Is double C2 protein (DOC2) expressed in bovine adrenal medulla? A commercial anti-DOC2 monoclonal antibody recognizes a major bovine mitochondrial antigen

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We have examined the expression in bovine adrenal medulla of double C2 protein (DOC2), a vesicular protein which associates with intracellular phospholipid and Ca^{2+} and is implicated in the modulation of regulated exocytosis. Extensive reverse transcription-PCR, Northern blot analyses and *in vitro* translation reactions have been combined with immunological studies to

provide data to suggest that neither DOC2 α nor DOC2 β is expressed at detectable levels in bovine adrenal chromaffin cells, and that a widely used monoclonal antibody directed against DOC2 also recognizes mitochondrial complex III core protein 2.

Key words: C2 domain, chromaffin cells, complex III, exocytosis.

INTRODUCTION

A large body of research carried out over recent years has identified a multitude of proteins involved in regulated exocytosis. A key event in this process is a transient increase in the level of intracellular Ca^{2+} , which provides the trigger for vesicle fusion and exocytosis. A number of candidate proteins have been implicated in Ca^{2+} binding at various stages throughout the exocytotic process. One common feature of these proteins is the C2 domain, first identified as the second of four conserved functional domains in protein kinase C (for a review, see [1]). The C2 domain has now been identified as a Ca^{2+} - and phospholipid-binding domain in a number of proteins, including Munc13 [2], synaptotagmin [3], rabphilin [4] and double C2 protein (DOC2) [5]. DOC2 was first identified in nervous tissue as DOC2 α [5,6], and subsequently as a widely expressed, but not ubiquitous, DOC2 β isoform [7]; for a review, see [8]. The DOC2 coding sequences share approx. 56% identity and are approx. 60% similar at the amino acid level, with highly conserved C2 and N-terminal regions, and no apparent transmembrane domains. DOC2 proteins have been implicated in the regulation of exocytosis by way of their interactions with other exocytotic proteins [8–10]. Furthermore, the overexpression of DOC2 in PC12 cells enhances exocytosis [11], whereas the microinjection of peptide fragments of DOC2 reversibly inhibits secretion [12]. DOC2 is a vesicular protein which associates with intracellular membranes by virtue of one or both of its C2 domains interacting with phospholipid [13–15], but it has also been shown to associate with the plasma membrane to a lesser extent [10,15].

Adrenal chromaffin cells have long been a model system of choice for studies of exocytosis. These neuroendocrine cells, like neurons, fire action potentials, exhibit calcium-dependent exocytosis and express many proteins closely related to synaptic proteins. A widely used monoclonal antibody directed against DOC2 recognizes a component of the expected molecular mass (~46 kDa) in bovine adrenal chromaffin cells which co-migrates with mitochondrial markers [16]. It has been suggested that this is the DOC2 β isoform; the DOC2 α isoform has not been detected in adrenal tissue [10,16]. We present data here to demonstrate that neither DOC2 α nor DOC2 β mRNA is expressed at detectable levels in bovine adrenal chromaffin cells, and that the

anti-DOC2 α monoclonal-reactive protein is bovine mitochondrial complex III core 2 protein. We conclude that the previously described isoforms of DOC2 proteins are not detectable in adrenal medulla cells.

MATERIALS AND METHODS

Reverse transcription-PCR (RT-PCR) amplification of DOC2 isoforms

An RT-PCR strategy was used to amplify a cDNA encoding the open reading frames of mouse brain DOC2 α and DOC2 β . A sample of 1 μg of mRNA was used as template in a first-strand cDNA synthesis directed from an anchored deoxyoligo d(T) [5'-TTCTAGAATTCAGCGGCCGC(T)₃₀N₁N₂] primer, using Superscript II reverse transcriptase (GibcoBRL Life Technologies, Paisley, Scotland, U.K.). A similar strategy was used to synthesize first-strand cDNA from mRNA purified from bovine adrenal medulla. For DOC2 α amplification, first-strand cDNA was diluted and used in a PCR with the following deoxyoligonucleotides: forward, 5'-GCTGCATGAGGGGCCGACGGGGCGATCGC; reverse, 5'-TCAAGCCAAAGGGTAAGCCCCTGCTGCAG. For DOC2 β amplification, the resultant cDNA was diluted and used in a PCR between forward (5'-CTGCCTGCATGACCCTCCGGC) and reverse (5'-TCAGTCGCTGAGYACAGCCCCTGGG) deoxyoligonucleotides, using Expand polymerase cocktail (Boehringer Mannheim). Alternative DOC2 β deoxyoligonucleotides were also used: forward, 5'-GACCAGCTCTTCGGAGCCTAC, reverse, 5'-ATGGACAGTCAGTCGCTGAG or 5'-AAAATAATCGGAGATCTGCTTGATAGGCCG. Initial attempts to amplify full-length cDNA encoding DOC2 from mouse brain were unsuccessful, but the inclusion of various concentrations of betaine hydrochloride [17,18] to assist template strand denaturation permitted efficient amplification (see Results section).

