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Characterization of the rat neutral and basic amino acid transporter utilizing anti-peptide antibodies

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ABSTRACT **High-titer, site-specific antibodies have been produced against the rat kidney broad-spectrum, sodium-independent neutral and basic amino acid transporter (NBAA-Tr) whose cDNA we cloned earlier. These antibodies have allowed us to characterize the transporter protein in normal rat tissues and in various cellular and *in vitro* expression systems. Western analysis detected 84- to 87-kDa glycosylated species enriched in rat renal and jejunal epithelial cell brush border membranes. *In vitro* translation of NBAA-Tr complementary RNA in the rabbit reticulocyte lysate system yielded a 78-kDa protein, a molecular mass that was predicted by the amino acid sequence deduced from the cloned cDNA. Translation in the presence of rough microsomal membranes yielded a glycosylated 89-kDa species. Glycosylated 87- to 89-kDa species were also expressed in *Xenopus* oocytes microinjected with NBAA-Tr complementary RNA and in COS-7 cells transfected with NBAA-Tr cDNA. Localization of NBAA-Tr in renal and intestinal brush border membranes is consistent with its proposed role in transepithelial transport of amino acids.**

Only recently have any of the mammalian nutrient transporters been characterized. Thus, a number of glucose transporters have been cloned and partially characterized (1). A murine ecotropic retrovirus receptor was subsequently shown also to transport cationic amino acids in a sodium-independent manner (2, 3). Recently, we reported the cloning of a protein from rat kidney that, when expressed in oocytes, mediates sodium-independent transport of neutral amino acids (4). Subsequently, it was shown that the same rat kidney protein, referred to as D_2, is also involved in the transport of cystine and dibasic amino acids (5). The cDNA for a comparable protein (rBAT) has also been cloned from rabbit kidney (6). The protein appears to be a broad-spectrum neutral and basic amino acid transporter (NBAA-Tr) resembling the $b^{0,+}$ transporter (7). In recent studies we found that mRNA encoding this transporter is present in only a limited number of rat tissues but is not present in cultured cells that are capable of transporting the same amino acids as are transported by NBAA-Tr (8). We now report the production of site-specific antibodies against NBAA-Tr and their use in the detection and characterization of the transporter protein in rat tissues as well as in a variety of translation/expression systems.

MATERIALS AND METHODS

Materials. Enzymes, detergents, and buffer components were obtained from commercial sources. Radiolabeled compounds were from Amersham. The *in vitro* translation kits were from Promega. Oocyte-positive *Xenopus laevis* females from Nasco (Fort Atkinson, WI) were maintained at 19°C, and the oocytes were isolated as described (9). Tissues were obtained from Wistar rats. Peptide *N*-glycosidase F was from Oxford Glycosystems (Rosedale, NY).

Peptide Synthesis and Coupling. Selection of peptides as synthetic epitopes was based on the amino acid sequence deduced from NBAA-Tr cDNA and on our postulated topographical model for the transporter in the membrane (4). Two of the selected peptide sequences were in the putative first extracellular domain. The structures of the synthetic peptides along with linkers that were added for coupling to carrier protein are as follows: (Cys)° (124–146)-NH_2 [i.e., (Cys)° (Tyr-Pro-Arg-Ser-Phe-Lys-Asp-Ser-Asp-Lys-Asp-Gly-Asn-Gly-Asp-Leu-Lys-Gly-Ile-Gln-Glu-Lys-Leu)-NH_2] and (Cys)° (357–375)-NH_2 [i.e., (Cys)° (Leu-Val-Arg-Asp-Phe-Arg-Gln-Thr-Met-Asn-Gln-Phe-Ser-Arg-Glu-Pro-Gly-Arg-Tyr)-NH_2]. These peptides were prepared by the Merrifield solid-phase methodology using the fluoren-9-ylmethoxycarbonyl (Fmoc)/*t*-butyloxycarbonyl (Boc) strategy (10). After cleavage from the resin, the crude peptides were purified to homogeneity by HPLC and gave the expected amino acid composition after acid hydrolysis. Further proof of structure was obtained by fast atom bombardment mass spectrometry. For immunization, each peptide (5 mg) was coupled through cysteine to the carrier protein, keyhole limpet hemocyanine (KLH; 5 mg), using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide as the linking agent. Conjugation and subsequent work-up were performed as described (11).

