cDNA Cloning and Sequence Analysis of a Novel Calcium Binding Protein with Oligoproline Motif

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Key words: Calcium binding protein/lectin binding site/oligoproline/Src homology region III

ABSTRACT. Using antibodies against pokeweed agglutinin-binding proteins from F9 embryonal carcinoma cells, we isolated a cDNA clone PW29 reacting with the antibody. mRNA hybridizing with the cDNA was 4.7 kb, and was strongly expressed in the testis, brain, kidney and heart as well as in F9 cells. The size of mRNA in the testis was heterogeneous. Sequencing the cDNA clone revealed a putative polypeptide of 70 kDa, which is rich in hydrophilic amino acids and has a characteristic sequence of (GluLys)₅. The protein also has an oligoproline motif, which conforms to the rule of binding capability to Src homology region III. The cDNA was translated as a fusion protein with glutathione-S-transferase, and was verified to have calcium binding activity, upon a calcium blot experiment.

Calcium binding proteins play important roles in regulation of cellular activities. They are classified into two groups; those with so-called EF-hand motif (4, 5) and those without it. An example of the latter is calreticulin, which is a calcium binding protein in endoplasmic reticulum (7). Calreticulin has segments rich in acidic amino acids or rich in acidic amino acids and proline, which serve as calcium binding sites (1). This paper describes a novel calcium binding protein, which lacks EF-hand motif, and is present in embryonal carcinoma cells. A novel calcium binding protein with EF-hand motif (12) and the one without it (10) were previously found by us also in embryonal carcinoma cells.

MATERIALS AND METHODS

Immunoscreening of an expression library. Glycoproteins which bind to pokeweed agglutinin (PWA)-agarose were isolated from F9 embryonal carcinoma cells grown subcutaneously in 129/sclp mice as described previously (9). Rabbit anti-serum against the PWA-binding glycoproteins were produced (10), and was absorbed by disrupted *Escherichia coli* Y1090, and used to screen λ gt11 cDNA library as described by Schuh

et al. (14). The library, constructed from mRNAs of F9 embryonal carcinoma cells, has been described (8).

Plaque screening. Approximately 1×10^6 phages were screened according to Benton and Davis (2) with *EcoRI/XhoI* fragment of PW29 cDNA (one of the positive clones obtained from λ gt11 cDNA library) as the hybridization probe. Filters were hybridized overnight at 42°C in hybridization solution containing 50% formamide, $5 \times$ SSC, 50 mM sodium phosphate buffer pH 6.5, $10 \times$ Denhardt's solution, 0.1% SDS, and 100 mg/ml heat-denatured salmon sperm DNA. The filters were washed twice with several changes of $2 \times$ SSC, 0.1% SDS at room temperature followed by several washes in $0.1 \times$ SSC, 0.1% SDS at 55°C. Phage DNAs were isolated by plate lysis (6).

Subcloning and DNA sequencing. Phage DNA from positive clones were digested with *EcoRI*, and the cDNA inserts were subcloned into the *EcoRI* site of plasmid vector pUC18. The inserts were further digested with various restriction enzymes, and the resulting fragments were subcloned into the plasmid vector pUC18 or pBluescript II (KS+). DNA nucleotide sequences of the fragments were determined by the dideoxy chain-termination method (13). Homology was searched by the program of Software Development, Tokyo, employing the data base of EMBL-GDB, LASL-GDB (GenBank) and NBRF-PDB.

RNA analysis. Total cellular RNA and total RNA from tissues were extracted by the acid guanidinium thiocyanate-phenol chloroform method (3). RNA was denatured with glyoxal, separated on a 1% agarose gel and transferred to ni-

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Abbreviations: G buffer; 50 mM Tris-HCl buffer, pH 9.6 containing 5 mM glutathione, GST; glutathione S-transferase, MBP; maltose binding protein, PBS(–); Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} ; PWA; pokeweed agglutinin, SSC; 0.15 M NaCl, 0.015 M sodium citrate.

trocellulose membrane in $20\times$ SSC. The membrane was baked for 2 h under vacuum at 80°C and treated in 20 mM Tris-HCl buffer, pH 8.0 at 100°C for 5 min. RNA blots were prehybridized for 2 h in 50% formamide, 50 mM sodium phosphate, pH 6.5, 200 $\mu\text{g}/\text{ml}$ heat denatured salmon sperm DNA, $10\times$ Denhardt's solution and 1% SDS at 42°C and hybridized for 20 h with [α - ^{32}P]-dCTP-labeled cDNA probes prepared by a random hexanucleotide priming method, at 0.5 – 2.0×10^7 cpm/ml under conditions identical to those used for prehybridization. The washing conditions of the membrane were the same as plaque hybridization. As a control, mouse β -actin cDNA was used.

