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Cloning of cDNAs encoding a rabbit renal brush border membrane protein immunologically related to band 3

Sequence similarity with microsomal dipeptidase

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Distinct anion transport processes have been identified in the mammalian renal proximal tubule, but none of the responsible proteins or genes have been isolated. A 43 kDa rabbit microvillus membrane protein that is immunologically related to the erythroid anion exchanger (band 3) was a candidate for a renal anion transporter. To examine the structural relationship with band 3, we cloned cDNAs encoding the 43 kDa protein. The 43 kDa band-3-like protein was purified, and a novel sequence of 24 amino acids was obtained from the *N*-terminus. Degenerate oligonucleotides were synthesized based on this sequence, and the polymerase chain reaction with single-sided specificity was used to amplify and clone a 1330 bp cDNA from rabbit renal cortex. Additional overlapping 272 bp and 1123 bp cDNAs were obtained by synthesizing and screening a rabbit renal cortical cDNA library. The composite sequence was 1483 bp, terminated with $(A)_{16}$, and was similar in size to the principal transcript expressed in rabbit renal cortex. The single long open reading frame was predicted to encode a protein composed of 410 amino acids with a molecular mass of 45193 Da; 15 amino acids predicted to reside at the *N*-terminus were absent in the mature protein and may constitute a signal peptide. There was only limited sequence similarity with human erythroid band 3. Rather, the sequence was highly similar to microsomal dipeptidase, including the presence of a signal peptide and a consensus sequence for covalent linkage to glycosylphosphatidylinositol. In summary, the 43 kDa protein from rabbit renal cortex that is recognized by a monospecific antibody to erythroid band 3 is most likely a microvillus membrane dipeptidase.

INTRODUCTION

Active transport of inorganic and organic anions in the proximal tubules of mammalian kidneys is mediated by carrier proteins that translocate one anion across the plasma membrane in exchange for another anion [1]. For example, the luminal (microvillus, brush border) membrane of proximal tubule epithelial cells contains distinct urate/OH^-, lactate/OH^-, Cl^-/formate, and Cl^-/oxalate exchangers [2–5] that participate in trans-epithelial solute secretion or reabsorption and regulation of intracellular pH. These transporters are functionally related by their specificity for anions, operation in the exchange mode, and characteristic sensitivity to inhibition by stilbene disulphonates such as DIDS or SITS. Although they may be structurally related as well, none of the proteins responsible for anion exchange in the proximal tubule have been isolated, nor have their cDNAs been cloned.

The best-characterized plasma membrane anion exchanger is the Cl^-/HCO_3^- exchanger in erythrocytes, also known as AE1 or band 3 [6,7]. Erythroid band 3 is functionally similar to proximal tubule anion exchangers since it also exchanges anions across the plasma membrane and is sensitive to inhibition by stilbene disulphonates. Indeed, putative anion exchangers that are structurally similar to erythroid band 3 have been described in kidney [8].

We have previously reported identification of a novel renal membrane protein that is immunologically related to erythroid band 3 and is a candidate for a plasma membrane anion transporter. A monoclonal antibody (called IIE1) was raised against the 17 kDa *N*-terminal chymotryptic fragment of the membrane-associated domain of human eythroid band 3. Monoclonal antibody IIE1 specifically recognized a 43 kDa protein in immunoblots of rabbit renal microvillus membranes [9]. Antisera that were raised against the purified 43 kDa renal protein also recognized erythroid band 3. Thus the 43 kDa renal protein was immunologically, and hence structurally, related to erythroid band 3. Since monoclonal antibody IIE1 was raised against the 52 kDa domain of band 3 which mediates anion transport [10], and specifically against a 17 kDa *N*-terminal peptide that contains a covalent binding site for stilbene disulphonates [11], the immunologically related 43 kDa renal protein was a candidate for a proximal tubule anion exchanger. To further examine its structural relationship with erythroid band 3, we cloned transcripts encoding the 43 kDa renal protein and inferred its primary structure.

MATERIALS AND METHODS

Materials

Oligo(dT)–cellulose (Type III), *Eco*RI linkers and random hexamers were from Pharmacia LKB Biotechnology (Piscataway, NJ, U.S.A.). Avian myeloblastosis virus (AMV) reverse tran-

Abbreviations used: DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; MDP, microsomal dipeptidase; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonate; SSC, standard sodium citrate (0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0); LB, Luria–Bertani medium (in 1 litre: 10 g of Bacto-tryptone, 5 g of yeast extract, 10 g of NaCl, pH adjusted to 7.5 with NaOH); BLOTTO, 5% non-fat dried milk/0.02% NaN_3/140 mM-NaCl/2.7 mM-KCl/8.1 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.4.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X61503.

scriptase was from Invitrogen (San Diego, CA, U.S.A.). RNAase inhibitor (RNAsin) and bacteriophage λ packaging extract (Packagene) were from Promega (Madison, WI, U.S.A.). *Taq* DNA polymerase and dNTPs were from Perkin–Elmer Cetus (Norwalk, CT, U.S.A.). Nylon filters for Northern and Southern blots (GeneScreen Plus) were from New England Nuclear (Boston, MA, U.S.A.). Nylon filters for library screening (Hybond-N), cDNA labelling kits, [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham (Arlington Heights, IL, U.S.A.). Low-melting agarose (NuSieve) and Sea-Kem GTG agarose were from FMC BioProducts (Rockland, ME, U.S.A.). Poly(vinylidene difluoride) (Immobilon) and nitrocellulose filters for Western blots were from Millipore Corp. (Bedford, MA, U.S.A.) and Schleicher and Schuell (Keene, NH, U.S.A.) respectively. *Eco*RI-digested dephosphorylated bacteriophage λgt11 arms were from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). *LacZ* primers were from New England Biolabs (Beverly, MA, U.S.A.). Calf intestinal alkaline phosphatase and Sephadex G-50 were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). T7 DNA polymerase (Sequenase) was from United States Biochemical Corp. (Cleveland, OH, U.S.A.). Restriction endonucleases and other DNA-modifying enzymes were from New England Biolabs or Promega. Molecular mass standards were from Bio-Rad (Richmond, CA, U.S.A.) and Bethesda Research Laboratories. pBluescript II KS(+) was from Stratagene (La Jolla, CA, U.S.A.). Alkaline-phosphatase-conjugated goat anti-guinea pig and anti-mouse IgG were from Boehringer Mannheim. 5-Bromo-4-chloro-3-indolylphosphate and Nitroblue Tetrazolium were from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD, U.S.A.). Other chemicals were of the highest purity available from Sigma (St. Louis, MO, U.S.A.), Bethesda Research Laboratories or U.S. Biochemicals Corp. Most solutions for use with RNA were treated with diethyl pyrocarbonate and autoclaved prior to use. *Escherichia coli* strains XL1-Blue (Stratagene), Y1090 and JM109 were maintained as frozen stocks and grown on Luria–Bertani medium (LB) plates. Strain XL1-Blue was maintained on media containing 100 μg of tetracycline/ml. Bacteria were rendered competent using a $CaCl_2$ method [12].

