A variant of the bovine noradrenaline transporter reveals the importance of the C-terminal region for correct targeting to the membrane and functional expression

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We have characterized a cDNA clone which encodes a variant (bNAT2) of the bovine noradrenaline transporter. This cDNA differs from the previously identified bovine noradrenaline transporter (bNAT1) in the sequence encoding part of the cytoplasmic-facing C-terminus and the 3′-untranslated region. The bNAT1 and bNAT2 cDNA clones are encoded by a 5.8 and 3.6 kb mRNA species respectively. The bNAT1 and bNAT2 proteins, which are identical apart from their C-terminal 31 and 18 residues, were stably expressed in HEK293 cells. Cells expressing bNAT1 showed a high level of desipramine-sensitive [^{3}H]noradrenaline uptake activity, whereas no activity was present in bNAT2 cells. The bNAT1 and bNAT2 proteins were present as major 80 and 50 kDa species respectively. Cells expressing bNAT1 showed strong immunostaining of the plasma membrane, whereas bNAT2 was present in the endoplasmic reticulum/Golgi region. Treatment of membrane samples from bNAT1 cells with peptide *N*-glycosidase F resulted in the formation of a predominantly 50 kDa species, but little effect was observed after similar treatment of bNAT2 cell membranes. These results indicate that bNAT2 is retained in the endoplasmic reticulum and that the glycosylation of this variant differs from that of bNAT1. The characterization of bNAT2 and its comparison with bNAT1 highlight the importance of the cytoplasmic-facing C-terminus for the intracellular trafficking of neurotransmitter transporters.

INTRODUCTION

The reuptake of neurotransmitters is important for regulating the amount of neurotransmitter available at the synapse. Rapid progress has been made in the molecular cloning of neurotransmitter transporters and their characterization by functional expression. It is known that the reuptake of extracellular neurotransmitters is due to a number of specific Na^+-dependent plasma-membrane transporters encoded by several gene families [1–3].

A transporter for the monoamine noradrenaline is found in the plasma membrane of only a few cell types. A cDNA encoding the noradrenaline transporter (NAT) was cloned from a human neuroblastoma cell line (SK-N-SH) and shown to be homologous to the cloned rat γ-aminobutyric acid (GABA) transporter, GAT1 [4,5]. A bovine noradrenaline transporter was cloned from the adrenal medulla and shown to be 93 % identical with the human transporter [6]. The noradrenaline transporter is also found in cells outside of the nervous system, since a cocaine-sensitive noradrenaline transporter with properties very similar to those of the neuronal transporter is present in maternal-facing brush-border membranes from the human placenta [7].

Two mRNA species corresponding to approx. 5.8 and 3.6 kb in size have been shown to hybridize specifically with a human noradrenaline transporter probe [4], and the expression of catecholamine transporter mRNA has been studied in rat brain by *in situ* hybridization and Northern-blot analysis [8]. The 5.8 kb mRNA species gave a stronger hybridization signal in PC12 cells, rat brain stem and adrenal gland. However, in some brain regions only the 3.6 kb species was detected, which is also the major species present in human placenta [7].

In the present work we have characterized a cDNA (bNAT2) that encodes a variant of the bovine noradrenaline transporter, differing only in the sequence of part of the predicted cytoplasmic-facing C-terminal region of the transporter. Comparison with the previously reported bovine noradrenaline transporter cDNA (designated bNAT1) has shown that they are encoded by separate mRNA species and have major differences in noradrenaline-transport activity after stable expression in HEK293 cells. This paper highlights the importance of the C-terminal region for the correct trafficking and functional expression of neurotransmitter transporters.

MATERIALS AND METHODS

Isolation of the bNAT2 cDNA clone

Two degenerate oligonucleotide primers forward 5′-CGAA(CT)-GT(AG)TGG(AC)G(GC)TTCCC(CT)TA(CT)CT-3′ and reverse 5′-CCGGAA(AG)AA(GT)ATCTG(AG)GT(GT) GC-(AG)GC-3′ based on the conserved sequences NVWRFPYL and DAATQIFF respectively, present in the human noradrenaline and rat GABA transporters [4,5], were used to amplify cDNA prepared from bovine adrenal medullary mRNA by PCR. A product encoding part of the bovine noradrenaline transporter was obtained and used to screen a bovine adrenal medullary plasmid cDNA library in the vector pCDSP6T7 (gift from Dr. M. Brownstein, Laboratory of Cell Biology, National Institute of Mental Health, NIH, Bethesda, MD, U.S.A.). A 3.3 kb cDNA was excised from the plasmid (pCDSP6T7-bNAT2) by *Xho*I digestion. Restriction fragments were subcloned into M13 mp18 and 19 and sequenced using an automated DNA sequencer (model 373A; Applied Biosystems Division, Perkin–Elmer).

Subcloning of the bNAT1 and bNAT2 cDNAs into expression plasmids

A 2 kb *Eco*RI–*Pvu*II fragment containing the entire bNAT1

Abbreviations used: GABA, δ-aminobutyric acid; GST, glutathione S-transferase; poly(A)$^+$, polyadenylated.