Preparation of fusion proteins. For producing maltose binding protein fusion protein (MBP-PW29), *EcoRI* fragment of PW29 was subcloned into a MBP fusion protein vector, pMAL-c (Bio-Labs.). *E. coli* TB1 cells were transformed with the recombinant plasmid, which encodes the fusion protein. The cells were cultured, induced for fusion protein production, and collected according to the protocol of the producer (Bio-Labs.). The collected cell pellet was suspended in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(–)] and disrupted by sonication. After centrifugation of the sonicate at 12,000 rpm for 30 min, the supernatant was applied to a column of amylose-resin (Bio-Labs.) (column volume = 1 ml), and the column was washed with 5–10 column volumes of PBS(–) containing 1% Tween 20 and with PBS(–) successively. The fusion protein was eluted with 10 mM maltose in PBS(–). About 5 mg of fusion protein was obtained from 1 liter of bacteria culture suspension.

For producing glutathione-S-transferase (GST) fusion protein, plasmid PW29 was digested with *AatII*, blunt-ended with T4 DNA polymerase, and then subcloned into *SmaI* site of glutathione-S-transferase fusion protein vector, pGEX-

2T. JM109 cells were transformed with the recombinant plasmid, which encodes the fusion protein. Forty-five ml of LB medium were inoculated with 5 ml of an overnight culture and allowed to grow to an OD of 1.0. Isopropylthio- β -D-galactoside was added to a final concentration of 1 mM, and the cultures were allowed to grow a further 6 h at 37°C . Cells were then pelleted, resuspended in 5 ml lysis buffer [50 mM Tris-HCl, pH 7.5, containing 25% sucrose 0.5% Nonidet P-40 and 5 mM MgCl_2]. After sonication, debris was pelleted by centrifugation for 15 min at 12,000 rpm. The supernatant was applied to a glutathione-agarose column (1 ml). Following three washes with 3 ml washing buffer [20 mM Tris-HCl, pH 7.5, containing 2 mM MgCl_2 and 1 mM dithiothreitol], the protein was eluted from the column with 3 ml of G buffer [50 mM Tris-HCl, pH 9.6 containing 5 mM glutathione]. Alternatively, pelleted cells grown in 1 liter of LB medium were resuspended in 100 ml of the lysis buffer, sonicated, and centrifuged at 10,000 rpm for 20 min. The supernatant was applied to 14 ml of glutathione-agarose column, followed by washing with 70 ml of washing buffer. Fusion protein was eluted by washing with 30 ml of G buffer as described previously. From 1 liter of bacterial culture, about 40 mg of fusion protein was obtained.

Preparation of anti-fusion protein antibodies. Anti-MBP-PW29 fusion protein antibodies were prepared as follows. MBP-PW29 fusion protein (50 μg), which was purified as described before, was emulsified with an equal volume of Freund's complete adjuvant. New Zealand White rabbits were immunized subcutaneously with the emulsion. After four booster injections at two-week intervals using the same amount of the antigen, the rabbits were bled and antisera were obtained.

Calcium blot. Glutathione-S-transferase (5 μg) and gluta-

Fig. 1. Restriction endonuclease map and sequencing strategy for the PW29 cDNA clones. The scale indicates nucleotide positions (in kilobase) beginning from the first base of the cDNA. The PW29 and extended PW29-16 cDNAs were shown under the scale. The endonuclease sites indicated above these two lines were used to generate cDNA fragments for sequencing. The arrows illustrate the direction and extent of DNA sequences obtained in each experiment.

thione-S-transferrase-PW29 fusion protein (5 μ g) were electrophoresed on 10% SDS-PAGE and were transferred to a nitrocellulose membrane. After washing the membrane with solution A (60 mM KCl, 5 mM MgCl₂, 10 mM imidazole-HCl,

pH 6.8) two to three times in 1 h, the membrane was then incubated with solution A containing ^{45}Ca ($10\ \mu\text{Ci}/\mu\text{g}$; New England Nuclear) at $1\ \mu\text{Ci}/\text{ml}$ for 10 min, rinsed with distilled water, dried, and then exposed to X-ray film for 24 h. The

[illegible]

Fig. 2. Sequence of cDNA encoding PW29 and deduced protein sequence. *, putative initiation site; ★, potential N-glycosylation sites; —, a segment composed of basic and acidic amino acid residues; =, pentaproline motif; ≡, polyadenylation signals. GenBank accession number is D49429.

same membrane was stained with Amino Black as described before (12).

