Oligomeric structure of a renal cystine transporter: implications in cystinuria

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Abstract Homologous proteins (NBAT) which mediate sodium-independent transport of neutral as well as basic amino acids and cystine when expressed in *Xenopus* oocytes were recently cloned from mammalian kidneys. Mutations in human NBAT have been implicated in cystinuria. Here, we show that rat kidney and jejunal brush border membrane NBAT (85 kDa) is found in association with a 50 kDa protein. The association involves one or more interprotein disulfide bonds. Rabbit kidney brush border membranes and membranes of NBAT cRNA-injected *Xenopus* oocytes also contain such heterodimers. Our data suggest that the heterodimer is the minimal functional unit of NBAT-mediated amino acid transport and that the NBAT-associated 50 kDa protein could play a role in cystinuria.

Key words: Cystinuria; Amino acid transport; Kidney; Oocyte; *Xenopus laevis*

1. Introduction

Recently, three laboratories, including ours, independently cloned homologous proteins from rat [1,2], rabbit [3], and human [4,5] kidneys which, when expressed in *Xenopus laevis* oocytes, induce Na^+-independent transport of neutral as well as basic amino acids and cystine (system $b^{o,+}$-like). The cloned proteins have been variously named (NAA-Tr,NBAT, D_2, rBAT). In this communication we will refer to it as NBAT (for neutral and basic amino acid transporter). NBAT is primarily localized in the brush border membranes (BBMs) of renal tubular and jejunal epithelial cells [6,7] where it appears to be involved in a major cystine transport system [4]. Recent studies provide strong evidence that mutations in NBAT gene are associated with the defects in cystine and basic amino acid transport in the inherited disorder, cystinuria [8]. The proposed membrane structure of NBAT, however, is unusual for a metabolite transporter. Thus, we proposed a four membrane-spanning domain (MSD) model [1] for which experimental evidence has recently been presented [9]. This topology is in marked contrast to that of other well-characterized mammalian metabolite transporters (including the amino acid transporters cloned to date) which appear to contain from 8 to as many as 12 MSDs [10–13]. The atypical topology of NBAT prompted the speculation that NBAT is not itself a transporter but is, in fact, a regulatory subunit of a larger transporter complex [2,3]. We, therefore, examined the oligomeric organization of NBAT and have obtained data which suggest that the minimal functional unit of NBAT-mediated transport in kidney and jejunal BBMs is a heterodimer containing NBAT (85 kDa) and another protein (approximately 50 kDa).

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Abbreviations: NBAT, neutral and basic amino acid transporter; BBM, brush border membrane; MSD, membrane-spanning domain; IgG, immunoglobulin; SDS-PAGE, sodium dodecylsulfate/polyacrylamide gel electrophoresis; MSH, 2-mercaptoethanol; NEM, *N*-ethylmaleimide; DMS, dimethylsuberimidate.

2. Materials and methods

Protein cross-linking reagents were purchased from Pierce. Six synthetic peptides representing sequences within rat NBAT were used as antigens for the production of rabbit antisera directed against NBAT. Details of peptide synthesis, production of the antisera, and evaluation of their specificity have been described previously [9,14]. The immunoglobulin (IgG) fraction from each antiserum was purified using a rProtein A antibody purification kit from Repligen (Cambridge, MA). The IgGs were used in our experiments either individually or as a mixture of all six IgGs (to increase the sensitivity of detection of immunoreactive proteins). Tissues were obtained from adult (200–250 g) male Sprague–Dawley rats.

Brush border membranes (BBMs) from rat kidney and jejunum were isolated by a calcium precipitation method [15]. The final BBM pellet was suspended in 0.01 M phosphate buffer/0.15M NaCl, pH 7.4, and stored as 50 μl aliquots (approx. 10 μg protein/μl) at −20°C. SDS–protein complexes were prepared by heating an aliquot of the BBM suspension at 100°C for 5 min in 0.05M Tris-HCl buffer (pH 8.0) containing 4% SDS (Tris-SDS) either in the absence or in the presence of 5% 2-mercaptoethanol (MSH). The samples (containing 5–10 μg of kidney and 50–75 μg of jejunal BBM proteins) were subjected to SDS-PAGE on either 8% or 10% gels and electrotransferred to nitrocellulose membranes [16]. The blots were blocked with 5% non-fat dry milk and then treated with anti-NBAT antibodies (each diluted 1:500). The immunoreactive species were detected with horseradish-conjugated goat or donkey anti-rabbit IgG using either the diaminobenzidine procedure or the ECL Western blotting procedure (as described in the protocol supplied by the manufacturer; Amersham). Protein molecular weight standards were either high M_r prestained standards from BRL-Gibco or the prestained kaleidoscopic standards from Bio-Rad.