N-Terminal protein sequencing

Male New Zealand White rabbits (2–3 kg) were killed by intravenous sodium pentobarbital, and kidneys were removed immediately. Renal cortices were dissected and microvillus membrane vesicles were prepared by magnesium aggregation and differential centrifugation [13]. Membrane vesicles were incubated with DNAase-I-coupled agarose beads to remove actin and actin-bound proteins as described previously [9]. Membrane proteins were size-fractionated by SDS/6–15%-PAGE, stained with Coomassie Blue and electroblotted on to poly(vinylidene difluoride) membranes as described by Matsudaira [14]. Strips of membrane containing 50 pmol of the purified 43 kDa protein were subjected to Edman degradation by the Yale University Protein and Nucleic Acid Chemistry Facility using a gas phase sequencer (model 470A; Applied Biosystems Inc., Foster City, CA, U.S.A.) equipped with on-line phenylthiohydantoin detection (model 120A amino acid analyser; Applied Biosystems Inc.).

RNA isolation and Northern blot analysis

Rabbit renal cortices were snap-frozen in liquid nitrogen, pulverized with a mortar and pestle and homogenized with a Polytron in 4 M-guanidinium isothiocyanate, 5 mM-sodium citrate, 0.1 M-2-mercaptoethanol and 0.5% Sarkosyl. The homogenate was layered over a 5.7 M-CsCl cushion and centrifuged with a Beckman SW41 rotor (126000 g_{av} for 23 h at 18 °C) as described by Chirgwin *et al.* [15]. The pellet was resuspended in water and extracted with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). RNA was precipitated from the aqueous phase using 2.5 vol. of ethanol at −70 °C. Poly(A)$^+$RNA was purified by two rounds of affinity chromatography over oligo-(dT)–cellulose as described by Aviv & Leder [16].

Total and poly(A)$^+$ RNA from rabbit renal cortex was electrophoresed in 1% agarose/0.66 M-formaldehyde gels [17] and transferred to nylon filters (GeneScreen Plus) by capillary action in 10 × SSC. Northern blots were baked for 2 h at 80 °C and then probed with PCR1.4 (corresponding to nucleotides 55–1385 in Fig. 2) labelled with [α-^{32}P]dCTP by random primer extension (specific radioactivity 6×10^9 c.p.m./μg). Filters were prehybridized in 1 M-NaCl, 1% SDS and 10% dextran sulphate at 65 °C, then hybridized overnight at 65 °C in the same medium containing 100 μg of denatured salmon sperm DNA/ml and ^{32}P-labelled PCR1.4 (10^6 c.p.m./ml). Filters were washed for 30 min in 2 × SSC at room temperature, 30 min in 1 × SSC containing 0.5% SDS at 60 °C, and 30 min in 0.5 × SSC containing 0.5% SDS at 65 °C, and exposed to film overnight at −70 °C.

cDNA synthesis and PCR

First-strand cDNA was synthesized from 1–4 μg of poly(A)$^+$ RNA using avian myeloblastosis virus reverse transcriptase primed by adapter-$(dT)_{17}$ (5′-GACTCGAGTCGACATCGA-T_{17}-3′). In addition to template RNA, each reaction contained 50 mM-Tris/HCl (pH 8.3), 6 mM-$MgCl_2$, 40 mM-KCl, 1 mM-dithiothreitol (DTT), 10 units of RNAsin; 1.5 mM each of dATP, dCTP, dGTP and dTTP, 100 pmol of adapter-$(dT)_{17}$ and 10 units of reverse transcriptase/μg. Reactions were performed at 42 °C for 2 h, and 1% of the products were used as the template for PCR.

Oligodeoxyribonucleotides (5′ primers, 5′-TTCCTSGACCA-GGC(I/C)GTSCAGAT-3′) were designed based on the sequence of the 43 kDa protein and preferred codon usage in the rabbit [18]. Oligonucleotides were synthesized by the Yale University Department of Pathology DNA Synthesizing Service using β-cyanoethylphosphoramidite chemistry (model 380A or model 391A DNA synthesizers; Applied Biosystems Inc.). Before use, oligonucleotides were deprotected, purified by denaturing PAGE or on oligonucleotide purification cartridges (Applied Biosystems Inc.), and 5′-end-phosphorylated with ATP using T4 polynucleotide kinase.

PCR [19] reactions contained 10 mM-Tris/HCl (pH 8.3), 50 mM-KCl, 1.5 mM-$MgCl_2$, 0.4 mM each of dATP, dCTP, dGTP and dTTP, 0.01% gelatin, 1 μl of diluted first-strand cDNA (containing 1% of the total cDNA product), 43 pmol of 5′ primer and 50 pmol of adapter oligonucleotide (5′-GACT-CGAGTCGACATCG-3′). Each reaction mixture was heated for 5 min at 95 °C, then cooled to 72 °C. *Taq* DNA polymerase (2.5 units) was added and the mixture was overlaid with mineral oil. Second-strand cDNA was synthesized by incubating for 2 min at 64 °C and then for 40 min at 72 °C. We then performed 40 cycles of PCR in a thermal cycler (Perkin–Elmer Cetus). Each cycle consisted of 1 min at 94 °C, 2 min at 64 °C and 3 min at 72 °C; a final incubation of 15 min was performed at 72 °C.