Detection of PW29 by antibodies against a fusion protein produced by MBP. Purified GST fusion protein (300 μ g) was dialysed against 0.5 M NaCl, 0.1 M NaHCO₃ (pH 8.0) and coupled to the CNBr-activated Sepharose 4B (0.5 ml) according to the method of the manufacturer. Two ml of anti-MBP-PW29 antiserum was applied to a column containing the fusion protein-Sepharose. Antibodies bound to the column were eluted with 0.1 M glycine-HCl (pH 2.5) and immediately neutralized with Tris-HCl, pH 8.8. Immunoblot analysis was carried out after F9 cells were separated by SDS-polyacrylamide gel electrophoresis on a 10% gel and transferred to the nitrocellulose membrane as described before (11). Antibodies eluted from a column containing GST-Sepharose were used as a control.

RESULTS AND DISCUSSION

Cloning and sequence analysis of PW29. A λ gt11 expression library constructed from F9 embryonal carcinoma cells was screened by antibodies against pokeweed agglutinin (PWA)-binding glyco-proteins isolated from

these cells. Three positive clones were obtained. Analysis by restriction endonucleases and Southern blotting revealed that the 3 clones were independent. Thus, we analyzed one of the clones, PW29 in detail. One of the other clones, PW19, encoded a calcium binding protein structurally unrelated to PW29 protein (10).

By Northern blot analysis, the PW29 probe (*Xho*I/*Eco*RI fragment of PW29) detected a single band of 4.3 kb. Since PW29 insert was about 3 kb (Fig. 1, 2), we analyzed the 5' end sequences by screening λ gt10 cDNA library constructed from F9 cells using PW29 probe (*Eco*RI-*Xho*I fragment of PW29). We got the clone PW29-16 and could extend the sequence about 0.9 kb (Fig. 1, 2). Thus, PW29 and PW29-16 yielded composite DNA sequence of 3.9 kb (Fig. 1, 2). This sequence comprised 84% of the size of PW29 mRNA in F9 cells (Fig. 3).

Sequencing data showed a single open reading frame, extending from the ATG at nucleotide 567 to the TGA stop codon at base 2486 encoding a polypeptide with a predicted molecular weight of 70 kD. The sequence has

Fig. 3. Expression of PW29 mRNA in F9 and PYS-2 cells. Poly(A)⁺ RNAs (10 μ g) were denatured, separated by 1% agarose gel electrophoresis, transferred to a nitrocellulose membrane and hybridized with an [α -³²P]dCTP-labeled probe (*Bam*HI-*Eco*RI restriction fragment of PW29 cDNA). 1) F9 cells and 2) PYS-2 cells. Lower lane: hybridization with β -actin probe.

Fig. 4. Expression of PW29 mRNA in different tissues from adult Mice. Twenty μ g of the total RNAs were denatured, separated by 1% agarose gel electrophoresis, transferred to a nitrocellulose membrane and hybridized with an [α -³²P]dCTP-labeled probe (*Bam*HI-*Eco*RI restriction fragment of PW29 cDNA). 1) muscle, 2) colon, 3) testis, 4) brain, 5) spleen, 6) ovary, 7) heart, 8) stomach, 9) liver, 10) lung, 11) kidney and 12) small intestine. Lower lane: hybridization with β -actin probe.

(Pro)₂₋₅, (LysArg)₂₋₃ and a long stretch of hydrophilic amino acids composed of (GluLys)₅(Glu)₇AspGluAsp. Although PW29 protein has no significant homology with other proteins, this feature of the protein has some resemblance to calreticulin, a calcium binding protein, which has a proline rich segment and C-terminal segments rich in poly-acidic amino acids (1). We also note that the proline cluster is present in the cytoplasmic portion of Fas ligand (15), and that pentaproline structure present in PW29 protein conforms to the consensus sequence for the binding site of Src-homology region III (16). The consensus motif is pΦPpXP (P represents proline, p represents residues that tend to be proline, Φ represents hydrophobic residues and X represents nonconserved residues). This observation raised a possibility that PW29 protein is involved in intracellular signal transduction system.