Production of Site-Directed Antibodies. These were produced in New Zealand White rabbits. KLH-conjugated peptide (250 μg) corresponding to the amino acid sequence 124–146 was injected intradermally in a 1:1 ratio with complete Freund's adjuvant followed by intradermal injections at 14-day intervals. The other KLH-conjugated peptide (1 mg) corresponding to aa 357–375 was injected into the lymph nodes, followed by intradermal injections (100–500 μg) at 14-day intervals. Antisera were collected after each injection. Three rabbits were immunized for each peptide, but generally only one rabbit in each group produced antisera of acceptable titer.

The various sera were tested for anti-NBAA-Tr activity as follows: NBAA-Tr complementary RNA (cRNA) was translated *in vitro* (see below) in a wheat germ system in the presence of [^{35}S]methionine to serve as a source of radiolabeled antigen. The labeled NBAA-Tr was denatured by boiling in 4% SDS/5% 2-mercaptoethanol, and aliquots were diluted (16-fold) with RIPA buffer (1% Triton X-100/0.5% deoxycholate/0.1% SDS/100 mM NaCl/1 mM EDTA/50 mM Tris·HCl, pH 7.5). The appropriate antiserum was then added to a final dilution of 1:70. After incubation overnight at 4°C, 30 μl of protein A-Sepharose bead suspension (Phar-

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Abbreviations: NBAA-Tr, neutral and basic amino acid transporter; TMD, transmembrane domain; RM, rough microsomal membrane; cRNA, complementary RNA.

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macia) was added to each sample, and incubation was continued for 60 min at room temperature. The beads were washed twice with 750 μl of RIPA buffer, and bound immune complexes were eluted in 25 μl of SDS/PAGE sample buffer. Samples were resolved by electrophoresis in 10% gels, which were fixed, treated with Amplify, dried, and exposed to XAR-2 film (Kodak). Radioactivity in each band was measured by a Betascope 603 blot analyzer (Betagen, Waltham, MA). The extent of immunoprecipitation was determined by comparison with an aliquot of the ^{35}S-labeled antigen subjected directly to SDS/PAGE without treatment with antisera.

***In Vitro* Translation and Processing of NBAA-Tr.** Cell-free translations of NBAA-Tr cRNA {180 ng per 25 μl of translation mixture containing 30 μCi (1 Ci = 37 GBq) of [^{35}S]methionine} using wheat germ extracts were carried out (90 min at 25°C) according to Promega's protocols. Translation using rabbit reticulocyte lysates and *in vitro* processing in the presence of rough microsomal membranes (RMs) from HeLa cells was performed as described by Kodukula *et al.* (12). After translation and processing, samples were immunoprecipitated and subjected to SDS/PAGE, as described above.

Deglycosylation. Deglycosylation of NBAA-Tr (either the *in vitro* translation/processing product or that expressed in *Xenopus* oocytes from NBAA-Tr cRNA or the endogenous transporter in rat kidney brush border membranes) was carried out as follows: aliquots of samples denatured in 2% SDS/5% 2-mercaptoethanol were diluted with the peptide *N*-glycosidase F buffer [20 mM sodium phosphate, pH 7.5/50 mM EDTA/0.5% octyl glucoside/0.02% sodium azide/protease inhibitors (aprotinin, antipain, bestatin, chymostatin, leupeptin, and pepstatin) each at 1 μg/μl] to a final SDS concentration of 0.4%. *N*-Glycosidase F (either 0.09 or 0.18 unit) was then added, and the mixture was incubated at 25°C for 3 h. After incubation, samples were boiled for 5 min and then subjected to SDS/PAGE.