Products of PCR were analysed by gel electrophoresis in 1.5% agarose and transferred to nylon filters (GeneScreen Plus) by capillary action in 10 × SSC. Southern blots were probed with internal oligonucleotides (5′-CASTGIGGICASTARCTRCC-3′) which were end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (specific radioactivity 8×10^7 c.p.m./μg). Filters were prehybridized in 1 M-NaCl, 1% SDS, 0.05 M-sodium phosphate (pH 6.8) and 10% dextran sulphate at 42 °C, and then hybridized for 6 h at 42 °C in the same medium containing 10^6 c.p.m. of ^{32}P-labelled internal oligonucleotide/ml. Filters were washed for

20 min in 2 × SSC at room temperature, 5 min in 2 × SSC containing 0.5 % SDS at 42 °C, and 5 min in 1 × SSC containing 0.5 % SDS at 42 °C, and then exposed to Kodak X-Omat AR film overnight at −70 °C.

PCR mixtures containing products that hybridized to the internal oligonucleotide were passed over Sephadex G-50 spin columns, digested with *Sal*I and electrophoresed in 1.5 % low-melting agarose gels (NuSieve). The hybridizing cDNA was excised from the gel and ligated to pBluescript II that had been digested with *Eco*RV and *Sal*I and dephosphorylated with alkaline phosphatase. Ligation reactions were used to transform competent *E. coli* strain XL1-Blue. Plasmids containing desired cDNA inserts were identified by hybridization to the ^{32}P-labelled internal oligonucleotide.

Synthesis and screening of a cDNA library

cDNA was prepared from 5 μg of rabbit renal cortical poly(A)$^+$ RNA using the method of Gubler & Hoffman [20] with random hexamers and a commercially available cDNA synthesis system (Amersham). Flush ends were generated with T4 DNA polymerase, and the cDNAs were treated with *Eco*RI methylase, ligated to phosphorylated *Eco*RI linkers and digested with *Eco*RI. After removal of linker fragments by repeated ethanol precipitation in the presence of 2.5 M-ammonium acetate, cDNAs were ligated to *Eco*RI-digested dephosphorylated λgt11 arms, packaged into phage particles and amplified in *E. coli* strain Y1090. The cDNA library contained 2 × 10^5 recombinants with an average insert size of 1.9 kb, and was validated by filter hybridization to a cDNA encoding the α1 subunit of mouse Na$^+$/K$^+$-ATPase.

The cDNA library was screened using the plaque-lift method [21]. The library was plated on *E. coli* strain Y1090, and duplicate nylon filters (HyBond-N) were prepared. A 349 bp *Bal*I fragment from the 5′ end of PCR1.4 was labelled with [α-^{32}P]dCTP by random primer extension (specific radioactivity 4 × 10^9 c.p.m./μg). Filters were prehybridized for 2 h at 68 °C in 0.5 M-sodium phosphate (pH 7.2), 1 mM-EDTA, 7 % SDS and 1 % BSA, then hybridized for 48 h at 68 °C in the same medium containing 100 μg of denatured salmon sperm DNA/ml and ^{32}P-labelled probe (2 × 10^6 c.p.m./ml). The filters were washed for 60 min in 2 × SSC at room temperature, and then for 60 min in 1 × SSC containing 0.5 % SDS at 68 °C. Plaques that gave positive signals on duplicate filters were purified through secondary and tertiary screenings. Bacteriophage DNA was isolated using poly(ethylene glycol) precipitation [22], and cDNA inserts were amplified using PCR with flanking *lacZ* primers [19]. Amplified inserts were digested with *Eco*RI, isolated on low-melting agarose gels and subcloned into pBluescript II.

DNA sequencing

Plasmids containing cDNA inserts were isolated by alkaline lysis mini-preps and sequenced using the dideoxynucleotide chain-termination method [23] with modified T7 DNA polymerase (Sequenase) and [^{35}S]dATP. Alternatively, cDNAs or restriction fragments were subcloned in M13mp19 or M13mp18 and amplified in *E. coli* strain JM109, and single-stranded sequencing templates were prepared. Sequencing reactions were analysed by electrophoresis on 6 % polyacrylamide gels containing 8.3 M-urea. dITP was substituted for dGTP to resolve band compressions. Overlapping sequence of both strands was obtained by directed subcloning and sequencing of appropriate restriction fragments or by using sequence-specific primers.

Fusion protein synthesis and immunoblot analysis

The 1184 bp *Sma*I fragment of PCR1.4 was subcloned in both orientations into the *Sma*I restriction site of pUC18. Orientations of the cloned cDNAs were verified by DNA sequencing. Transformants (*E. coli* strain XL1-Blue) were grown in LB medium for 8 h, then incubated in the presence or absence of 5 mM-isopropyl-β-D-thiogalactopyranoside (IPTG) for an additional 2 h. A 1.5 ml sample of culture was lysed by treatment for 3 min at 100 °C in 125 mM-Tris/HCl (pH 6.8), 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 2 mM-phenylmethanesulphonyl fluoride and 2 mM-EDTA, and sheared by passage through a needle. Lysates were fractionated by SDS/10 %-PAGE and electroblotted on to nitrocellulose filters [24]. Filters were blocked with Blotto [25] and incubated overnight with a 1:1000 dilution of guinea pig antiserum to the purified rabbit renal 43 kDa protein. After further washing with Blotto, filters were incubated with a 1:1000 dilution of alkaline-phosphatase-conjugated goat anti-(guinea pig IgG), and then developed with 5-bromo-4-chloro-3-indolylphosphate and Nitroblue Tetrazolium. Similar analyses were performed using the monoclonal antibody IIE1 (1:100 dilution) and goat anti-(mouse IgG) secondary antibody.