Northern blot experiment indicated that PW29

mRNA was strongly expressed in the testis, brain, kidney and heart of adult mice (Fig. 4) as well as in F9 cells (Fig. 3). The size of the mRNA was heterogeneous. In PYS-2 cells, which represent parietal endoderm cells differentiated from F9 cells, the PW29 mRNA was only weakly expressed (Fig. 3).

PWA binding sites comprise many glycoproteins of different molecular weights. We examined what molecular species corresponded to the protein specified by the cDNA clone. For that purpose, we isolated the MBP-PW29 fusion protein and immunized it to a rabbit. F9 cells were separated by SDS gel electrophoresis, transferred to nitrocellulose membranes, and stained with anti-fusion protein anti-serum which was purified by GST fusion protein coupled to CNBr-activated Sepharose 4B. We detected a band of apparent molecular weight 120 kDa (Fig. 5); this band was not detectable upon Western blotting using a control antibody purified by GST column. The difference of the predicted molecular weight (70 kDa) and the observed molecular weight de-

Fig. 5. Immunoblotting analysis of PW29 using antibodies raised against MBP-PW29 fusion protein. Proteins from 1×10^5 F9 cells were separated by SDS gel electrophoresis on a 10% gel, and electrophoretically transferred to a nitrocellulose membrane and were reacted with antibodies against MBP-PW29 fusion protein. Lane 1: reaction with the antibodies purified by GST-PW29 fusion protein. Lane 2: control run: reaction with the antibodies purified by GST. Molecular weight markers were myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa).

Fig. 6. Calcium blotting analysis. Glutathione-S-transferase (5 μ g) (lane 1) and glutathione-S-transferase-PW29 fusion protein (5 μ g) (lane 2) purified on glutathione agarose column were electrophoresed and transferred to a nitrocellulose membrane. After incubation with Ca^{2+} , the membrane was exposed to x-ray film (B). The same membrane was stained with Amino Black to detect proteins (A). GST migrated as a huge band of molecular weight 27.5 kDa. Because of the instability of the fusion protein, a large amount of GST was detected in the lane of the fusion protein (A, lane 2).

tected upon SDS gel electrophoresis is best explained by unusual electrophoretic behavior due to the presence of highly charged segments in the polypeptide. Indeed, calreticulin which has a molecular weight 45.5 kDa migrates as a 60 kDa protein upon SDS-PAGE (1). However, the possibility is not excluded that the PW29 cDNA so far cloned represent a truncated minor component originated by alternative splicing.

Calcium binding property of PW29 protein. We examined whether PW29 protein can bind Ca^{2+} . For that purpose, glutathione-S-transferase fusion protein was produced, and was purified by affinity chromatography on glutathion agarose column. The purified fusion protein, but not glutathione-S-transferase reacted with Ca^{2+} upon calcium blotting (Fig. 6). In addition to the 90 kDa fusion protein band, a 55 kDa band, which was considered to be partially degraded fusion protein, also reacted with calcium.

As above, PW29 is a calcium binding protein strongly expressed in several adult organs as well as in a line of embryonal carcinoma cells. Since the sequence lacks a signal sequence and a hydrophobic region, PW29 is likely to be a cytoplasmic protein. However, PW29 as well as endoplasmic reticulum resident calcium binding protein CBP-140 (10) were cloned by using antibodies to teratocarcinoma glycoproteins purified by PWA lectin. By using antibodies to teratocarcinoma glycoproteins, which were isolated by affinity chromatography on another lectin, *Dolichos biflorus* agglutinin (DBA), we also cloned reticulocalbin, a calcium binding protein principally located in the luminal side of the endoplasmic reticulum (12). It is possible that these calcium binding proteins are bound to the cytoplasmic region of the membrane glycoproteins, and that after detergent solubilization and lectin affinity chromatography, the calcium binding protein co-purifies with membrane glycoproteins. Furthermore, we noted that the PW-29 cDNA sequence is almost identical to a galactosyltransferase-related antigen isolated in our laboratory (Uehara and Muramatsu, unpublished results). Since this antigen expressed in *E. coli* did not express enzyme activity, we considered that PW-29 is a calcium binding protein and antibodies against the enzyme reacted with PW-29 by antigenic cross reactivity. In any event, a novel calcium binding protein, PW29, is an interesting molecule for further study, because of its possible role in cell regulation.

Acknowledgments. This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan. We thank Kumiko Sato for her expert secretarial assistance.

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(Received for publication, May 17, 1995

and in revised form, May 25, 1995)