Identification and characterisation of a homologue of p64 in rat tissues

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Abstract Previous work has suggested that the gene encoding p64, a component of a bovine kidney intracellular chloride channel, may be a member of a gene family. We have raised a polyclonal antibody to an *E. coli* fusion protein which has sequence similarity to p64. Immunoblotting detected a protein in rat brain, kidney, liver and lung. In rat brain, the protein was enriched in cerebellar microsomal membranes. Western blot analyses of denaturing and blue native polyacrylamide gels indicated that the protein is a single non-disulphide-linked polypeptide chain with an apparent M_r of 43 kDa that contributes to a native protein complex with an apparent M_r of 130 kDa.

Key words: Chloride channel; p64; RS43; Rat cerebellum

1. Introduction

Chloride channels are found in the plasma membrane of many cell types (for reviews see [1] and [2]) and this is where most research concerning Cl^- channel structure and function has focussed. However, over the last two decades, it has become apparent that Cl^- channels are not restricted to the plasma membrane. They are also found in the membranes of intracellular organelles [3–5] where they appear to have important roles in cell function.

In lysosomes, Golgi and endosomes intracellular Cl^- channels play an important role in regulating the intraorganelle pH [6]. This is achieved by shunting the electrogenic membrane potential generated by the H^+-ATPase, to enable greater acidification of the intracellular compartment than would otherwise be generated. Intracellular Cl^- channels may also be involved in the control of organelle volume [7]. In brain microsomal membranes, intracellular Cl^- channels are co-localised with ryanodine-sensitive Ca^{2+}-release channels [8] located in the endoplasmic reticulum [9]. As a result, it has been suggested that they may maintain charge balance during, and thereby regulate, intracellular Ca^{2+} release [8].

p64 is a component of a Cl^- channel and is found in intracellular membranes [10]. The cDNA encoding the protein was cloned from bovine kidney [11]. The predicted secondary structure from the deduced amino acid sequence suggests that p64 spans the lipid bilayer two or possibly four times. We have previously shown that p64 may be part of a gene family by isolating two partial gene homologues from rat brain [12]. Here we have raised a polyclonal antibody to an expressed region of one of these p64 homologues and used it to identify and characterise a protein (RS43) ubiquitously expressed in rat tissues. The identification of a rat brain protein related to bovine kidney p64 makes the possibility of an extensive gene family seem all the more likely.

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Abbreviations: SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; M-MuLV-RT, Moloney murine leukaemia virus reverse transcriptase; dNTP, 2′-deoxynucleotide 5′-triphosphate; IPTG, isopropyl-β-thiogalactopyranoside; NBT/BCIP, Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyphosphate *p*-toluidine salt-stable mix

2. Materials and methods

2.1. Materials

2.1.1. Antibodies. A polyclonal antiserum recognising a fusion protein (see below) was generated by subcutaneous injection of 200 μg of a partially purified fusion protein into rabbits at two-weekly intervals in suspension with Freund's incomplete adjuvant (50% v/v). Following four injections the animal was sacrificed and IgG-containing serum isolated. Horseradish peroxidase-conjugated donkey anti-rabbit IgG was purchased from Pierce (Chester, UK).

2.1.2. Molecular biology reagents. Moloney murine leukaemia virus (M-MuLV) reverse transcriptase and dNTPs were purchased from Pharmacia Biotech (St. Albans, UK). Restriction endonucleases, *Taq* DNA polymerase and isopropyl-β-thiogalactopyranoside (IPTG) were purchased from Promega UK (Southampton, UK). The expression vector pAX11 was a generous gift from Dr. Alan Boyd, University of Edinburgh, UK. Vector pBluescript II KS(+) and XL-1 Blue cells were purchased from Stratagene Inc. (La Jolla, CA).

2.1.3. Tissues. Tissues were obtained from Sprague-Dawley rats supplied by MFAA (Edinburgh, UK).

2.1.4. Other. 4-(2-Aminoethyl)-benzensulfonylfluoride (AEBSF) was obtained from Calbiochem (Nottingham, UK). Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyphosphate *p*-toluidine salt-stable mix (NBT/BCIP) was from Gibco BRL (Paisley, UK). All other materials were purchased from Sigma.