Translation *in vitro*

PCR products encoding DOC2 β were ligated to the T/A vector pCR2.1 (Invitrogen), and then subcloned as an *Eco*RI–*Bam*HI restriction fragment into pcDNA3.1(+) (Invitrogen). DOC2 α PCR products were ligated to pcDNA3.1V5HIS-TOPO accord-

Abbreviations used: CNS, central nervous system; DOC2, double C2 protein; RT-PCR, reverse transcription-PCR.

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ing to the manufacturer's instructions (Invitrogen). All cDNA inserts were sequenced to confirm their identities and orientations. A portion of 1 µg of each recombinant plasmid was used in an *in vitro* coupled transcription/translation reaction (TnT T7; Promega, Southampton, U.K.). Radiolabelled reaction products were detected by SDS/PAGE [19] followed by autoradiography.

Subcellular fractionation and blue native gel electrophoresis

Bovine adrenal chromaffin granule membranes were purified as described elsewhere [20]; mitochondria were obtained by centrifuging the 'large granule fraction' over a cushion of 1.6 M sucrose (60 min; 160 000 g) to remove chromaffin granules. Bovine heart mitochondria were prepared by the method of Smith [21]. Blue native PAGE and two-dimensional gel electrophoresis (blue native PAGE followed by SDS/Tricine/PAGE) were performed as described by Schägger et al. [22,23]. Adrenal medullary mitochondria were washed by suspending them at a concentration of 2.7 mg of protein/ml in 0.1 M Na₂CO₃, pH 11.0, containing 5 mM EDTA; after 30 min at 0 °C, the membranes were collected by centrifugation, and the supernatant dialysed against three changes of distilled water, before freeze-drying and electrophoresis. Fractionation of adrenal medullary mitochondria with Triton X-114 was performed as described by Bordier [24]. All mitochondrial subfractions were resuspended in their original volume, before dilution with sample buffer for electrophoresis.

Antibodies and immunoblotting

Approx. 10–20 µg of protein per sample (10 µl for *in vitro* translated proteins) was electrophoresed in an SDS/10% polyacrylamide gel using the method of Laemmli [19]. Proteins were transferred to PVDF using a Trans-Blot SD (Bio-Rad, Lewes, E. Sussex, U.K.) semi-dry electroblotter or an LKB Transphor wet blotter. Monoclonal anti-DOC2 antiserum (Transduction Laboratories, Lexington, KY, U.S.A.; catalogue no. D45720) was diluted 1:500 in blocking buffer and incubated with the membranes for 1 h. A rabbit polyclonal antiserum directed against bovine mitochondrial complex III core 2 was donated by Dr Hermann Schägger (Johann Wolfgang Goethe-Universität, Frankfurt-am-Main, Germany), and was used at a dilution of 1:20 000. Membranes were washed extensively with PBS/0.05% (v/v) Tween 20 before incubation with horseradish peroxidase-conjugated second antibody, diluted in blocking buffer as before. Blots of two-dimensional gels were also made on PVDF, but using a wet blotter. Decorated proteins were revealed by ECL[®] (Amersham-Pharmacia Biotech, Little Chalfont, Bucks., U.K.).

MS analysis

Mitochondrial proteins were prepared and proteins were separated by SDS/PAGE and visualized by Gelcode (Pierce) Coomassie Blue staining. Bands of interest were excised and subjected to reductive carboxyamidomethylation prior to in-gel digestion with trypsin (Promega; sequencing grade). Peptides were desalted using a C8 Microguard column (Presearch) and eluted directly into an Econo12-coated glass (New Objective Inc, Cambridge, MA, U.S.A.) nanospray needle as described previously [25]. Analysis was carried out on an LCQ mass spectrometer (ThermoQuest, San Jose, CA, U.S.A.) equipped for nanoelectrospray ionization, operating in positive-ion mode. Selected ions were subjected to collision-induced dissociation

MS/MS with optimized collision energy. Uninterpreted MS/MS spectra were searched against an NCBI database using the SEQUEST program (J. Eng/J. Yates, University of Washington; licensed to Finnigan MAT).

