Characterization of a polyclonal antiserum against the purified human recombinant calcium binding protein calretinin

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Abstract — We have purified recombinant human calretinin (CR) from Escherichia coli lysates and have produced a polyclonal antiserum against it. The antiserum recognizes determinants conserved in fish, chicken, rat, monkey and human CR. We show its use in the qualitative detection of CR by different methods of immunohistochemistry as well as in the detection of CR on immunoblots.

Calretinin (CR) is a calcium binding protein (CaBP) and a member of the widely distributed family of EF-hand proteins [1, 2]. CR was first identified by cloning a cDNA fragment from isolated RNA of the chicken retina [3]. The protein is mainly found in neurons of the central nervous system, particularly in the retina and in the sensory pathway [4]. CR was first detected on immunoblots of the rat brain as a protein band of 29 kD [5] cross-reacting with an antibody against another CaBP, calbindin-D28k [6]. Indeed, both proteins show a 60% identical amino acid sequence. CR expression was discovered in the human colonic WiDr cell line by immunohistochemical and molecular biological methods [7]. The screening of the cDNA library from these cells led us to the identification of several cDNA clones, identical to the cDNA of human brain CR as described by Parmentier [8] with the exception of codon 235 which is ATG in our clone as compared to ATC [8].

Only 2 different antisera have been available until now: one against CR purified from the brain of guinea pigs, where it was first termed 'protein 10' [9, 10]. The second antiserum was raised in rabbits and rats against an Escherichia coli derived β-galactosidase-calretinin fusion protein using a chick cDNA fragment [11]. Here we report on the production of human recombinant CR in E. coli and its purification. The purified protein was used to immunize rabbits. The polyclonal serum (code 7696) was characterized in Western blots and in immunohistochemical assays. The polyclonal antibody 7696 specifically recognizes CR from human, monkey, rat, chicken and fish tissues and does not cross-react with calbindin-D28k in these species. Due to its low background in immunohistochemical staining and its high specificity, the antibody may serve as a useful tool to study the localization of CR in species as distant in evolution as human and fish. Comparison of the cells expressing CR in different species
may lead to the identification of functional specializations which might help to elucidate the role of this highly conserved protein.

Materials and methods

Cloning of CR cDNA

Poly A⁺-enriched RNA was isolated from the human colon cell line WiDr [12] and used to synthesize cDNA as described [13]. Briefly, the ends of the double stranded cDNA were polished using T4 DNA polymerase and the resulting blunt-ended DNA was ligated into the Smal site of pUC18. Electroporation of 1 μg of ligated cDNA into E. coli HB101 yielded a cDNA library with a complexity of 1.5 x 10⁶ independent clones. The cDNA library was screened with 2 sense oligonucleotides corresponding to positions 25-44 and 75-94 of human brain CR cDNA [8]. After 2 rounds of screening, 5 of the clones remained positive.

Expression and purification of CR from E. coli

cDNAs from the 5 positive clones were sequenced by standard techniques. Clones 3.6, 3.9, and 16.17 in pUC18 were cut with NcoI (which naturally overlaps the ATG initiation codon of human CR) and HindIII (cutting in the pUC polylinker). The resulting fragment was cloned into the E. coli expression vector pDS56/RBSIN/NcoI [14]. Since translation in this vector starts at the ATG codon contained in the NcoI site, the resulting protein is devoid of erroneous amino acids at its N-terminus. The recombinant CR plasmids were used to transform competent E. coli M15 cells that contain the lac repressor encoding plasmid rep4 [14]. The 3 clones were grown in TB media [15] to a density of 0.6 (OD₅₅₀nm). Expression of CR was induced by isopropyl-thio-β-D-galactoside (IPTG) which was added to a final concentration of 2 mM. Cells were grown for an additional 3 h, centrifuged (10 000 g, 20 min) and the cell pellet was washed once with TBS, pH 7.4. The pellet was resuspended in a cell breaking buffer (50 mM HEPES, 150 mM NaCl, 1 mM MgSO₄, 5 mM EGTA, 2 mM DTT, pH 8.0) containing a protease inhibitor cocktail (soybean trypsin inhibitor, antipain, pepstatin, leupeptin, chymostatin, 10 μg/ml final, each, and PMSF 1 mM) and the cells were disrupted mechanically with a French press. The homogenate was centrifuged twice (10 000 g, 20 min, followed by an ultracentrifugation at 30 000 g, 90 min) and the supernatant containing the recombinant CR was used for purification. Protein was enriched by ammonium sulphate precipitation (60% saturation) and dialyzed against several changes of buffer (13.8 mM Tris, 120 mM NaCl, 4.7 mM KCl, 1 mM β-mercaptoethanol (β-ME), pH 7.4). For the whole purification procedure, a FPLC system (Pharmacia) was used. The protein mixture was first purified on a Superoxose 12 column (Pharmacia). After a 10-fold dilution with buffer A (10 mM Tris, 1 mM EDTA, 1 mM β-ME, pH 7.4) the fraction containing CR was directly applied to a Mono Q column, which had been equilibrated with buffer A. The gradient was developed with buffer B (10 mM Tris, 1 M NaCl, 1 mM EDTA, 1 mM β-ME, pH 7.4) as shown in Figure 3B. CR was specifically eluted from the column at 3% buffer B by injecting 5 ml 6% buffer B additionally containing 2 mM Ca²⁺. Purity was assessed by SDS-PAGE using the silver staining technique.