2.2. Cloning of a partial cDNA and generation of a fusion protein showing homology to p64

Degenerate oligonucleotide primers were designed based on the published amino acid sequence of bovine p64 ([11], GenBank Accession number L16547), with the addition of terminal recognition sequences for the restriction endonuclease *Eco*RI. Total RNA was extracted from whole rat brain as previously described [13]. The 'reverse' primer (DGDE, 5′-CACGAATTCAAYTCRTCNCCRTC-3′) was used to direct first-strand cDNA synthesis from 1 μg rat brain total RNA in a buffer containing 10 mM Tris-HCl, pH 9.0, at 25°C, 50 mM KCl, 2.5 mM $MgCl_2$, 1% (v/v) Triton X-100, with 0.5 mM of each dNTP and 5 units M-MuLV reverse transcriptase (Pharmacia). This reaction mixture was used directly for amplification after addition of the 'forward' primer (FMILW, 5′-CACGAATTCTTYAT-GATHYTNTGG-3′), additional $MgCl_2$ to 2 mM and 1.25 units *Taq* polymerase (Promega). Thermal cycling conditions were as follows; 95°C, 1 min, 55°C, 1 min, 72°C, 2 min, 30 cycles in an Omnigene Thermal Cycler (Hybaid). The resultant amplification product (BS2) was digested with *Eco*RI and ligated into pBluescript II KS (Stratagene). Dideoxy sequencing of both strands (Sequenase II, Amersham) was carried out on two independent recombinant plasmids. The deduced amino acid sequence shows approximately 70% identity in an optimal alignment to bovine p64 (see Fig. 1).

The nucleotide sequence of BS2 revealed an *Eco*RI recognition site at position 346 which is not present in p64 (not shown). This 346 bp

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*Eco*RI restriction fragment was ligated into the expression vector pAX11 [14] to create an 'in-frame' fusion with the sequence encoding IgG-binding domains of staphylococcal Protein A. After transformation of *E. coli* (XL1-Blue, Stratagene), expression of a unique peptide of the predicted size, inducible with 1 mM IPTG, was detected by SDS-PAGE followed by Coomassie Brilliant Blue staining (data not shown). Further analysis revealed this protein to be insoluble at 37°C, allowing it to be partially purified by isolating 'inclusion bodies' as previously described [15]. These purified inclusion bodies were used to immunise rabbits.

2.3. Tissue homogenisation and fractionation

Tissues were removed from SD rats within 2 min of humane sacrifice and placed immediately into ice-cold homogenisation buffer (5 mM Tris-HCl, 0.32 M sucrose, 1 mM EDTA, 1 μg/ml Pepstatin A, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin, 0.2 mM AEBSF, pH 7.4). Tissues were rinsed in homogenisation buffer and then homogenised in 9 volumes of the same buffer. In some experiments tissue homogenates were fractionated by differential centrifugation to yield microsomal membranes as described previously [8].

2.4. Denaturing and blue native polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE) was performed using the method of Laemmli [16]. In order to assess the presence of interprotein disulphide links the reducing agent (dithiothreitol) was omitted in some experiments.

To determine the apparent molecular weight of the native protein complex, blue native gel electrophoresis was undertaken [17,18]. Protein samples were separated according to a charge shift induced by Serva blue G binding. Briefly, membrane proteins were solubilised in 1% dodecylmaltoside in aminocaproic acid. Serva blue G was added and the proteins were subjected to electrophoresis on 6, 7, 8, 9 and 10% (w/v) polyacrylamide gels. Non-denatured protein molecular weight markers (Sigma) were run in parallel and the apparent native molecular weights of samples were determined according to the manufacturer's instructions.