Expression of NBAA-Tr in COS-7 Cells. COS-7 cells (American Type Culture Collection) cultured in Iscove's modified Dulbecco's medium supplemented with 10% (vol/vol) fetal calf serum were seeded (2–4 × 10^6 cells per 100-mm tissue culture plate) 24 h prior to transfection with plasmid DNA [full-length NBAA-Tr cDNA subcloned into pSV-SPORT (8); 2 μg per plate] by the DEAE-dextran method (13). Two days after transfection, cells were preincubated for 1 h in methionine-free Dulbecco's minimal medium supplemented with 10% fetal calf serum. Then 5 ml of the medium containing [^{35}S]methionine (470 μCi) was added per plate, and the cells were incubated for an additional 14 h at 37°C. After incubation, the labeled medium was removed; cells were rinsed with cold phosphate-buffered saline and harvested by scraping. Harvested cells from two similarly treated plates were pooled, pelleted at 2000 rpm for 3 min at 4°C in a Sorvall SS-34 rotor, and washed once with phosphate-buffered saline. The pellets were resuspended in 10 mM triethanolamine (pH 7.5) (a volume 7.5 times the volume of the cell pellet) and incubated for 15 min at 4°C. An equal volume of 600 mM sucrose/6 mM dithiothreitol was then added to the suspension, and cells were homogenized with 10 strokes in a Dounce homogenizer. The homogenates were sequentially centrifuged at 4°C at 8000 rpm for 10 min, 8000 rpm for 20 min, and 12,000 rpm for 10 min in an SS-34 rotor. The final supernatant was centrifuged at 70,000 rpm for 60 min at 4°C in a Beckman tabletop (TL-100) ultracentrifuge with a TLA-100.2 rotor. The pellet (crude membrane fraction) was suspended in 300 μl of 2% SDS/5% 2-mercaptoethanol and boiled for 5 min. An aliquot of the supernatant was then immunoprecipitated and subjected to SDS/PAGE as described above.

Western Analysis. Brush border membranes from rat kidney and jejunum were isolated by the calcium precipitation method of Malathi *et al.* (14). The final brush border membrane pellets (generally from three rats, about 150 g each) were each suspended in 2% SDS/5% 2-mercaptoethanol (500 μl for kidney membranes and 250 μl for jejunal membranes) and boiled for 5 min. Aliquots of the supernatant (e.g., 2 μl of the kidney brush border membrane extract containing about 14 μg of total protein and 12.5 μl of jejunal membrane extract containing about 400 μg of protein) were subjected to SDS/PAGE on 10% gels and electrotransferred to nitrocellulose membranes (ECL grade; Amersham) as described by Tsang *et al.* (15). Subsequent processing of the membranes was carried out essentially as described in the ECL Western blotting protocols supplied by the manufacturer (Amersham). The blot was treated with the antiserum (diluted 1:5000) against the peptide corresponding to sequence 357–375 (Ab 357–375), and the antigen–antibody complex was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG, using standard protocols.

Expression of NBAA-Tr in *Xenopus* Oocytes. *Xenopus* oocytes were microinjected with either water (control) or 10 ng of NBAA-Tr cRNA (per oocyte), and the expressed L-leucine transport was assayed as described by Tate *et al.* (4). For radiolabeling of the transporter, oocytes were selected 2 h after injection of the cRNA and cultured in 2 ml of oocyte medium (modified Barth's saline) containing [^{35}S]methionine (15 oocytes per 2 ml of buffer containing 150 μCi of [^{35}S]methionine). They were then washed several times with buffer, homogenized in 500 μl of 2% SDS/5% 2-mercaptoethanol, boiled for 5 min, and centrifuged for 15 min at 25°C. Aliquots (50 μl) of the supernatants were taken for immunoprecipitation as described above.