Mutagenesis of clone 3.9

Initial CR expression experiments showed that aside from CR a second protein with a Mr of about 25 kD was produced. N-terminal protein sequence analysis of this second protein revealed that it arose from an internal start of translation at ATG codon 68 of the CR mRNA. In order to remove the preceding cryptic ribosome binding site, this region of CR was mutagenized without changing the encoded amino acid sequence. An oligonucleotide of 37 base pairs (position 172-208, sequence shown in Fig. 2) containing 2 mismatches and the universal primer were used to amplify a fragment of clone 3.9 by PCR. The amplicon contains the sequence from nucleotide 1 to 208 so that the fragment ends blunt at the XmnI site at position 208. The fragment was isolated from a low melt agarose gel, the ends were filled by Klenow fragment and it was further digested by NcoI. Clone 3.9 in pUC was digested with XmnI and HindIII and the CR fragment (position 208 –
HindIII site of the polylinker) was isolated from a low melt agarose gel. The 2 purified fragments were again ligated into the NcoI–HindIII digested expression vector pDS56/RBSII,NcoI [14]. Transformation, induction of protein production and lysis of cells was done as described above.

**Gel electrophoresis**

2 different systems of one-dimensional SDS polyacrylamide gels were used. Normal slab gels (12% or 15% acrylamide) [16] were used to monitor the induction and expression of the recombinant protein. For assaying the purification procedure and for the Western blot experiments, a Phast System (Pharmacia, Uppsala, Sweden) was employed. Gels were either stained by Coomassie Blue or the more sensitive silver stain. For molecular weight determinations, marker proteins (Pharmacia) were used.

**Immunization of rabbits**

For the immunization of 2 rabbits, 20 μg purified CR (280 μg/ml) in complete Freund’s adjuvant was injected. In 4 week intervals, rabbits were boosted with CR emulsified with incomplete Freund’s adjuvant and were bled 2 weeks after each boost to check the titer by dot blot analysis of the serum.

**Isolation of the cytosolic fractions of brains of monkey, rat, chicken and fish**

An equal volume of buffer (2 mM EDTA, 3 μg/ml aprotinin (Trasylol®, Bayer, Germany) was added to freshly isolated brain from rat, chicken and fish. The tissues were disrupted by ultrasonication (Micro Ultrasonic Disruptor, Kontes) for 20 s. For the monkey brain, a small piece of cortex (0.5 g) which had been stored at –80°C was used for the isolation, but an otherwise identical isolation procedure was used. The homogenate was centrifuged (18 000 g, 4°C, 30 min) and the supernatant was used for Western blotting. Protein concentrations of the different samples were determined by the method of Bradford [17] using the reagents of Bio Rad. Samples were diluted to the same protein concentration and used for the Western blot.

**Western blotting**

Protein samples (4 μg) from the brain extracts of the different species were separated by SDS-PAGE and transferred onto Zeta Probe membranes (Bio Rad). A parallel gel was stained with Coomassie Blue. Membranes were saturated with blocking buffer (1%, w/v, bovine serum albumin and 10%, v/v, fetal calf serum), incubated with either CR antibody (1:1000) or a mixture of CR and calbindin-D28k antibodies (mAb 300, 1:1000 [18]) and were then further processed by the avidin-biotin method as described earlier [19], using 4-chloronaphthol/ hydrogen peroxide as a chromogen.