2.5. Western blotting

Proteins were transferred onto Hybond-C membrane (Amersham) at 0.5 mA/cm^2 in a Tris/glycine/methanol buffer using a Semiphor™ blotting apparatus (Hoeffer). Following transfer excess binding sites on the membrane were blocked by incubation in 10 mM Tris-HCl, 140 mM NaCl, 0.1% (v/v) Tween-20, pH 7.4 (TTBS) containing 1% (w/v) BSA for 30 min. Polyclonal antibody (1 : 2000 dilution in TTBS) was incubated with the membrane for 60 min after which membranes were washed in TTBS and incubated for 30 min in horseradish peroxidase-conjugated donkey anti-rabbit IgG (1 : 4000 dilution in TTBS). Membranes were washed extensively in TTBS and protein bands revealed with NBT/BCIP substrate.

2.6. Other methods

Protein was measured by the bicinchoninic acid method [19] using a micro BCA protein assay reagent kit (Pierce) according to the manufacturer's instructions.

3. Results

3.1. Identification of a homologue of p64 in rat tissues

Various rat tissue homogenates were subjected to SDS-PAGE (50 μg/lane). Following Western blotting, immunoreactive proteins were detected using the polyclonal antibody raised against the p64 homologue. In rat brain, kidney, liver and lung homogenates, only one protein species could be detected (Fig. 2). In all cases the protein (named RS43) migrated with an apparent M_r of 43 kDa. The recognition of only a single species on Western blots from protein gels loaded with a mixture of proteins demonstrates the high specificity of the antibody.

3.2. Localisation of RS43, a homologue of p64, in rat brain

Rat brain was chosen for further study. Classical differential centrifugation was carried out on whole rat brain and the membrane fractions obtained were subjected to SDS-PAGE and Western blotting. These fractions have previously been extensively characterised by measuring the enrichment of intracellular organelles and marker enzymes (e.g. [20]). The fraction P1 is enriched in cell nuclei, P2 is enriched in mitochondria and P3 is enriched in microsomes. RS43 was enriched in the microsomal fraction, with a smaller amount found in the P1 fraction (Fig. 3a). No immunoreactive material was seen in the final supernatant (containing soluble proteins not pelleted by centrifugation at 100,000×g for 60 min), strongly suggest-

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P64BOV.PRO FMILWLKGVVFNVTTVDLKR 294
           FMILWLKGVVF:VTTVDLKR
BS2.PRO    FMILWLKGVVFSVTTVDLKR 20

P64BOV.PRO KPADLHNLAPGTHPPFLTFN 314
           KPA:L:NLAPGTHPPF:TFN
BS2.PRO    KPAHLQNLAPGTHPPFITFN 40

P64BOV.PRO GDVKTDVNKIEEFLEETLTP 334
           ::VKTDVNKIEEFLEE.L P
BS2.PRO    SEVKTDVNKIEEFLEEVLCP 60

P64BOV.PRO EKYPRLAAKHRESNTAGIDI 354
            KY :L::KH.ESNTAG:DI
BS2.PRO    PKYLKLSPKHPESNTAGMDI 80

P64BOV.PRO FVKFSAYIKNTKQQSNAALE 374
           F.KFSAYIKN::.::N.ALE
BS2.PRO    FAKFSAYIKNSRPEANEALE 100

P64BOV.PRO RGLTKALKKLDDYLNTPLPE 394
           RGL K:L:KLD:YLN:PLP.
BS2.PRO    RGLLKTLQKLDEYLNSPLPG 120

P64BOV.PRO EIDADTRGDDEKGSRRKFLD 414
           EID.::. :D K:S R:FLD
BS2.PRO    EIDENSM-EDIKSSTRRFLD 139

P64BOV.PRO GDE 417
           GDE
BS2.PRO    GDE 142
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Fig. 1. Alignment of amino acid sequence of bovine p64 with that deduced from a partial cDNA encoding a rat brain homologue. Bovine p64 (p64bov) was optimally aligned with BS2 using the Lipman-Pearson method within Megalign of Lasergene (DNAStar Inc., Madison, WI). The consensus can be seen in the middle. Colons represent conservative changes, periods indicate neutral changes and spaces represent unfavourable substitutions.