RESULTS AND DISCUSSION

Characterization of the *in Vitro* Translation Products of NBAA-Tr cRNA. The same two proteins were precipitated from the products of *in vitro* translation of NBAA-Tr cRNA in a wheat germ system by the antisera produced against either of the two peptides (Ab 124–146 or Ab 357–375) (Fig. 1*A*, lanes 2 and 3). The major protein band was at ≈76 kDa and the minor one was at ≈72 kDa. Specificity of the antisera was determined by showing that with each antiserum precipitation of the 76- and 72-kDa species could only be blocked by addition of the specific peptide against which that antiserum was produced (data not shown). Since only a fraction (<10%) of the translated product was precipitated by each of the antisera under the conditions employed, in subsequent studies, we used a 1:1 mixture of the two antisera (comb-Ab) at a 1:70 dilution to increase the efficiency of immunoprecipitation (Fig. 1*A*, lane 4). Immunoprecipitation of both the 76- and 72-kDa species by comb-Ab was specifically inhibited by a mixture of the two peptides against which the antibodies had been made but not by peptides corresponding to NBAA-Tr sequences 26–41 and 620–632 (Fig. 1*B*, lanes 3 and 4). The latter two peptides were synthesized by the same procedures described in *Materials and Methods*. *In vitro* translation of NBAA-Tr cRNA in rabbit reticulocyte lysate system produced a protein of ≈78 kDa (Fig. 2, lane 1). However, when translation was carried out in the presence of HeLa cell RMs, two larger products appeared (≈89 and 87 kDa) (Fig. 2, lane 2). They apparently represent glycosylated forms of NBAA-Tr since treatment with *N*-glycosidase F reduced their masses to ≈84 and 80 kDa (Fig. 2, lanes 3 and 4). It should be noted that the deduced amino acid sequence of NBAA-Tr contains seven possible N-glycosylation sites (4). The molecular mass of the species synthesized in the rabbit reticulocyte lysate system in the absence of RMs (78 kDa) is close to that predicted by the amino acid sequence deduced from NBAA-Tr cDNA (4). However, the molecular masses of the species translated in the wheat germ system are significantly less than expected. It may be that alternate

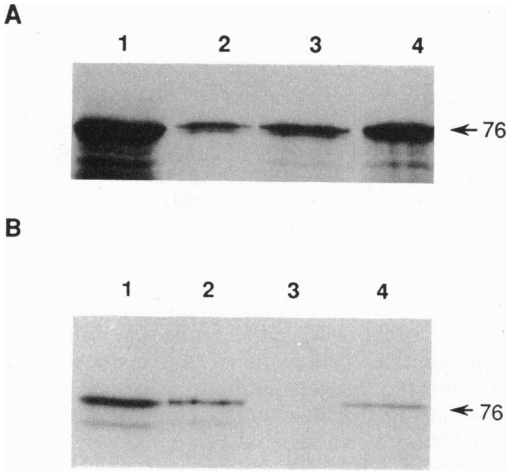

FIG. 1. Immunoprecipitation of *in vitro* translation products of NBAA-Tr cRNA. A wheat germ system was used for the translation of NBAA-Tr cRNA in the presence of [^{35}S]methionine. (*A*) An aliquot of the translation mixture was directly subjected to SDS/PAGE (lane 1). Lanes 2 and 3, species immunoprecipitated by Ab 124–146 and Ab 357–375, respectively; lane 4, immunoprecipitation carried out by a mixture (1:1) of Ab 124–146 and Ab 357–375 (comb-Ab). (*B*) A separate experiment in which an aliquot of the translation mixture was run directly on SDS gel (lane 1). Lane 2, immunoprecipitation with comb-Ab; lane 3, immunoprecipitation with comb-Ab in the presence of peptides (124–146 and 357–375; 0.5 μg of each) used to produce the antisera; lane 4, immunoprecipitation with comb-Ab in the presence of unrelated peptides (representing sequences 26–41 and 620–632 of NBAA-Tr; 0.5 μg of each). Numbers at right represent the approximate molecular masses of immunoreactive species. The molecular masses shown in this and subsequent figures were estimated from the following standards that were run simultaneously: phosphorylase b (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa).

initiation sites are employed for translation in the wheat germ system. Thus, the 76-kDa species might be translated using either Met-13 or Met-15 as initiation sites, and the 72-kDa species might represent a product using Met-65 to initiate synthesis (see sequence in ref. 4).

Characterization of NBAA-Tr Expressed in *Xenopus* Oocytes. Translation of NBAA-Tr cRNA in *Xenopus* oocytes yielded products of ≈89 kDa and 87 kDa (Fig. 3*A*, lanes 1 and 3). Immunoprecipitation of both of these species by comb-Ab was specifically inhibited by addition of the two peptides that had been used as antigens for producing the antisera (data not shown). The two species represent differentially glycosylated forms of NBAA-Tr since *N*-glycosidase F treatment decreased their masses to ≈83 and 79 kDa (Fig. 3*A*, lane 4). It is of interest that two distinct entities remained even after extensive *N*-glycosidase F treatment. The two products may contain *N*-glycosidase F-resistant sugar residues (i.e., O-linked oligosaccharide chains), or they may represent products of alternate translation initiation sites. It is also significant that considerably more *N*-glycosidase F-treated NBAA-Tr protein was precipitated by the comb-Ab compared to the untreated antigen (compare lanes 4 and 3 in Fig. 3*A*), indicating that glycosyl residues inhibit antibody recognition. These two peptide sequences were chosen for antibody production on the basis of hydrophobicity (4) and their linear distance from possible N-glycosylation sites. The fact that antibody recognition is so markedly inhibited by glycosyl residues may be due to some residual structure that withstands SDS and high temperature.