Northern blotting

Total RNA from mouse whole brain, bovine adrenal medulla or bovine adrenal chromaffin cells was prepared as described previously [26]. A sample of 15 µg of total RNA, or 5 µg of poly(A)⁺ RNA, was electrophoresed in a fully denaturing formaldehyde/1% (w/v) agarose gel and transferred to a Hybond N nylon membrane (Amersham-Pharmacia Biotech). Random-primed, α-³²P-labelled probes were synthesized from restriction fragments of DOC2α and DOC2β cDNAs (see Figure 2A). After hybridization, membranes were washed under moderate-stringency conditions [final wash: 2×SSPE (1×SSPE is 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA), 65 °C, 30 min] or low-stringency conditions (final wash: 2×SSPE, 55 °C, 15 min) before autoradiography.

RESULTS

Amplification of *DOC2*

A titration of betaine hydrochloride concentrations from 0.25 M to 2.5 M (final concentrations) in the presence of 1.3% (v/v) DMSO revealed that *DOC2β* was only amplified from mouse brain cDNA on the inclusion of 2.25 M betaine, with no detectable amplification at betaine concentrations other than this. Likewise, no *DOC2α* amplification could be detected in the absence of betaine hydrochloride. Amplification of murine brain *DOC2α* occurred in the presence of 0.25–1.75 M betaine hydrochloride [final concentrations; also with 1.3% (v/v) DMSO], with maximal amplification at 1.25 M betaine. The integrity of the cDNA used was confirmed by successful amplification of a fragment of Munc18-1 [27] from the same preparations of bovine adrenal medulla and mouse brain cDNAs (results not shown). These conditions provide a protocol for the efficient amplification of full-length *DOC2* cDNA species, which until now have proved to be resistant to amplification, presumably because of their unusually high GC content [5–7]. In contrast, no *DOC2α* or *DOC2β* could be amplified using any primer combination with bovine adrenal medulla cDNA. It is possible that the PCR deoxynucleotides have mis-matches at, or near, their 3'-termini when annealing to bovine *DOC2* species, which would prevent primer extension when using *Taq* polymerase alone. For this reason, we routinely used a cocktail of *Taq* polymerase and *Pwo* polymerase (Expand; Boehringer Mannheim), which has an inherent 3' → 5' exonuclease 'proofreading' property.

DOC2 mRNA is not present at detectable levels in bovine adrenal medulla cells

In order to examine the expression of *DOC2* RNA transcripts in bovine adrenal medulla, we performed low-stringency RNA blots. Northern blot analyses using a wide variety of probes covering the full length of *DOC2α* and *DOC2β* coding regions consistently detected single transcripts of the predicted sizes in mouse brain RNA samples (see Figures 1C and 1D for examples). However, in agreement with the RT-PCR studies presented above, no RNA species hybridized with any *DOC2*-specific probe in parallel experiments using total RNA or poly(A)⁺ RNA from bovine adrenal chromaffin cells, even under conditions of reduced stringency and with extended exposure times of up to 14 days (results not shown). We estimate that transcripts with as

Figure 1 Northern blot analysis of DOC2 RNAs from mouse brain and bovine adrenal medulla

(A) Diagrammatic representation of the domain structures of mouse DOC2 proteins, with the approximate bp indicated (not to scale). (B) Summary of the restriction fragments used in the present study to direct the synthesis of probes for Northern analyses. (C) A representative Northern blot of DOC2 β , using probe h, washed at low stringency and exposed for 15 h. A single transcript of the expected size of 5.4 kb is revealed only in mouse brain. No hybridizing species are revealed in the bovine adrenal medulla sample. The corresponding Methylene Blue-stained filter is shown alongside the autoradiogram to confirm loading and sample integrity. (D) A similar, representative Northern blot of DOC2 α using probe e. Again, a single transcript of the predicted size (2.2 kb) is revealed only in the murine brain sample. Extended exposures (up to 14 days) revealed no hybridizing species in bovine adrenal medulla mRNA samples. The corresponding Methylene Blue-stained filter is shown alongside the autoradiogram. Lanes: 1, bovine adrenal medulla RNA; 2, murine brain RNA; 3, bovine adrenal medulla RNA, stained with Methylene Blue; 4, murine brain RNA, stained with Methylene Blue.

little as 50% identity would hybridize under these conditions [28]. The integrity of the RNA was confirmed by Methylene Blue staining of the transfer membranes (Figures 1C and 1D).