Covalent cross-linking of proteins in BBMs was carried out in 0.1 M triethanolamine-HCl buffer (pH 8.5). In a typical experiment, 5 μl of the BBM suspension (about 50 μg of protein) was mixed with 25 μl of triethanolamine buffer and 10 μl of 20 mM dimethyl suberimidate (DMS; prepared in the triethanolamine buffer just before use). Following incubation (60 min at 25°C), the mixture was centrifuged at 43,000 × *g* for 15 min. The membrane pellet was suspended in 15 μl of Tris-SDS containing 5% MSH and heated at 100°C for 5 min. The sample was then subjected to SDS-PAGE followed by Western analysis as described above.

Details of the synthesis of NBAT cRNA from NBAT cDNA, isolation of *Xenopus laevis* oocytes, and microinjection of the oocytes with NBAT cRNA have been described previously [1]. Three days after the injection (10 ng NBAT cRNA per oocyte), 25 oocytes were homogenized in 1 ml of TNE buffer (0.05 M Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM *N*-ethylmaleimide (NEM), and 1 mM phenylmethyl sulfonyl fluoride) and the homogenate centrifuged at 2,000 × *g* for 10 min. The supernatant was in turn centrifuged at 140,000 × *g* for 30 min and the membrane pellet so obtained was suspended in 40 μl of Tris-SDS either in the absence of MSH or in the presence of 5% MSH and heated at 100°C for 5 min. The SDS extracts were then subjected to SDS-PAGE and Western analysis as described above.

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3. Results and discussion

3.1. Oligomeric organization of NBAT in brush border membranes

To investigate the possibility that NBAT might be part of a larger complex, we subjected rat kidney BBMs to SDS-PAGE under reducing and non-reducing conditions followed by Western analysis using NBAT-specific antibodies. Under non-reducing conditions (i.e. when the BBMs were treated with SDS in the absence of MSH), Western analysis gave two major immunoreactive bands, one at 85 kDa corresponding to the glycosylated NBAT monomer [14], and another, representing about 20% of the total NBAT-containing species, at about 135 kDa (Fig. 1A, lane 1). When BBMs were treated with SDS in the presence of MSH all of the NBAT was quantitatively recovered in the 85 kDa band (Fig. 1A, lane 2), indicating that the 135 kDa species contains disulfide-linked proteins. The relative amount of 135 kDa species varied from about 15–50% of the total NBAT-containing species in separate batches of kidney BBMs (data not shown). Independent of such variation, only the 85 kDa NBAT monomer band was detected in all BBM preparations under reducing conditions. To determine whether the variation in the relative amounts of the 135 kDa species was because the disulfide bonds linking individual proteins in the 135 kDa species are readily reduced by sulfhydryl compounds normally present in rat kidney (e.g. glutathione, concentrations of which can be as much as 5 mM [17]), a thiol-blocking reagent, NEM, was included in the buffer used for homogenization of kidneys. Western analysis (under non-reducing conditions) of BBMs prepared in this manner showed that the 135 kDa species now consistently accounts for as much as 90% of the total NBAT-containing molecules (Fig. 1B, lanes 1 and 2), and only the NBAT monomer (85 kDa) was seen when these BBM samples were subjected to SDS-PAGE under reducing conditions (Fig. 1B, lanes 3 and 4). In all of the Western blots shown in Fig. 1, an equimixture of the six anti-NBAT IgGs was used. Qualitatively similar results were obtained when each of the six IgGs was used individually (data not shown).