Data analysis

Analyses of nucleotide and amino acid sequences and searches of GenBank, NBRF and EMBL databases were performed using the Genetics Computer Group Sequence Analysis software on the Yale Biomedical Computing Unit VAX 8800.

RESULTS AND DISCUSSION

Partial amino acid sequence of the 43 kDa protein

To examine the structural relationship between erythroid band 3 and the immunologically related 43 kDa renal protein, we cloned cDNAs encoding the 43 kDa protein and inferred its amino acid sequence. The 43 kDa protein was purified from renal microvillus membrane vesicles by treatment with DNAase I and SDS/PAGE. Purified protein (50 pmol) was transferred to poly(vinylidene difluoride) filters and then subjected to gas phase Edman degradation. The yield on the second cycle of sequencing was 35 pmol (see Fig. 2 for the *N*-terminal protein sequence obtained). Amino acids could not be unambiguously identified at three positions (corresponding to Asp-17, Arg-28 and His-36). This novel sequence of 24 amino acids was not present in the sequence of human erythroid band 3, and had no matches in the NBRF protein database.

Cloning of a cDNA using the PCR

To clone a cDNA encoding the partial amino acid sequence of the 43 kDa band-3-like protein, we utilized PCR with single-sided specificity. This approach was selected because efforts at screening the cDNA library with single long oligonucleotides derived from the amino acid sequence were unsuccessful. Similarly, screening of the cDNA expression library with antisera to the 43 kDa protein or monoclonal antibody IIE1 did not yield meaningful clones. Accordingly, we adapted the method of Frohman *et al.* [26] to specifically amplify a cDNA encoding the 43 kDa band-3-like protein. The adaptation consisted of the use of degenerate oligonucleotides based on partial amino acid sequences, rather than unique oligonucleotides as in the original description. From the partial amino acid sequence of the 43 kDa protein and preferred codon usage in the rabbit, we synthesized oligonucleotides (5′ primers) that were 8-fold degenerate 23-mers, included deoxyinosine at some third codon positions [27], and corresponded to the coding strand from Phe-3–Ile-10 of the

N-terminal sequence. First-strand cDNA was synthesized using oligo(dT) coupled to an adapter containing a *SalI* restriction site. The 5′ primer was annealed to the first-stand cDNA, and second-strand cDNA was synthesized. We then performed PCR using the 5′ primer and adapter oligonucleotides. Whereas all cDNAs synthesized would have the adapter sequence, only the cDNA specific for the 43 kDa protein would also have the 5′ primer sequence and should be amplified by PCR. We obtained major species of 0.6, 0.8 and 1.4 kb (results not shown). Of these, only a 1.4 kb cDNA would be sufficiently long to encode the 43 kDa protein. To determine which, if any, of these cDNAs encoded the 43 kDa protein, we performed Southern blot analysis using an internal oligonucleotide that was completely independent from the primers used for PCR. The internal oligonucleotide was a 16-fold degenerate mixture of 20-mers corresponding to Val-13–Gly-19 of the *N*-terminal sequence and hybridized strongly only to the 1.4 kb cDNA (results not shown). To verify that it contained the sequence which encoded the *N*-terminus of the 43 kDa band-3-like protein, the 1.4 kb cDNA was cloned and sequenced. The nucleotide sequence of the resultant cDNA (PCR1.4) contained an open reading frame which, when translated, gave an amino acid sequence that was identical to the actual amino acid sequence obtained directly by Edman degradation, verifying that PCR1.4 encoded the 43 kDa renal protein. The opposite end of PCR1.4 contained a poly(A) tail.

Northern blot analysis

To verify that the cloned transcripts were expressed in rabbit kidney and to determine the size of the transcript(s), we performed Northern blot analysis. Using PCR1.4 as the hybridization probe we detected a major transcript of 1.6 kb in rabbit renal cortex (see Fig. 1). A transcript of similar size was also expressed in the epithelium of rabbit ileum (results not shown). A minor transcript of 4 kb was detected in poly(A)$^+$ RNA from rabbit kidney cortex. The nature of the minor transcript is not known (splicing variant, homologous transcript, etc.), but it was not detected when Northern blots were hybridized with an antisense oligonucleotide corresponding to the *N*-terminal sequence of the protein.

cDNA library screening

The 1.4 kb cDNA appeared to contain almost the entire coding region and the 3′ untranslated region of the 43 kDa band-3-like protein. In order to clone the remainder of the transcript, we synthesized a cDNA library in λgt11 from rabbit renal cortex and screened it with a restriction fragment from the 5′ end of PCR1.4. We screened 9×10^5 recombinants and obtained four positive clones. These clones were plaque-purified and contained either a 1123 bp cDNA (λ5Z) or a 272 bp cDNA (λ4). The sequence of λ4 was contained entirely within the sequence of PCR1.4. λ5Z overlapped by 955 bp with PCR1.4 and extended an additional 168 bp upstream. Overlapping sequence was obtained from 100 % of both strands of the clones, and 64 % of the complete sequence, including 77 % of the coding sequence, was obtained from the two independent clones, PCR1.4 and λ5Z. The overlapping sequence was completely identical between these two cDNAs, indicating the absence of misincorporations during PCR of this region.

Nucleotide and amino acid sequence

Fig. 2 shows the composite nucleotide sequence of the cDNAs and the inferred amino acid sequence of the 43 kDa band 3-like protein. The sequence consists of 1483 nucleotides followed by $(A)_{16}$, which is similar in size to the principal transcript expressed in rabbit renal cortex. There is a 5′ untranslated region of 114 nucleotides, a single long open reading frame of 1230 nucleotides, and a 3′ untranslated region of 155 nucleotides. The initiation codon was identified as the first in-frame ATG of the long open reading frame. There is no in-frame stop codon further upstream in the cDNA clone. Thus we cannot definitely identify the initiator codon, although the methionine indicated as the initiator

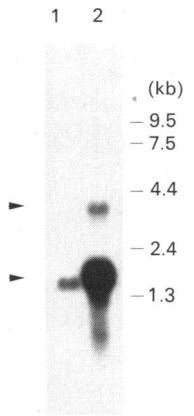

Fig. 1. Hybridization of a cDNA encoding the 43 kDa protein to rabbit renal cortical RNA

Total RNA (18 μg; lane 1) or poly(A)$^+$ RNA (28 μg; lane 2) from rabbit renal cortices were subjected to Northern blot analysis and hybridized with ^{32}P-labelled PCR1.4 as described in the Materials and methods section. Arrowheads indicate the positions of 1.6 kb and 4 kb hybridizing transcripts. The positions and sizes (in kilobases) of RNA markers are shown on the right.