**Immunohistochemistry**

Rat and monkey brain tissues have been used to characterize the immunoreactivity patterns of the new polyclonal antibody 7696 directed against human recombinant CR. 2 rats (Zur-Siv, Institut für Tierkunde, Universität Zurich, Switzerland) were deeply anesthetized with ether and transcardially perfused with ice cold 4% paraformaldehyde (PFA) in phosphate buffer (PB) pH 7.4. Saturated picric acid and 0.4% glutaraldehyde were added in one case; one additional rat was perfused with Bouin fixative. Blocks of 2 monkey brains were taken from animals, used for an earlier study [20] where the tissue had been fixed by transcardial perfusion with 4% PFA in PB. All brains were immediately removed, cut in smaller blocks, and post fixed in the same fixative for at least 5 h with gentle agitation. The fixed brain blocks were stored at 4°C in Tris buffered saline (TBS), pH 7.3. Small blocks from the occipital lobes were washed several times in 18% sucrose in TBS, subsequently frozen in liquid nitrogen and sliced in 40 μm thick sections using a cryostat. For the incubation with the new CR antibody, we used a dilution between 1:5000 and 1:10 000 in TBS, with 10% bovine serum. We incubated floating sections with the first antibody for 72 h at 4°C with gentle agitation. The bound antibody was detected using the avidin-biotin kit (Vector Lab, Burlingame, CA, USA) and diaminobenzidine as the chromogen. We tested the specificity of the new antiserum by preadsorption with 10 μg highly purified (FPLC) CR protein per ml antibody.
solution. This mixture was incubated for 12 h before use.

Results

Purification of recombinant CR and production of antibodies

CR cDNA was synthesized from isolated RNA of the human adenocarcinoma cell line (WiDr) as described earlier [7]. After sequencing several clones, our CR cDNA sequence was found to be identical to that published by Parmentier [8] with the exception that in our clone codon 235 is ATG instead of ATC [8]. This cDNA was cloned into the expression vector pDS56/RBSII [14], which has an IPTG-inducible promoter. SDS polyacrylamide gel analysis revealed that the induced E. coli cells synthesized 2 additional proteins (M_r 31 000 and 25 000) when compared to non-induced control (Fig. 1). The 2 protein bands were blotted onto PVDF membranes and used directly for N-terminal Edman degradation. The sequence of the first 10 amino acids of the larger protein (M_r 31 000) was found to be identical to human brain calretinin [8]. The amino acid sequence analysis additionally revealed that the N-terminal methionine was cleaved off by E. coli. The second protein (M_r 25 000) was also a calretinin-like protein that, however, lacks the first 67 amino acids and starts with Met 68 (Figs 1 & 2). Since the shorter protein starts with Met and since 4 nucleotides upstream there is a sequence that could serve as an E. coli ribosome binding site, we assumed that this protein arose by an internal start of translation. To eliminate the expression of the smaller protein, the putative ribosome binding site in calretinin clone 3.9 was mutated without changing the encoded amino acid sequence. The strategy of the mutagenesis is depicted in Figure 2 and the two point mutations were introduced by PCR. E. coli transfected with the mutated construct only produced the M_r

Fig. 2 Mutation to eliminate internal ribosomal binding sites. To eliminate the lower molecular weight expression product (Fig. 1; calretinin starting with Met 68) the region preceding the ATG codon (solid bar) at position 202–204, 2 mutations were introduced in the putative ribosomal binding site (hatched bar). For this purpose an oligonucleotide of 37 base pairs (bottom row) containing these 2 mutations and half of the XmnI site (position 204–208, checkered bar) was synthesized and used for PCR as described in the text.
Fig. 3  Purification of CR from E. coli lysate. (A) The lysate from E. coli was applied on a Superose 12 column and the fraction containing CR (arrow) was further purified on a Mono Q column in 10 mM Tris, 1 mM EDTA, pH 7.4. The gradient was developed from 0–1 M NaCl (0–100% buffer B) as seen in (B) Specific elution of CR (arrow) was performed by injecting with 6% buffer B + 2 mM Ca²⁺. In the inset, a silver stained SDS gel of: (1) the peak fraction of the Mono Q column (single band at 29 kD); and (2) a crude E. coli lysate is shown.

31 000 form of CR, confirming the assumption that the small form of CR produced was due to an internal start of translation at Met 68. The introduced mutations were confirmed by sequencing the mutated clone. Nevertheless, the yield of the upper band (full length calretinin) was not significantly increased after the mutation (Fig. 1).