Chemical cross-linking has been widely used to investigate the subunit organization of membrane-bound proteins [18]. Fig. 1C is a representative Western blot of kidney BBMs (prepared in the absence of NEM) cross-linked with DMS and then subjected to SDS/PAGE (under reducing conditions). The major cross-linked species exhibits a molecular mass of about 135 kDa, a size equal to that of the disulfide-linked species shown in Fig. 1B. Longer incubations with DMS did not influence the relative amount of the cross-linked species produced. Qualitatively similar results were obtained when cross-linking with DMS was carried out on BBMs pretreated with Lubrol WX, a non-ionic detergent (data not shown), indicating that the components of the 135 kDa species remain associated even when the membrane structure is disrupted by a detergent. The 135 kDa cross-linked species was also produced when the BBMs were treated with bis-(sulfosuccinimidyl)suberate (BS^3), a membrane-impermeant cross-linking reagent. Thus, the proteins constituting the 135 kDa complex are at least partially exposed on the outer surface of the BBMs.

In addition to kidney BBMs, NBAT is also concentrated in jejunal BBMs [6]. Rat jejunal BBMs were isolated in the presence of NEM and subjected to SDS/PAGE and Western analysis. Under non-reducing conditions, almost all NBAT migrated as the 135 kDa species (Fig. 2A, lane 1). Under reducing conditions, only the 85 kDa NBAT monomer band was seen (Fig. 2A, lane 2). Furthermore, Western analysis of rabbit kidney BBMs indicated that, in this species also, a large fraction of NBAT (the glycosylated NBAT monomer in rabbit is about 90 kDa) migrates as a complex of about 150 kDa when the BBMs are subjected to SDS-PAGE under non-reducing conditions (Fig. 2B). For detection of rabbit NBAT, the anti-IgG directed against rat NBAT sequence 527–539 (Ab527) was used (the corresponding sequence in rabbit NBAT differs in only the first residue, which in rat is Asn [1] and in rabbit is Ser [3]). As in rat kidney, the relative amounts of the disulfide-linked species in different batches of the rabbit kidney BBMs also varied, and when NEM is included in buffers during BBM isolation, the 150 kDa species (presumably analogous to the 135 kDa

Fig. 1. Western analysis of NBAT from rat kidney brush border membranes. (A) Aliquots of the BBM suspension (about 50 μg of protein) were heated with SDS either in the absence (lane 1) or in the presence (lane 2) of 2-mercaptoethanol (MSH), subjected to SDS-PAGE followed by electroblotting of the proteins on a nitrocellulose membrane. The transferred proteins were detected, in this experiment and in experiments shown in panels B and C, using a mixture of all six anti-NBAT IgGs and the diaminobenzidine procedure. Arrowheads indicate position of the immunoreactive 135 (upper band) and the 85 kDa (NBAT subunit; lower band) species. The protein standards a, b and c are, respectively, 200, 97.4, and 68 kDa. (B) Western analysis of rat kidney BBMs isolated using buffers containing either 5 mM NEM (lanes 1 and 3) or 10 mM NEM (lanes 2 and 4). Lanes 1 and 2 show results obtained with membranes treated with SDS without MSH; lanes 3 and 4 show results of experiments in which the BBMs were treated with SDS in the presence of MSH prior to SDS/PAGE. Arrowheads mark the position of the 135 (upper band) and 85 kDa (lower band) species. Standards a, b, and c are, respectively, 202, 133, and 71 kDa. The band above the 202 kDa standard in lanes 1 and 2 are proteins at the top of the gel (presumably higher aggregates containing NBAT). (C) BBM proteins were cross-linked with either 5 mM or 2.5 mM dimethyl suberimidate (DMS) (lanes 1 and 2, respectively). Following DMS treatment (for 60 min at 25°C), the membranes were heated with SDS in the presence of 5% MSH, subjected to SDS-PAGE and Western analysis. Lane 3 is an untreated BBM control. The protein standards a, b, and c are, respectively, 200, 97.4, and 68 kDa.