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                                                    CAGGAGGGCTCCGGCCGCAATCTCCTCGACCAAAGGCCT -76
CTGCGCTCCCTCCCGGCAGGTGCGGGCTTCTCTGCGGGCTCCAGGGCAGTGAGGGAGAGGTCGCAGGAGCCGGTC -1
                          10                  20
 M  W  T  S  W  W  L  W  P  L  V  A  V  C  T  A  D  S  F  L  D  Q  A  V  Q
ATGTGGACCAGCTGGTGGCTGTGGCCCCTGGTGGCCGTCTGCACAGCGGACTCATTCCTGGACCAGGCAGTGCAA 75
                          35                  45
 I  L  R  V  T  P  V  I  D  G  H  N  D  L  P  W  Q  L  L  N  K  F  N  N  R
ATCCTGCGGGTCACGCCGGTCATCGACGGGCACAATGACCTGCCCTGGCAGCTGCTGAACAAGTTCAACAATCGG 150
                          60                  70
 L  Q  D  S  R  A  N  L  T  V  L  A  D  T  H  T  N  I  P  K  L  R  A  G  F
CTGCAGGACTCGAGGGCCAACCTGACCGTTCTGGCCGACACCCACACCAACATCCCCAAGCTGAGGGCTGGCTTT 225
                          85                  95
 V  G  G  Q  F  W  S  A  Y  T  P  C  D  T  Q  N  K  D  T  V  R  R  T  L  E
GTGGGGGGCCAGTTCTGGTCTGCGTACACGCCCTGCGACACCCAGAACAAAGACACGGTGAGGAGGACACTGGAG 300
                          110                 120
 Q  M  D  V  V  H  R  M  C  Q  L  Y  P  E  T  F  L  C  V  T  D  S  A  G  I
CAGATGGACGTGGTCCACCGCATGTGTCAGCTGTACCCCGAGACCTTCCTGTGCGTCACCGACAGTGCAGGCATC 375
                          135                 145
 Q  Q  A  F  R  E  G  K  V  A  S  L  I  G  V  E  G  G  H  S  I  D  S  S  L
CAGCAGGCCTTCCGGGAGGGGAAGGTGGCCAGTTTGATTGGTGTGGAGGGCGGTCACTCCATTGACAGCAGCCTG 450
                          160                 170
 G  V  L  R  A  L  Y  R  L  G  M  R  Y  L  T  L  T  H  N  C  N  T  P  W  A
GGCGTCCTGCGGGCCCTCTACCGCCTGGGCATGCGCTACCTCACCCTCACCCACAACTGCAATACGCCCTGGGCT 525
                          185                 195
 D  N  W  L  V  D  R  G  D  D  E  A  Q  S  G  G  L  S  V  F  G  Q  R  V  V
GATAACTGGCTGGTGGACAGAGGGGACGACGAGGCCCAGAGCGGAGGGCTGTCAGTCTTCGGGCAGCGCGTGGTG 600
                          210                 220
 R  E  M  N  R  L  G  V  M  I  D  L  A  H  V  S  V  A  T  M  K  A  A  L  Q
AGGGAGATGAACCGCCTGGGTGTCATGATCGACTTGGCCCACGTGTCTGTGGCCACCATGAAGGCCGCTCTGCAG 675
                          235                 245
 L  S  T  A  P  V  I  F  S  H  S  S  A  F  T  V  C  A  H  K  R  N  V  P  D
TTGTCCACGGCCCCGGTTATCTTCAGCCACTCTTCCGCCTTCACCGTGTGTGCACACAAGCGCAACGTGCCGGAC 750
                          260                 270
 D  V  L  Q  L  V  K  E  T  G  S  L  V  M  V  N  F  Y  N  D  Y  V  S  C  A
GACGTGCTGCAGCTGGTGAAGGAAACGGGCAGCCTGGTGATGGTGAACTTCTACAACGACTATGTTTCCTGTGCA 825
                          285                 295
 S  E  A  T  L  S  Q  V  A  D  H  L  D  Y  I  K  N  V  A  G  A  A  A  V  R
AGTGAGGCCACCCTGTCCCAAGTGGCAGACCACCTGGACTACATCAAGAATGTGGCAGGAGCTGCGGCCGTACGG 900
                          310                 320
 F  G  G  D  F  D  G  V  T  R  L  P  V  G  L  E  D  V  S  K  Y  P  D  L  V
TTCGGTGGGGACTTTGACGGTGTTACAAGGCTCCCTGTGGGGCTGGAGGACGTGTCCAAGTACCCAGACCTGGTC 975
                          335                 345
 A  E  L  L  R  R  G  W  T  E  A  E  V  R  G  A  L  A  E  N  L  L  R  V  F
GCTGAGCTGCTCAGGAGGGGCTGGACGGAGGCGGAGGTCAGGGGTGCGCTGGCTGAGAACCTGTTGAGGGTCTTC 1050
                          360                 370
 R  E  V  E  Q  V  S  N  Q  A  Q  V  P  E  E  E  P  I  S  L  E  Q  L  G  G
CGGGAAGTGGAGCAGGTGAGCAACCAGGCGCAGGTGCCCGAGGAGGAACCCATCTCACTGGAGCAGCTGGGCGGC 1125
                          385                 395
 S  C  R  T  Q  Y  G  Y  S  E  A  P  S  L  H  R  R  P  G  A  L  L  A  S  L
TCCTGCAGGACGCAGTACGGCTACTCGGAAGCCCCCAGCCTCCACCGCCGGCCAGGGGCTCTGCTGGCCTCCCTC 1200
                          410
 S  L  L  L  L  S  L  G  L  L  *
AGTCTCCTGCTGCTCAGCCTGGGTCTCCTGTAATCCCCGGGGACTGGGGGCCGCGTGCACTTACGCACCTGCTGC 1275
CCCCACATCAGCCCTGGGCCCACCAACACCGACAGGCCTGCTCAGGACACGAGCAAACCACAACCCTCAATAAAA 1350
GCAAGAGCCCTGCAAGTTCAAAAAAAAAAAAAAAA 1385
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Fig. 2. Nucleotide sequence of the full-length cDNA and deduced amino acid sequence of the 43 kDa protein