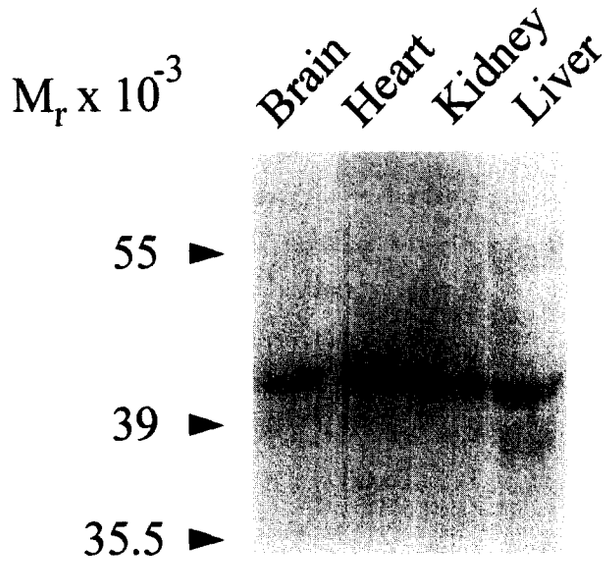

Fig. 2. Identification of a homologue of p64 in rat tissues. Tissues were homogenised as described in Section 2 and 50 μg subjected to SDS-PAGE. Proteins were transfered to Hybond-C membrane and detected with the polyclonal antibody recognising the p64 homologue.

ng that RS43 is a membrane protein. P3 membranes were prepared from rat cerebellum and rat forebrain. Western blot analysis of protein subjected to SDS-PAGE can be seen in Fig. 3b. Densitometric scanning revealed approximately ive-fold enrichment of RS43 in P3 from rat cerebellum, compared with rat forebrain (result not shown).

3.3. Characterisation of RS43

In order to obtain more information regarding the subunit makeup of RS43, SDS-PAGE was carried out under both reducing and non-reducing conditions, using the P3 fraction from rat cerebellum. Immunodetection showed that under both conditions RS43 migrated as a single protein species of apparent M_r 43 kDa (Fig. 4). This suggests that RS43 exists as a single non-interdisulphide-linked polypeptide chain in microsomal membranes.

Many ion channels are known to consist of various subunits that together form the native protein complex. Blue native PAGE was performed at different polyacrylamide concentrations using P3 membranes from rat cerebellum. Electrophoresed proteins were transferred to Hybond-C membrane and RS43 was detected using the polyclonal antibody described earlier. A single immunoreactive species could be detected at all polyacrylamide concentrations (data not shown). The molecular weight of the native protein complex was determined using a modification of the methods of Bryan [21] and Davis [22], as described in the Sigma Technical Bulletin No. MKR-137. Briefly, $100.\log(R_f \times 100)$ was plotted against the percentage gel concentration for each protein sample (either a protein standard, or the novel protein detected by immunoblotting), where R_f is the electrophoretic mobility. For each protein standard the logarithm of the negative slope was plotted against the logarithm of the M_r. The M_r of the unknown protein was then determined from the standard curve. Using this method RS43 was predicted to form a native protein complex with apparent M_r 130 kDa.

4. Discussion

Our previous work has suggested that p64 may be a member of a family of intracellular Cl^- channels, with at least two homologues in rat brain [12]. Further evidence for this is shown here, with the identification of a homologue of p64 at the protein level. We believe that RS43 is distinct from p64 for a number of reasons. Firstly, p64 migrates with an apparent M_r of 64 kDa following SDS-PAGE [10], whereas RS43 migrates with an apparent M_r of 43 kDa. This large difference in M_r makes it unlikely that the two are the same protein. Furthermore, Northern analyses of brain mRNA failed to detect a message for p64, although its mRNA was detected in other tissues (Q. Al-Awqati, personal communication). However, we have previously identified homologues of p64 in neuronal tissue [12]. It is interesting to note that during the drug-affinity purification of p64, a protein of approximately 40 kDa was co-purified [10,23]. Whether RS43 and this 40 kDa protein are related remains to be seen.