Characterization of a widely used anti-DOC2 monoclonal antibody

Immunoblot analyses using a commercially available monoclonal anti-DOC2 antibody (see the Materials and methods section) revealed decorated protein species of the appropriate size in the mouse olfactory bulb, cerebellum and frontal cortex (Figure 2A). Likewise, a protein species of approximately the predicted size could be detected in bovine adrenal medulla lysates. To characterize this antibody further, we performed immunoblot analysis using *in vitro*-translated DOC2 α and DOC2 β . Coupled *in vitro* transcription/translation reactions synthesized DOC2 α and DOC2 β efficiently (Figure 2B). Both DOC2 α and DOC2 β migrated with apparent molecular masses of approx. 45–47 kDa, as described previously for the native protein [5–7]. Occasionally, *in vitro* translation reactions using DOC2 α resulted in two

Figure 2 Both DOC2 α and DOC2 β cDNAs direct the *in vitro* translation of proteins with the predicted molecular masses

(A) Immunoblot analysis of mouse brain and bovine adrenal medulla cells demonstrates that the anti-DOC2 monoclonal antibody recognizes proteins in the mouse olfactory bulb, frontal cortex and cerebellum, and a protein of a similar molecular mass in bovine adrenal medulla. Abbreviations: Bam, bovine adrenal medulla; Olf, mouse olfactory bulb; Cb, cerebellum; Ctx, mouse frontal cortex. (B) *In vitro*-translated murine DOC2 β has a molecular mass of 45500 Da, and shows no change in apparent mass when translated in the presence of canine pancreatic microsomes. In the absence of a recognizable cleavable signal sequence, this indicates that DOC2 is not glycosylated on the consensus sites *in vitro*. A similar *in vitro* translation experiment using murine DOC2 α also revealed a translated product of the predicted mass and no apparent glycosylation. Lanes: 1, DOC2 α , no microsomes; 2, DOC2 α , microsomes; 3, DOC2 β , no microsomes; 4, DOC2 β , microsomes. (C) Demonstration of the specificity of the monoclonal anti-DOC2 antibody for murine DOC2 α . *In vitro*-translated DOC2 α and DOC2 β were probed with monoclonal anti-DOC2. Decorated protein was detected only in the DOC2 α sample. Lanes: 1, DOC2 α , no microsomes; 2, DOC2 α , microsomes; 3, DOC2 β , no microsomes; 4, DOC2 β , microsomes.

products: one of the predicted molecular mass, and a smaller, presumably truncated, protein. When canine pancreatic microsomes were included during the *in vitro* translation, there was no shift in the apparent molecular mass of either DOC2 α or DOC2 β after electrophoresis, indicating that DOC2 proteins are not post-translationally modified *in vitro*.

The monoclonal antibody did not cross-react with *in vitro*-expressed DOC2 β , but did detect DOC2 α (Figure 2). As the central nervous system (CNS)-specific DOC2 α is not expressed in adrenal chromaffin cells [10], these data, taken together with the mRNA expression studies, suggest that the monoclonal antibody cross-reacts with a DOC2-like epitope within another protein in these cells. To address this question, we used isopycnic fractionation of adrenal medulla homogenates on sucrose gradients, followed by SDS/PAGE and blotting of individual fractions. This confirmed the finding of Charvin et al. [16] that the component recognized by the anti-DOC2 monoclonal antibody equilibrated at the same position in the gradient as mitochondria. We therefore isolated mitochondria from bovine adrenal medullae, and subfractionated them in order to identify the component recognized by the monoclonal antibody.