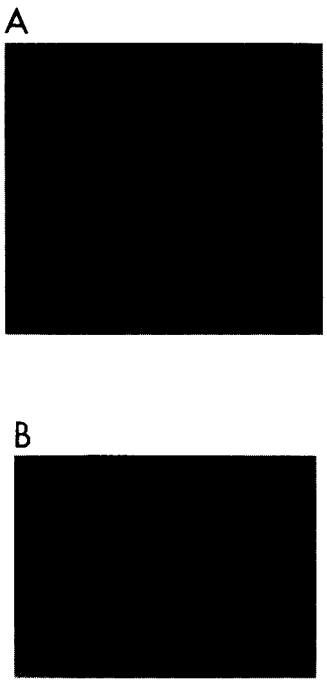

Fig. 2. Western analysis of NBAT in rat jejunal and rabbit kidney BBMs. (A) Aliquots of rat jejunal BBMs (about 75 μg protein), isolated using buffers containing NEM , were subjected to SDS-PAGE followed by Western analysis using a mixture of all six anti-NBAT IgGs and the diaminobenzidine procedure for detection of the immunoreactive species. Lane 1 = BBMs treated with SDS without MSH; lane 2 = BBMs treated with SDS plus MSH. Arrowheads mark the position of the 135 kDa (upper band) and the 85 kDa (NBAT subunit; lower band) species. (B) Aliquots of rabbit kidney BBMs, isolated in the absence of NEM (about 10 μg of protein), were subjected to SDS-PAGE followed by Western analysis using Ab527 [9]. Left lane = SDS treatment in the absence of MSH; right lane = SDS treatment in the presence of MSH. Immunoreactive species were detected usingthe ECL procedure. Arrowheads mark the 150 kDa (upper band) and the 90 kDa (NBAT subunit; lower band) species. Molecular mass standards a, b, and c are, respectively, 202, 133, and 71 kDa. Identity of the species in the 75 kDa region seen in both lanes is not known. Its intensity is not affected by presence or absence of MSH. It might represent a degradation product of NBAT.

complex in rat kidney) accounted for about 80% of all NBAT-containing moieties (data not shown).

The size of the disulfide-linked (as well as the chemically cross-linked) species in rat kidney and jejunum (135 kDa) is significantly less than that expected for a homodimer containing two NBAT subunits (expected size, about 170 kDa). We have never observed species, either disulfide-linked or chemically cross-linked, intermediate in size between 85 kDa and 135 kDa. The most likely structure, therefore, for the 135 kDa species is that of a heterodimer consisting of an NBAT subunit and a 50 kDa protein. The finding that NBAT in two different tissues of the rat (kidney and jejunum) and in kidneys from two different species (rat and rabbit) is associated with a smaller protein indicates that such a heterodimer most probably represents the minimal functional unit for the NBAT-mediated amino acid transport. Elucidation of the precise role of protein(s) associated with NBAT will require its isolation and molecular characterization. It would also be of interest to determine whether NBAT found in locations other than the kidney and intestine is also part ofheterooligomeric complexes similar to those found in these two organs. Such sites include certain enteroendocrine cells and enteric neurons [6], chromaffin cells of the adrenal medulla, and some of the catecholamine-containing neurons of brainstem and spinal chord [19]. Further studies are also required to determine whether the proteins found in association with NBAT can also occur on their own and/or in association with other proteins and, thus, possibly participate in additional transport and other functions.

3.2. *Relationship of the heterodimer to transport*

The heterodimeric model for NBAT-mediated transport, however, does not satisfactorily provide an explaination for the observations that system $b^{o,+}$-like amino acid transport can be expressed in *Xenopus* oocytes [1–5] from NBAT cRNA alone. We considered the possibility that NBAT, synthesized in oocytes from injected cRNA, associates with a protein (or proteins) endogenous in oocytes to generate the functional heterodimer. Fig. 3 shows a Western analysis of oocyte membranes (isolated in the presence of NEM) 3 days after injection of NBAT cRNA. The membranes were subjected to SDS-PAGE under both reducing and non-reducing conditions. Under reducing conditions, only the 87 and 89 kDa bands representing the glycosylated NBAT monomers produced in oocytes [14] are seen (Fig. 3, lane 1). Under non-reducing conditions, however, additional bands are seen in the 180–200 kDa region (presumably representing homodimers of NBAT subunits) (Fig. 3, lane 2, open circles), and at about 135 and 140 kDa (Fig. 3, lane 2, arrowheads). Since NBAT cRNA can direct synthesis of only the 87–89 kDa NBAT subunits, it follows that the formation of 135 and 140 kDa species must involve association of NBAT with approximately 50 kDa protein(s) of oocyte origin. The resultant heterodimers are similar in size to those seen in rat kidney and intestine and, thus, might perform similar function(s) in oocytes (i.e. system $b^{o,+}$-like amino acid transport activity). If formation of such a heterodimer is, indeed, a prerequisite for expression of transport activity, the upper limit of induced transport must then depend upon the available amounts of the 50 kDa protein in oocytes. Such a scenario