RS43 is enriched in microsomes isolated from whole rat brain, suggesting that it may be localised in an intracellular compartment of the cell. The protein was also unaffected following incubation with endoglycosidase F (our unpublished results), suggesting that it is not glycosylated. In a similar way, p64 protein was purified from microsomal membranes enriched for Golgi markers, and in CFPAC cells it has been immunolocalised to perinuclear vesicles [10]. Finally, there is increasing evidence that Cl^- channels in endosomes and clathrin-coated vesicles can be activated by protein kinase-A, and phosphorylation of membrane proteins in these locations has caused the appearance of a phosphorylated 65–70 kDa protein [24]. Clearly, further studies on possible RS43 phosphorylation, both in vivo and in vitro, will be of interest.

The relative abundance of RS43 in rat cerebellum agrees well with intense immunohistochemical staining in this area (R.R. Duncan and R.H. Ashley, unpublished work). Isolating microsomes from this RS43-rich region has allowed us to further characterise the protein. Western blot analyses of proteins following reducing, non-reducing and native gel electrophoresis suggested that RS43 exists as a single non-disulphide-linked polypeptide chain with an apparent M_r of 43 kDa that forms a native protein complex with an apparent M_r of approximately 130 kDa. There are a number of possibilities that can give rise to a native protein complex of 130 kDa. RS43

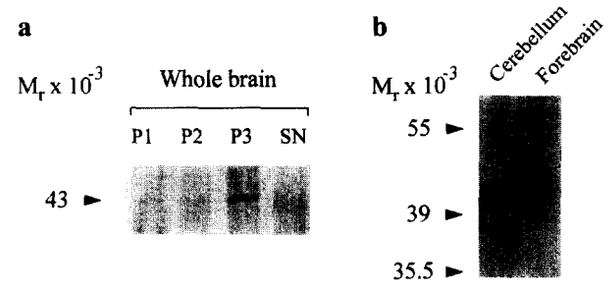

Fig. 3. Rat brain localisation of RS43. (a) Whole rat brain was homogenised and differential centrifugation undertaken to produce enriched membrane fractions [8,20]. SDS-PAGE (10 μg protein/lane) and Western blotting were carried out on the membrane pellets and the final supernatant. The deduced M_r for the band is indicated on the left-hand side. (b) P3 membranes were prepared from rat brain cerebellum and rat forebrain. 10 μg of protein was then subjected to SDS-PAGE and Western blotting.

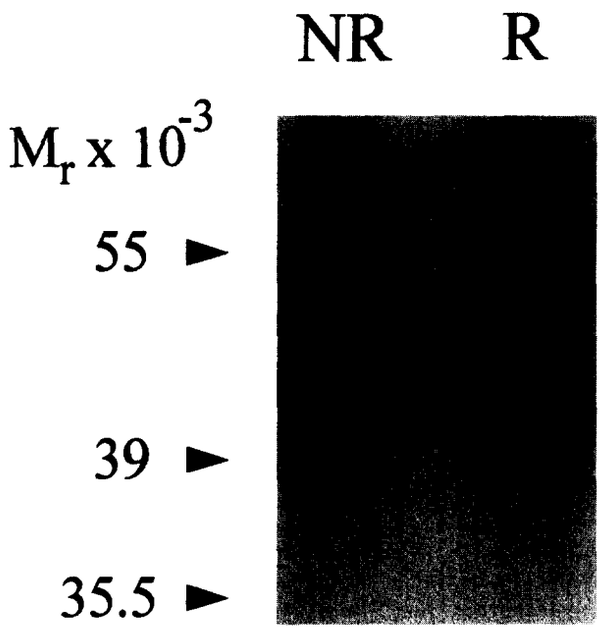

Fig. 4. SDS-PAGE and Western blotting of reduced and non-reduced RS43. P3 from rat cerebellum was subjected to SDS-PAGE either in reducing (R) or in non-reducing conditions (NR). Following Western blotting, proteins were detected using the polyclonal antibody as described in Section 2.

may form a homotrimeric or homotetrameric native complex, although the association of other proteins with a monomer or dimer of RS43 cannot be ruled out. We are currently investigating these possibilities.

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