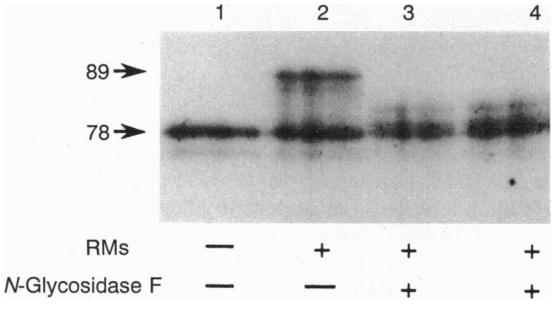

FIG. 2. *In vitro* translation and processing of NBAA-Tr. NBAA-Tr cRNA was translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine either in the absence (lane 1) or in the presence of RMs from HeLa cells (lane 2). After translation in the presence of RMs, aliquots of the labeled extracts were treated with either 0.09 (lane 3) or 0.18 unit (lane 4) of *N*-glycosidase F. Immunoprecipitations were carried out with comb-Ab.

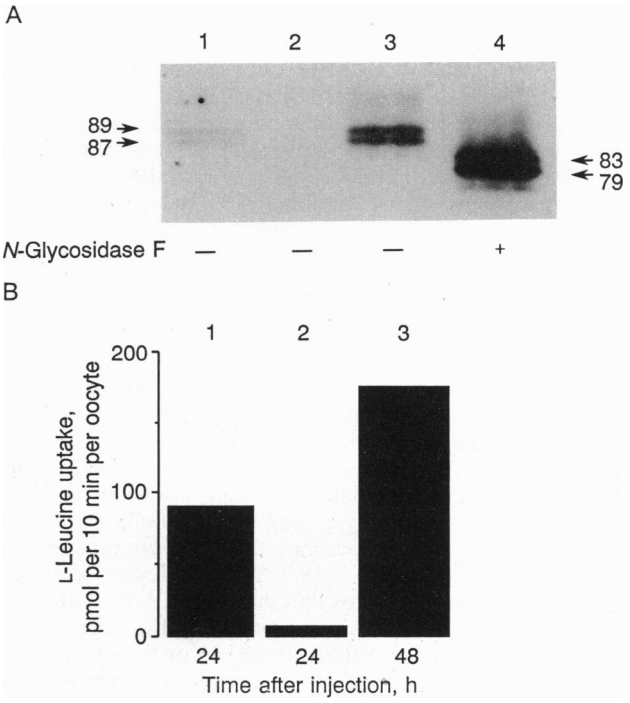

FIG. 3. Immunological characterization of NBAA-Tr expressed in *Xenopus* oocytes. The oocytes were injected with NBAA-Tr cRNA (10 ng per oocyte). The injected oocytes were then cultured in modified Barth's saline either in the presence or absence of [^{35}S]methionine. (*A*) At 24 and 48 h after injection, ^{35}S-labeled oocytes were homogenized, and aliquots of the extracts were taken for immunoprecipitation with comb-Ab (lanes 1 and 3, respectively). Lane 2, immunoprecipitation of an extract of [^{35}S]methionine-labeled, water-injected oocytes (control). Lane 4, an aliquot of the same oocyte extract, used to obtain the data in lane 3, was treated with *N*-glycosidase F prior to immunoprecipitation with comb-Ab. (*B*) The corresponding unlabeled, injected oocytes were used for determination of expressed L-leucine transport activity at 24 h (bar 1) and 48 h (bar 3) after injection. Bar 2, transport of L-leucine in unlabeled control oocytes.