Fig. 3. Western analysis of NBAT and NBAT-containing species in membranes of *Xenopus* oocytes injected with NBAT cRNA. Three days after the injection of NBAT cRNA (10 ng per oocyte), the oocyte membranes were isolated (in the presence of NEM), aliquots treated with SDS either in the presence of MSH (lane 1) or in its absence (lane 2), subjected to SDS-PAGE and Western analysis using a mixture of the six anti-NBAT IgGs. The immunoreactive species were detected using the ECL procedure. X marks the position of NBAT subunits (two closely spaced bands at about 87 and 89 kDa, respectively); arrowheads indicate the NBAT-containing species, approximately 135 kDa (lower band) and 140 kDa (upper band), respectively; and open circles denote the NBAT-containing oligomers, approximately 180–200 kDa (presumably homodimers of NBAT subunits). The standards a, b, and c are, respectively, 202, 133, and 71 kDa.

could explain the previously reported results which showed that, while the amount of the total NBAT synthesized in oocytes increased greater than 10-fold between 24 and 48 h following the injection of NBAT cRNA, the rate of amino acid uptake increased only about 1.5-fold [14]. Note that the higher M_r aggregates seen in oocyte membranes, corresponding in size to that expected for homodimers of NBAT subunits (Fig. 3, lane 2, open circles), were absent in rat kidney and jejunal BBMs, and, thus, must be presumed to have no physiological significance.Their formation in oocytes may be a consequence of the greater density achieved by the expressed NBAT in oocyte membranes.

We had previously found that although NBAT can be expressed in COS-7 cells transfected with NBAT cDNA, no significant increments in amino acid uptake clearly ascribable to the expressed NBAT could be demonstrated [14]. The explanation given then was that the basal endogenous rate of amino acid uptake by COS-7 cells is already so high that the increase in transport due to the expressed NBAT is difficult to distinguish. An alternate explanation for the failure to detect induction of transport activity despite synthesis of NBAT could be that the 50 kDa or similar proteins required for the formation of functionally competent heterodimers are absent in COS-7 cells. This, indeed, appears to be so since Western analysis of membranes from NBAT cDNA-transfected COS-7 cells, under non-reducing conditions, failed to reveal NBAT-containing species in the 135–140 kDa range, although intense bands corresponding to the NBAT monomers (about 87 kDa) and higher M_r species in the 200 kDa range, presumably homodimers, were readily visible (data not shown).

NBAT has recently generated considerable interest in wake of the finding that mutations in human NBAT gene segregate with cystinuria [8]. Cystinuria is an autosomal recessive disease caused by an amino acid transport defect in renal and intestinal BBMs resulting in excessive urinary excretion of cystine and basic amino acids [20]. It is one of the most common genetic disorders, the clinical manifestation of which is the development of kidney stones resulting from the poor solubility of cystine. Yan et al. [21] localized human NBAT gene to the p21 region of chromosome 2; this is consistent with the earlier finding that the gene causing cystinuria maps to chromosome 2p markers [22]. These observations, along with discovery of mutations within the NBAT gene amongst cystinurics [8], indicate that NBAT is most likely a cystinuria gene. However, the possibility that other genes might also be involved must be considered in view of the fact that at least three types of cystinurias have been described, each with a characteristic biochemical and genetic pattern [20]. Furthermore, a case associated with cystinuria and mental retardation has been described involving a de novo balanced translocation [14,20], suggesting that one of these breakpoints (14q22 or 20p13) might also lead to cystinuria [23]. In view of this, our finding that NBAT-associated transport might require another protein (50 kDa) acquires special significance. Molecular characterization of this protein and elucidation of its function in cystine transport will be required to define its role in cystinuria.

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