The nucleotides are numbered to the right of the sequence, with the first nucleotide corresponding to the translation initiation site. Amino acids are numbered above the sequence. Solid-underlined amino acids correspond to the sequence obtained from Edman degradation of the purified protein. Dotted-underlined amino acids indicate a probable signal peptide. ● is a possible site of *N*-linked glycosylation; underlined nucleotides are a consensus poly-adenylation signal; * is the termination codon.

in Fig. 2 does reside in a consensus sequence for initiation of translation [28]. The poly(A) tail is preceded 20 nucleotides upstream by a consensus eukaryotic polyadenylation signal [29], indicating that the location of the poly(A) tail in the clone is likely to be genuine.

The single long open reading frame was translated, and the cDNA is predicted to encode a protein that is composed of 410 amino acids, with a molecular mass of 45193 Da. The deduced amino acid sequence contained the exact *N*-terminal sequence of the 43 kDa protein that was obtained directly from Edman degradation. The first in-frame methionine was not located in the expected position adjacent to the first amino acid of the *N*-terminal sequence obtained from Edman degradation. Rather, it was displaced 15 amino acids further upstream. The 15 amino acids were all hydrophobic, which suggested that they might comprise a signal peptide (leader peptide) that could be involved in co-translational insertion of the protein into the membrane bilayer and would be removed post-translationally. Indeed, when the predicted amino acid sequence was analysed with the weight-matrices provided by von Heijne [30], a maximum score of +7.6 was obtained, with a cleavage site located between Ala-16 and Asp-17. This exactly matched the observed *N*-terminal sequence of the mature protein. Moreover, the score was in the range expected for signal sequences and not for *N*-terminal regions of cytosolic proteins. The calculated molecular mass of the mature protein was 43378 Da. Additional features of the amino acid sequence included the presence of a consensus sequence for *N*-linked glycosylation (Asn-Xaa-Ser/Thr) at residue 57, consistent with the observation that the 43 kDa protein binds lectins [9]. Although monoclonal antibody IIE1 was raised against a proteolytic fragment of band 3 that is covalently labelled with DIDS [11], a consensus sequence for binding to stilbene disulphonates, Lys-Leu-Ile-Lys [6], was not identified in the 43 kDa protein.

Comparison with human erythroid band 3

To ascertain the structural relationship with erythroid band 3, we compared the deduced amino acid sequence of the 43 kDa protein with the sequence of human erythroid band 3 inferred from its cDNA sequence [31]. A comparison was performed with the human sequence, since the monoclonal antibody that detected the 43 kDa protein was raised against a region within the human membrane-associated domain. Overall, there was no significant sequence similarity with human erythroid band 3: 23% sequence identity and 45% sequence similarity (conservative substitutions) using the BESTFIT algorithm. The longest sequences with at least 50% amino acid identity were of 12 and 14 residues. Dot matrix analysis also revealed no large areas of sequence similarity (results not shown). The longest continuous sequences that were identical in the two proteins consisted of five amino acids. There were also two regions in which five amino acids out of six were identical.

Fig. 3 shows the six regions of greatest sequence similarity between human erythroid band 3 and the 43 kDa renal protein. Erythroid sequences A, B, C and E are predicted to reside in the cytoplasmic domain of band 3. Sequence F is at the junction between the first two putative membrane-spanning helices, and sequence D resides within the second putative membrane-spanning segment. Sequences that are similar to human erythroid sequences A–F are also present in band 3 from chicken [32] and mouse [33] and in two other mouse band-3-related proteins, AE2 [8] and AE3 [34]. Whether these short regions of sequence identity are responsible for the immunological cross-reactivity between erythroid band 3 and the 43 kDa protein is not known. Since monoclonal antibody IIE1 recognizes only the 43 kDa protein in renal microvillus membranes, and antibodies to the 43 kDa protein react with band 3, if the basis for structural

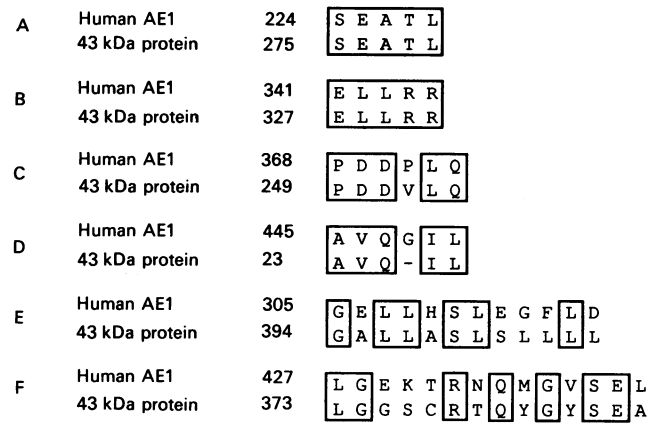

Fig. 3. Comparison of deduced amino acid acid sequences of human erythroid band 3 and the 43 kDa renal protein

A–F depict sequences of human erythroid band 3 (Human AE1 from Tanner *et al.* [31]) aligned with deduced amino acid sequences of the 43 kDa renal protein. Boxes enclose amino acids that are identical in both sequences. – is a gap introduced to optimize the alignment. Amino acids are numbered to the left of the sequences.

relatedness between the two proteins resides in these short amino acid sequences, they must be dominant epitopes. Alternatively, the antibodies might recognize a discontinuous epitope common to both proteins that is not evident from analysis of the amino acid sequences alone, e.g. a tertiary structure.