To purify the recombinant CR, a 2-step procedure using a FPLC system has been developed. The first step consisted of a size exclusion chromatography on a Superose 12 column, which separated proteins in the range 10–100 kD. The elution diagram is shown in Figure 3A. Fractions containing CR were pooled, diluted with buffer A and added directly to a Mono Q (anion exchange) column. The gradient was developed from 0–1 M NaCl. Under these conditions, minor protein bands were always detected in SDS-PAGE and highly purified CR could not be obtained (data not shown). Even decreasing the slope of the gradient did not completely eliminate contaminating proteins. Therefore, a slightly different approach was chosen. It is well known for CaBPs that their conformations can change in the presence or absence of Ca²⁺ which can even effect their migration in SDS polyacrylamide gels [21]. We determined in earlier runs on the Mono Q column the concentration at which CR elutes (6–7% buffer B). For the calcium specific elution, the gradient was developed to 3% buffer B and an isocratic phase at 3% buffer B was introduced as shown in Figure 3B. Specific elution of CR was achieved by injecting a buffer of 6% buffer B containing 2 mM Ca²⁺. The elution diagram is shown in Figure 3B. Purity of the eluted protein was found to be greater than 95% as estimated on SDS-PAGE using the sensitive silver staining technique (Fig. 3B, inset). This purified protein was used to immunize rabbits using standard methods. The polyclonal serum was tested for its specificity in Western blots and in immunohistochemical stainings of cryostat sections of monkey and rat brain.

Western blots

Cytosolic fractions of brains from monkey, rat, chicken and fish were isolated. The cytosolic fraction from WiDr cells was used as a positive control. From each brain fraction 4 μg, and 0.5 μg protein
Fig. 4 Western blot of cytosolic fractions of WiDr cells and brain from monkey, rat, chicken and fish. (A) Coomassie Blue stain of cytosolic extracts (left to right) WiDr cells and brain extracts from monkey, rat, chicken and fish. Right lane: molecular weight marker (97, 68, 43, 31, 21, 14 kD). (B) Western blot with the same fractions as in (A) stained with anti-CR antiserum (1:1000). (C) Western blot as in (B) immunostained with an antibody mixture of anti-CR antiserum (1:1000) and monoclonal antibody mAb 300 [17] against calbindin-D28k.

from the WiDr cell lysate, were separated on a polyacrylamide gel (12%). One portion of gel was stained by Coomassie Blue (Fig. 4A) and the identical proteins from the second portion of the gel were transferred onto Zeta Probe membranes. The membranes were blocked and incubated either with the rabbit polyclonal serum (7696) against CR (1:1000) or with a mixture of polyclonal serum 7696 and a monoclonal antibody mAb 300 against calbindin-D28k (CB). In the WiDr cell lysate, a single band at 29 kD was detected (Fig. 4B, lane 1). The same protein band was also visible in monkey, rat, chicken and fish brain (lanes 2–6). Minor bands (Mr 115 kD and 78 kD) could be seen in the cytosolic brain extracts of chicken and fish. No cross-reactivity with CB was visible in all of the species tested. To confirm the presence of CB in our sample preparations, a Zeta Probe membrane containing the identical fractions was incubated with a mixture of antibodies against CR and CB. As seen in Figure 4C, a second band at 27 kD was present in monkey, rat and chicken brain, whereas only one band was visible in the WiDr cell lysate and in the fish brain fraction.

**Immunohistochemistry**

We tested different protocols for the fixation of tis-
Fig. 5  Low power histograms of the monkey occipital cortex. (A) Immunostaining with anti-CR antisera 7696 (1:5000 in TBS, pH 7.4, 10% cow serum). In the grey matter only subsets of neurons are stained. In the white matter fibre tracts as well as glial cells are stained. The boundary between Areas 17 (left side) and 18 (right side) is clearly visible and marked with arrowheads. (B) Adjacent section incubated with a solution containing anti-CR antisera 7696 (1:5000) and 10 μg/ml purified CR protein. The characteristic staining patterns of the CR antibodies are completely blocked. The immunoreaction product in both figures is white, due to the reproduction technique. Scale bar 300 μm.

Discussion

Winsky et al. [10] have described a procedure for purifying CR from the brain of guinea pigs. However, for further biochemical and biophysical analysis of this protein, larger amounts of pure protein are required. With human CR, in particular, the tissue for isolation is difficult to obtain. A convenient source of CR was therefore found by expressing the protein in E. coli and purifying the protein from cell lysates.