Fig. 3*B* shows that enhancement of L-leucine transport into NBAA-Tr cRNA-injected oocytes very roughly parallels the amount of newly formed transporter protein. These results are in accord with the proposed role of NBAA-Tr in amino acid transport.

Expression of NBAA-Tr in COS-7 Cells. In previous studies we had shown that NBAA-Tr-related mRNA can be detected in NBAA-Tr cDNA-transfected COS-7 cells and that this mRNA, when injected into *Xenopus* oocytes, directed the expression of L-leucine, L-phenylalanine, and L-arginine transport (4, 8). NBAA-Tr cDNA-transfected COS-7 cells expressed a protein of about the same mass (88 kDa) (Fig. 4,

lane 1) as that of the glycosylated product made either in *Xenopus* oocytes (Fig. 3*A*) or translated *in vitro* from NBAA-Tr cRNA in the rabbit reticulocyte lysate system in the presence of RMs (Fig. 2, lane 2). Immunoprecipitation of the expressed protein was inhibited by addition of the specific peptides for comb-Ab (Fig. 4, lane 2). All of the NBAA-Tr protein made in transfected COS-7 cells was located, as expected, in a crude membrane fraction (data not shown).

Characterization of NBAA-Tr in Rat Tissues. Preliminary studies utilizing Western analysis, with Ab 357–375, showed that NBAA-Tr-related protein could be detected in extracts of rat kidney and jejunum but not in rat liver (data not shown). It was previously shown that rat liver contains no NBAA-Tr mRNA (8). For more precise characterization of NBAA-Tr proteins in rat tissues, we isolated brush border membranes from kidney and jejunum. Western analysis indicated that, indeed, specific immunoreactive NBAA-Tr-related protein is enriched in these membrane fractions (Fig. 5, lanes 1 and 2) and that kidney brush border membranes contain far more NBAA-Tr than do membranes from the jejunum. Extracts of jejunal brush border membranes yielded a single immunoreactive species of ≈84 kDa, whereas those from kidney brush border membranes showed two bands: the major one was at ≈87 kDa and the minor band was at ≈84 kDa. *N*-Glycosidase F treatment of kidney brush border membrane extracts, prior to Western analysis, reduced the molecular masses of the two species to ≈83 and 80 kDa (Fig. 5, lane 3), indicating that NBAA-Tr in these membranes is glycosylated. The extent and nature of glycosylation of NBAA-Tr requires further study.

A summary of our results on the molecular characteristics of NBAA-Tr in rat tissues and in the various *in vitro* and cellular expression systems is given in Table 1. Note that the major glycosylated species in rat kidney and in other expression systems exhibit approximately the same molecular mass (range between 87 and 89 kDa) and that the major unprocessed product synthesized *in vitro* in the rabbit reticulocyte system corresponds in mass to that predicted from the deduced amino acid sequence of NBAA-Tr [i.e., 78 kDa (4)].

Availability of specific antibodies against NBAA-Tr will permit more detailed tissue and cellular localization studies. In addition to the two peptide antigens described in this report, we have synthesized four additional peptides representing sequences in both the putative cytoplasmic domains and the putative extracellular domains of NBAA-Tr based on a topological model that was proposed in a previous report (4). High-titer antisera to all these peptides have now been produced. In our original report, we proposed a four-transmembrane-domain (TMD) topological model for NBAA-Tr (4). In subsequent reports on the same protein from rat kidney (5) and a similar one from rabbit kidney (6), only one transmembrane sequence (near the N-terminal region) was postulated. Availability of a battery of site-directed antibodies should permit direct experimental determination of the precise topology of NBAA-Tr in plasma membranes. Regardless of which topological model of NBAA-Tr eventually turns out to be correct, this amino acid transporter stands in sharp contrast to other mammalian nutrient transporters that have been characterized so far. Thus, the mammalian Na^+/glucose (1) and rabbit intestinal Na^+/nucleoside (16) cotransporters exhibit significant homology to each other and have been proposed to contain 12 TMDs. The model proposed for Na^+-independent glucose transporters predicts 11 TMDs (1) and that for the mouse Na^+-independent cationic amino acid transporter predicts 14 TMDs (2, 3). Also, the closely related Na^+-dependent transporters for neurotransmitters such as γ-aminobutyric acid, glycine, noradrenaline, serotonin, and dopamine have each been proposed to contain 12 TMDs (17–23). Because of its unusually small number of TMDs, Bertran *et al.* (6) have raised the question as to whether NBAA-Tr is itself the transporter or part of a transporter complex.