One observation which suggests that these amino acid sequences might be responsible for the immunological cross-reactivity is that some are located within the portion of band 3 that is known to contain the epitope to monoclonal antibody IIE1. The epitope in human erythroid band 3 that is recognized by monoclonal antibody IIE1 has been mapped to a 17 kDa proteolytic fragment containing the *N*-terminus of the 52 kDa membrane-associated domain [35]. The 17 kDa fragment is produced by cleavage of erythroid band 3 with internal trypsin at Lys-360 [36] and external chymotrypsin at Tyr-553 [31]. Moreover, the epitope is present in a 12 kDa fragment that is produced by *S*-cyanylation of the 52 kDa domain [35], probably at Cys-479. Thus the epitope for monoclonal antibody IIE1 resides between Lys-360 and Tyr-553, and probably between Lys-360 and Cys-479. Sequences C, D and F in Fig. 3 reside within this region, whereas the others do not.

Comparison with microsomal dipeptidase (MDP)

In contrast to the limited sequence similarity with human erythroid band 3, a GenBank database search revealed that the nucleotide sequence of the cDNA and deduced amino acid sequence of the 43 kDa protein were highly similar to that of MDP (EC 3.4.13.11), which was recently cloned from human placenta and kidney [37] and pig kidney cortex [38]. Within the coding region, the nucleotide sequence of the cDNA encoding the 43 kDa protein was 84% identical to that of human MDP. Fig. 4 aligns the deduced amino acid sequence of the 43 kDa rabbit protein with those of human and pig MDP. The primary structure of the 43 kDa protein was 81% and 82% identical to human and pig MDP respectively. The high degree of identity throughout most of the sequence indicated that the cDNAs which we cloned were very likely to encode rabbit renal MDP. All three sequences contain an *N*-terminal signal peptide, and one of four potential sites for *N*-linked glycosylation is conserved in the rabbit sequence. A leucine-rich hydrophobic sequence of 18 amino acids at the *C*-terminus is probably too short to span

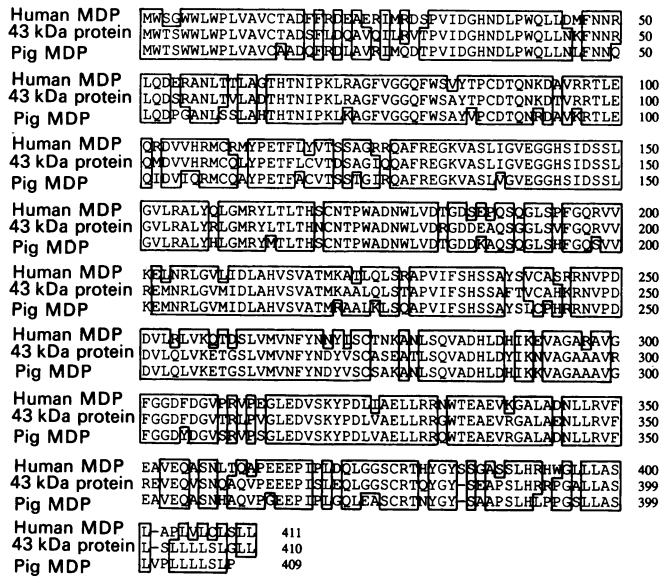

Fig. 4. Comparison of deduced amino acid sequences of MDP and the 43 kDa renal protein

The deduced amino acid sequences of human MDP (from Adachi *et al.* [37]), the 43 kDa protein and pig MDP (from Rached *et al.* [38]) are aligned. Boxes enclose amino acids that are identical with those of the 43 kDa protein. – indicates gaps introduced to optimize the alignment. Amino acids are numbered to the right of the sequences.

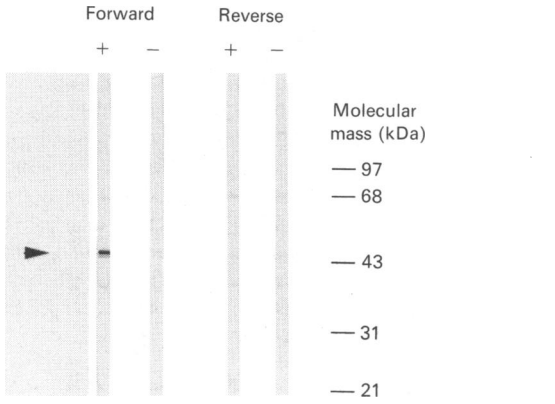

Fig. 5. Immunoblot of fusion proteins probed with antisera to the 43 kDa protein

A 1.2 kb *Sma*I fragment of PCR1.4 was cloned into pUC18 in either the forward or the reverse orientation and grown in the presence (+) or absence (–) of IPTG. Lysates of *E. coli* transformants were electrophoresed, blotted and probed with guinea pig antiserum as described in the Materials and methods section. The arrowhead indicates the position of a 43 kDa immunoreactive protein. The positions and sizes of molecular mass standards are shown on the right.

the membrane bilayer. Moreover, a similar sequence is present in many proteins that are anchored to the plasma membrane by glycosyl-phosphatidylinositol [39], and is removed post-translationally. Since pig MDP is released from the membrane by phosphatidylinositol-specific phospholipase C [40], the 43 kDa rabbit protein may also be anchored in the plasma membrane by covalently bound phosphatidylinositol. Taken together, these results suggest that the cloned cDNAs probably encode a rabbit renal dipeptidase.

