 11 are well conserved. That the role of CRVP in snake venom might be related to its capacity to bind metals appears to be an attractive supposition. Because the consensus sequence of the zinc-binding site of metalloproteinases is found in the protein structure of Taiwan habu (Iha *et al.*, 1995), sequence homology of the C-terminal region of CRVP among these zinc-binding proteins is intriguing and may offer a clue to the characterization of CRVP.

The biological function of CRVP is unclear, but the sequence similarity of CRVP to lizard helothermine of defined function is of interest (Fig. 2). In the consensus sequence of CRVP-HELO, CRVP either has the same residue or this is replaced by a positively related amino acid at each position in the helothermine. Helothermine, a venom protein isolated from Mexican beaded lizard (*Heloderma horridum horridum*), is capable of blocking ryanodine receptors and Ca^{2+} release in cardiac and skeletal sarcoplasmic reticulum (Morrissette *et al.*, 1995). In addition, Crotalinae venom exhibits related enzymes that could act as blockers of the Ca^{2+} influx in the sarcoplasmic reticulum (Muszkat *et al.*, 1994). Furthermore, the venom glands in snakes and lizards originated from the salivary gland (Kent, 1983), and the activity of CRISP in mouse salivary gland has been reported (Haendler *et al.*, 1993). The sequence similarity of CRVP to the helothermine may be related to the fact that the CRVP might possess Ca^{2+} channel-blocking properties.

Insect allergic venom protein of vespids (Lu *et al.*, 1993) and plant pathogenesis-related proteins from tobacco, tomato and barley (Cornelissen *et al.*, 1986; Lucas *et al.*, 1985; Muradov *et al.*, 1993) exhibit weaker but significant similarities with CRVP. These insect and plant proteins lack the cysteine-rich C-terminal moiety characteristic of the CRISP family. However, the 16 cysteine residues of the C-terminal moiety of CRVP are invariant in the family of CRISP, suggesting a common pattern of disulfide bridges. All of these proteins share a common stretch (Gly-His-Tyr-Thr-Gln-Val-Val-Trp), corresponding to residues 76–83 of the CRVP amino acid sequence. Analysis of reptile, rat, mouse and human proteins indicates that CRVP and helothermine are products of the same gene family and the mammalian CRISPs are in a distinct but closely related gene family.

CRVP was identified as a new member of the CRISP family of proteins, suggesting that this protein might exert the blocking activity in striated muscle. Because CRISP proteins are found in mammalian tissues, it is possible that CRISP-like proteins might endogenously regulate ryanodine receptor (Morrissette *et al.*, 1995). Furthermore, the expression and purification of CRVP was essential for the characterization of their biological functions. The CRVP expressed in *E. coli* was purified by a Ni-NTA ligand affinity resin through the in-frame expressed histidine tag. Affinity gel-purified CRVP can be recognized by the specific antibody of *T. mucrosquamatus* at a mol. wt of 20,000, indicating that it exhibited the same antigenicity as the native toxin [Fig. 4(B)]. This investigation strongly suggests that CRVP may be a venom metalloprotein targeted against ryanodine receptors and Ca^{2+} release. Further studies are required to establish whether these observations on sequence similarity and function are of significance in the elucidation of the effects of CRVP venom protein.

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