When expressing CR in E. coli, surprisingly, two bands having molecular weights of 31 000 and 25 000 were observed on SDS gels. The larger protein corresponded to the full length protein (without the first methionine, which is cleaved off by E. coli), while the smaller protein band consisted of a truncated form starting with amino acid Met 68. This start was due to an internal ribosomal binding site in proximity to ATG 68. Although the putative ribosome binding site (GGAGAA, position 192–197) only distantly resembles the canonical ribosomal binding site [22] and also the distance to the ATG 68 (position 202) is very close, it seemed that the ribosomes preferentially bound to the internal site since the small CR form was predominant. Mutation of the putative internal ribosome binding site resulted in the disappearance of the smaller form of CR, which shows that the smaller form of CR was indeed due to an internal start of translation at Met 68. The relative abundance of full length CR did not increase after the mutation of the internal ribosomal binding sites, suggesting that the two sites did not compete with each other. Whereas the intro-
duced mutations (not altering the amino acid sequence) did not increase the expression level, they proved to be very helpful in the purification procedure. Due to the similar physical and chemical behavior of the full length and the truncated form of calretinin, purification to homogeneity was extremely difficult to achieve using chromatographic methods. Parmentier and Lefort [8] expressing human brain calretinin also observed 2 CR forms. From the calculated molecular weights and the identification of another putative canonical ribosomal binding site the authors suggested an internal start of translation at Met 57 and/or 58.

Few requirements for a purification procedure for CR can be posed: (a) the procedure should include as few steps as possible; and (b) sufficient quantities should be producible. By expressing CR in E. coli and by destruction of the internal ribosome binding site that gives rise to a smaller CR form, a suitable raw extract in large quantities can easily be prepared. From this extract, pure calretinin can be isolated in a 2-step procedure. In our purification procedure, we take advantage of the fact that calcium binding proteins can change their conformation depending on their binding of Ca$^{2+}$. By first selecting the molecular weight range by a normal size exclusion chromatography (Superose 12), followed by an anion exchange coupled to an affinity chromatography, the purification is both fast and easy, and yields protein in large amounts and in a very high purity.

The serum obtained from the rabbit immunized with CR was first tested in a Western blot using either E. coli lysate or a cytosolic fraction of WIDr cells, from which the original cDNA was isolated. In both cases, the antiserum reacted with a single protein of 29 kD (data only shown for WIDr cell lysate). In Western blots of cytosolic fractions from brain of monkey, rat, chicken and fish, the antiserum recognized a band of approximately 29 kD. When the nitrocellulose was concomitantly incubated with an antibody against calbindin-D28k, a second band of 27 kD appeared in the brain extract of monkey, rat and chicken. In fish and WIDr cells, only one band was present. Northern blot analysis has shown the lack of calbindin-D28k (CB) mRNA
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in WiDr cells [23] and, therefore, the 27 kD band (CB) should not appear in the WiDr cell lysate. In fish, the existence of CB immunoreactivity has been demonstrated by Parmentier et al. [24], but since the antibody used by this group cross-reacts with CR, the existence of CB in fish is uncertain. Also the fact that they have found the immunoreactive band only in brain and not in intestine implies that they might have looked at CR which is found almost exclusively in neuronal cells, while the CB is present in intestine and brain in almost all non-mammalian species. Western blot experiments of cytosolic extract from fish brain with either the monoclonal antibody mAb 300 or a polyclonal serum against calbindin-D28k (kind gift of Dr K.G. Baimbridge) showed no immunoreactive band in the expected molecular weight region, thus supporting the above findings (data not shown). Since the close relationship of CB and CR suggests that they have arisen from a common ancestral gene, it can not be excluded that only this precursor protein is present in fish and therefore contains epitopes which are recognized by the CR antisem 7696 and by the antisem as described by Parmentier et al. Cloning of the CR or CB analogue in fish will help to clarify this point.

The results from the Western blot experiments using our CR antibody 7696 demonstrated that under the conditions where both proteins are present in amounts typical for whole brain extracts, no cross-reaction between CB and CR is observed.

The new CR antibody 7696 is also a very useful tool for neuroanatomical studies, and CR was specifically detected in most brain areas. CR immunoreactivity filled almost all of the cellular processes. CR labelled neurons in the cerebral cortex have predominantly a vertical orientation of their processes. This precise outlining of the shape will help to further classify neuronal subtypes by their biochemical characteristics. In the monkey cerebral cortex CR immunoreactivity revealed distinct cyto- and myeloarchitectural boundaries. Further studies comparing these boundaries with those described with classical Nissl or myelin staining techniques, could give new insight into the neuronal organization. The fact that CR antibodies were directed against human CR, will make them an excellent tool for neuropathological studies.

In conclusion, we describe a simple procedure for the isolation of pure human recombinant CR from E. coli lysates. The antisem which has been raised in rabbits was characterized in Western blots and immunohistochemical stainings. Both methods confirmed that the antisem specifically recognizes CR and does not cross-react with the closely related protein, calbindin-D28k. Since CR is very highly conserved, it is not surprising that the antisem recognizes epitopes of CR conserved in human, monkey, rat, chicken and fish.

References


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