MDP (also known as renal dipeptidase or dehydropeptidase-I) is a 40–50 kDa protein that normally exists as a homodimer and comprises 0.2–3 % of microvillus membrane protein [41–43]. Renal MDP mediates the catabolism by urinary glutathione [43,44], leukotriene D_4 [43,45] and certain β-lactam antibiotics [46]. Although it does not directly mediate membrane transport, MDP hydrolyses dipeptides to their constituent amino acids, which are then reabsorbed via sodium-dependent processes. Since band 3 and MDP are functionally unrelated, the structural similarities responsible for immunological cross-reactivity are probably coincidental.

Synthesis of fusion protein

Because the cDNAs that we cloned were dissimilar from erythroid band 3 and unexpectedly encoded a known protein, MDP, it was important to verify that they actually encoded a protein with reacted with antibody to erythroid band 3. We synthesized fusion proteins composed of β-galactosidase and the 43 kDa protein, and reacted them with guinea pig antiserum to the purified 43 kDa protein from rabbit kidney. A 1.2 kb *Sma*I fragment of PCR1.4 containing most of the coding region was subcloned into the *lacZ* gene of pUC18 in either the forward or the reverse orientation, and synthesis of fusion protein was induced with IPTG. Fig. 5 shows an immunoblot of the *E. coli* lysates. Antiserum against the 43 kDa protein from rabbit kidney recognized a fusion protein of 45 kDa expressed in *E. coli* only when the cDNA was subcloned in the correct, forward, orientation but not in the incorrect, reverse, orientation. Moreover, synthesis was increased by treatment with IPTG. The 45 kDa protein was not recognized by pre-immune serum or secondary antibodies alone. Monoclonal antibody IIE1 did not react with the fusion protein (results not shown), possibly because it recognizes a tertiary structure which is altered by the fusion with β-galactosidase or retention of the *C*-terminus that is normally removed post-translationally.

Whereas it was not possible to demonstrate directly that the cloned cDNAs encoded a protein which was immunologically related to erythroid band 3, indirect evidence suggested that this was the case. An alternative possibility was that the 43 kDa protein which was recognized by the antibody to band 3 differed from the 43 kDa protein that was sequenced. However, the following observations militate against this alternative possibility. First, the measured amino acid content of the 43 kDa band was nearly identical to the deduced composition of the protein encoded by the cloned cDNAs (except for more Glx in the deduced composition, which probably reflects the known instability of the phenylthiohydantoin derivative of glutamic acid [47]). Secondly, the molar yield on the second cycle of Edman degradation was 70 %, indicating that the protein which was sequenced and cloned was the major, if not the sole, protein in the 43 kDa band. Thirdly, the protein that reacts with monoclonal antibody IIE1 was also the major constituent of the 43 kDa band: when SDS/polyacrylamide gels were run under non-reducing conditions, the entire 43 kDa band shifted to a higher apparent molecular mass, and no visible proteins remained at the 43 kDa position [9]. By immunoblot analysis, the protein that reacted with guinea pig antiserum and the protein that was recognized by monoclonal antibody IIE1 co-migrated with the 43 kDa protein and also completely shifted under non-reducing conditions to the same higher molecular mass (Fig. 6). Taken together, these findings indicate that the 43 kDa protein which is recognized by the anti-(band 3) antibody is the major, if not the

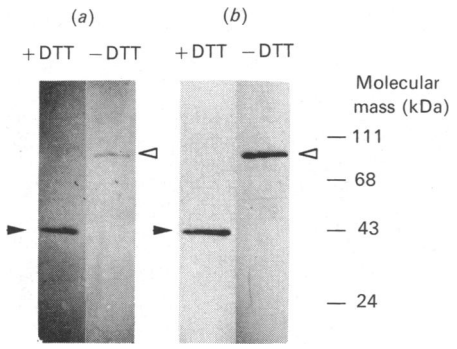

Fig. 6. Co-migration of the 43 kDa protein and band-3-related protein

Prior to electrophoresis on an SDS/polyacrylamide gel, solubilized DNAase-I-purified microvillus membranes were boiled for 3 min in sample buffer containing 3% SDS, 17% glycerol, 0.125% Bromophenol Blue and 125 mM-Tris/HCl (pH 6.8) in the presence or the absence of 40 mM-DTT. To avoid diffusion of the reducing agent into lanes without DTT (−DTT), these lanes were not run adjacent to lanes containing DTT (+DTT). (*a*) Immunoblot with monoclonal antibody IIE1; (*b*) immunoblot with guinea pig antiserum. Closed arrowheads show the positions of immunoreactive proteins in reducing gels; open arrowheads indicate the positions of immunoreactive proteins in non-reducing gels. The positions and sizes of molecular mass standards are shown on the right.

sole, constituent of the 43 kDa band, and this same protein was sequenced and cloned.

What are the implications of these findings for the study of renal anion exchangers? Examples exist of non-erythroid proteins that immunologically cross-react with band 3 and mediate anion transport. For example, antibodies to erythroid band 3 label the basolateral membranes of type A intercalated cells in the collecting tubule [48]. These cells are known to express a Cl^-/HCO_3^- exchanger in this location, and transcripts identical to erythroid band 3 (or differing only at their 5′ termini) have recently been cloned from kidney [49,50]. In addition to the 43 kDa protein, other species that are immunologically related to erythroid band 3 have been identified and are candidate anion exchangers. These include a 117 kDa protein in the Golgi of osteosarcoma cells [51] and a 44 kDa protein in the mitochondria of Type A intercalated cells [52]. The results of the present study suggest that cloning cDNAs encoding these Golgi and mitochondrial proteins may help to clarify their structural relationship with erythroid band 3 and whether they are likely to participate in anion transport.

In summary, rabbit renal microvillus membranes contain a 43 kDa protein that is immunologically related to erythroid band 3 and is a candidate for an anion transporter. PCR and cDNA library screening were used to clone a full-length cDNA encoding this protein. The cDNA is predicted to encode a 410 amino acid protein containing a signal peptide and a consensus sequence for membrane anchoring by glycosyl-phosphatidylinositol. Despite the immunological cross-reactivity, there is little similarity in primary structure with human erythroid band 3. Rather, the 43 kDa microvillus membrane protein that is immunologically related to erythroid band 3 appears to represent a rabbit renal dipeptidase.

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