The localization of NBAA-Tr in brush border membranes is consistent with its proposed role in the transport of amino acids across epithelial cells (8). In more recent studies, V. M. Pickel, M. J. Nirenberg, J. Chan, R.M., S.U., and S.S.T. (unpublished work) have utilized two of the site-directed antibodies in immunocytochemical studies and localized NBAA-Tr to microvilli of proximal convoluted tubules in the rat kidney. Recently, it was also shown that mRNA from rabbit intestinal mucosa induces a $b^{0,+}$ uptake activity similar to that of NBAA-Tr (24). Further studies are, however, needed to precisely define the contribution of this transporter to the overall uptake of amino acids by mammalian cells. In

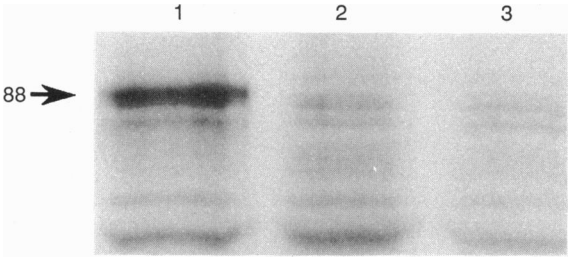

FIG. 4. Expression of NBAA-Tr in transfected cells. COS-7 cells were transfected with NBAA-Tr cDNA and labeled with [^{35}S]methionine. Lane 1, immunoprecipitation of the ^{35}S-labeled transporter from extracts of transfected COS-7 cells using comb-Ab; lane 2, inhibition of immunoprecipitation by a mixture of the corresponding peptides 124–146 and 357–375 (0.5 μg each). Lane 3, immunoprecipitation of an extract of mock-transfected (control) COS-7 cells.

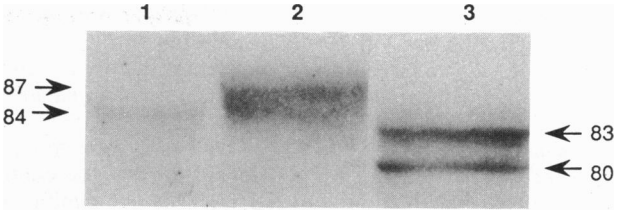

FIG. 5. Western analysis of NBAA-Tr from rat kidney and jejunal brush border membranes. Aliquots of brush border membrane extracts representing about 400 μg of total jejunal membrane protein (lane 1) and 14 μg of kidney membrane protein (lane 2) were subjected to SDS/PAGE followed by electroblotting of the proteins on a nitrocellulose membrane. The transferred proteins were detected using Ab 357–375 and horseradish peroxidase-conjugated goat anti-rabbit IgG. Lane 3, rat kidney brush border membrane extract after treatment with 0.18 unit of *N*-glycosidase F.

Table 1. Immunoreactive species of NBAA-Tr normally present in rat tissues or synthesized in translation/expression systems

Source	Size of immunoreactive species, kDa	
	As isolated	*N*-Glycosidase-treated
Rat kidney	87*, 84	83*, 80
Rat jejunum	84	ND
Expressed in *Xenopus* oocytes	89, 87	83, 79
Transfected COS-7 cells	88	ND
In vitro translation		
Rabbit reticulocyte lysate without RMs	78	ND
Rabbit reticulocyte lysate with RMs	89	80
Wheat germ system	76*, 72	ND

ND, not determined.
*Major species.

this respect, we note that, although NBAA-Tr protein was readily identified in COS-7 cells transfected with NBAA-Tr cDNA (Fig. 4), we have thus far not succeeded in detecting a significant enhancement of uptake of neutral amino acids in these cells (8). It may be that the basal endogenous rate of neutral amino acid uptake by COS-7 cells is already so high, apparently via a typical L-type mechanism, that the contribution due to transfection with NBAA-Tr is not significant. Alternatively, if NBAA-Tr is part of a heterodimer as suggested by Bertran *et al.* (6, 25), its expression may be dependent on the status of a complementary monomer in the transfected cells.

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