On washing the mitochondria with sodium carbonate at pH 11, the antigen remained membrane-bound (Figure 3A, lanes 1–3). On fractionation of the mitochondria with Triton X-114

Figure 3 The anti-DOC2 reactive protein is bovine complex III core 2

Western blot of mitochondrial fractions, run on a 9% (w/v) polyacrylamide gel. Lanes 1 and 7, adrenal medullary mitochondria (27 μ g of protein); lane 2, sodium carbonate-washed adrenal mitochondrial membranes; lane 3, supernatant from sodium carbonate washing of adrenal mitochondria; lane 4, detergent-rich phase from Triton X-114 fractionation of adrenal mitochondria; lane 5, aqueous phase from Triton X-114 fractionation of adrenal mitochondria; lane 6, bovine heart mitochondria (20 μ g). (A) Blot decorated with anti-DOC2 monoclonal antibody (dilution 1:500); (B) the same blot re-probed with a polyclonal antiserum against complex III core 2 (dilution 1:20 000).

[24], the antigen was found in the detergent-rich phase, and was completely absent from the aqueous phase (Figure 3A, lanes 4 and 5). The behaviour of the antigen in these experiments suggested that it might be an intrinsic membrane protein; as noted above, DOC2 contains no predicted transmembrane sequences. We next used one-dimensional blue native gel electrophoresis [22,23] to separate the intact respiratory components of adrenal mitochondria. Immune blots of these gels (results not shown) suggested that the monoclonal antibody recognized mitochondrial respiratory complex III (ubiquinol:cytochrome *c* reductase), and we further found that the antigen was present in isolated bovine heart mitochondria, with a slightly greater abundance than in adrenal mitochondria (Figure 3A, lane 6).

In order to identify the subunit of complex III that was recognized by the antibody, we ran a preparative gel of sodium carbonate-washed adrenal mitochondrial membranes, stained it with colloidal Coomassie Blue, cut out the candidate band and electroeluted the protein. This protein was analysed by MS after re-running on a thin gel and checking its reaction with the monoclonal anti-DOC2 antibody. An ion which was present in both heart and adrenal medulla samples was subjected to collision-induced dissociation MS/MS fragmentation. The resultant spectrum corresponded to a tryptic peptide comprising residues 43–60 (LPNGLVIASLENYAPASR; molecular mass 1885 Da) from ubiquinol:cytochrome *c* reductase complex core protein 2 (reference: UCR2-bovin; P23004; when searched with SEQUEST).

A polyclonal antiserum raised against bovine heart complex III core 2 was used to re-probe the blot shown in Figure 3(A), and was found to exactly overlay the staining pattern produced by the monoclonal antibody (Figure 3B).

DISCUSSION

We provide molecular biological evidence that neither DOC2 α nor DOC2 β RNA transcripts are present at detectable levels in bovine adrenal chromaffin cells, even when screening at reduced stringencies or using RT-PCR with a 'proof-reading' polymerase. We have characterized a commercially available monoclonal anti-DOC2 antibody and demonstrated that it reacts

specifically only with the murine DOC2 α isoform and not with the widely expressed DOC2 β isoform. This may not be surprising, as the monoclonal antibody was raised using a brain DOC2 antigen.

DOC2 proteins have been shown to be localized to intracellular structures, both in neuronal tissue and in PC12 and bovine adrenal chromaffin cells overexpressing DOC2 [5,9,10,14,15]. These intracellular structures are most likely to be secretory vesicles, as indicated by their size, acidic contents and immunological markers [10,14,15]. Recently it has been reported that the monoclonal anti-DOC2 antibody recognizes a protein of the expected size in bovine adrenal chromaffin cells; however, this immunoreactivity co-migrates with mitochondrial markers [16]. Our data suggest that the antigen recognized by the anti-DOC2 monoclonal antibody in bovine adrenal cells is an intrinsic membrane protein. MS indicated that this was core protein 2 of respiratory complex III, and this was confirmed by the use of a polyclonal antibody directed against this protein. The molecular mass of this protein, 46 kDa, is coincidentally very similar to that of DOC2. It is likely that bovine adrenal chromaffin cells express another isoform or a paralogue of DOC2, distinct from DOC2 α or DOC2 β .

In summary, we have characterized a monoclonal anti-DOC2 antibody and shown it to react with the CNS-specific murine DOC2 α isoform and to cross-react with bovine mitochondrial complex III core protein 2. Furthermore, we have shown that DOC2 α and DOC2 β RNAs cannot be detected in bovine adrenal medulla cells, even when using conditions which would permit hybridization to species with as little as 50% identity. Therefore we conclude that previously described isoforms of DOC2 proteins are not expressed at detectable levels in bovine adrenal medulla cells.

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