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cDNA was excised from the plasmid pSG5 [6] and ligated into pcDNA3.1+ (Invitrogen), cut with *Eco*RI and *Eco*RV (pcDNA3.1-bNAT1). The bNAT2 cDNA was originally subcloned into pcDNA1neo (Invitrogen) by inserting a *Hin*dIII site upstream from the ATG start site. A 400 bp fragment was generated by PCR using two primers, 5-CCCAAGCTTAACC-GCACCCATGCTTCTG-3′ (forward) and 5′GGCAGATCTT CCACACGGT GG-3′ (reverse) and the pCDSP6T7-bNAT2 DNA as template. The product was cut with *Hin*dIII and *Bgl*II and ligated along with a 1.4 kb *Bgl*II–*Bst*EII fragment into *Hin*dIII and *Eco*RV-cut pcDNA1neo (pcDNA1neo-bNAT2). The sequence of this construct was confirmed by DNA sequencing. The bNAT2 cDNA was subsequently isolated from this plasmid by digestion with *Hin*dIII and *Xho*I and ligated to pcDNA3.1+ DNA cut with the same enzymes. Plasmid DNA was purified using a plasmid purification kit (Qiagen; QIA12143) according to recommendations of the manufacturer.

Stable expression of bNAT1 and bNAT2

HEK293 cells were grown in minimal essential medium containing 10 % fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100 U/ml) and plated out at a density of 2×10^5 cells/well in six-well (3.5 cm) plastic culture dishes (Falcon) 24–48 h before transfection experiments. Cells were transfected with 1 μg of the plasmid and 8 μl of lipofectamine in 1 ml of Opti-MEM (Gibco/BRL Life Technologies) according to the manufacturer's recommendations. After 5 h, the DNA/lipofectamine suspension was removed from the cells and replaced with minimal essential medium/10 % fetal bovine serum without antibiotics. After 48 h the cells were treated with trypsin, and aliquots representing 1/50th to 1/500th of the original cells plated into 10 cm dishes in medium containing G418 (Gibco/BRL Life Technologies; active concentration 0.5 mg/ml). After 7–10 days, colonies were picked using cloning rings and the cells transferred to 24-well dishes. The cells were screened for [^{3}H]noradrenaline-uptake activity and by immunofluorescence staining with anti-bNAT serum. HEK293 cells were also stably transfected with empty pcDNA3.1-vector, and a population of G418-resistant cells (293-PC) was selected for use in control experiments.

[^{3}H]Noradrenaline-uptake experiments

Uptake assays were carried out essentially as described by Richards and Sadee [9]. Cells were plated (3×10^5/well) into 12-well culture dishes (Falcon) and grown until confluence. Cells were washed once with Krebs–Ringer–Hepes (KRH) buffer, resuspended in 1 ml of KRH containing 20 nM [^{3}H]noradrenaline (NEN; Net-678; 71.7 Ci/mmol) and incubated for 10 min at 37 °C. The incubation mixture was then rapidly aspirated and the cells washed several times with ice-cold KRH buffer. The cells were solubilized with 0.5 % Triton X-100 and aliquots taken for scintillation counting and protein assays (Bio-Rad; Detergent Compatible Protein assay). All uptake experiments were carried out in quadruplicate.

Preparation of antisera against a glutathione S-transferase (GST)–noradrenaline transporter fusion protein

A 147 bp fragment encoding a 49-residue segment of the predicted extracellular region of the bovine noradrenaline transporter was synthesized by PCR using the bNAT2 cDNA as a template. The forward primer 5′-CGGGATCCACAGACCCCAAGCTCC T-CAACAG-3′ and the reverse primer 5′-GGAATTCCTGGGG-CAGGCCAA TGTCATGAAT-3′ encoded sites for *Bam*HI and *Eco*RI (underlined) to facilitate subcloning into the vector pGEX-2T (Pharmacia Biotech). This positioned the noradrenaline transporter sequence downstream and inframe from the sequence of GST. The fusion protein was expressed in *Escherichia coli* (DH5α or BL21) and purified on glutathione–agarose. The purity of the fusion protein was assessed by SDS/PAGE. Rabbits were injected subcutaneously with 250 μg of the GST–NATM2 fusion protein in Freund's complete adjuvant and boosted three times at monthly intervals with 200 μg of protein in Freund's incomplete adjuvant. One week after the final immunization, the rabbits were deeply anaesthetized and bled out by cardiac puncture. The serum was collected and stored at −20 °C. Before use, the antiserum was depleted of antibodies against GST. The serum (10 ml) was diluted with an equal volume of PBS and applied twice to a column containing 8 ml of GST linked to CNBr-activated Sepharose 4BCL (5.7 mg of GST/ml). This 'depleted' antiserum was used for all experiments.

Preparation of HEK293 cell membranes and peptide *N*-glycosidase F treatment

HEK293-PC, -NAT1 and -NAT2 cells, grown to confluence in 10 cm dishes, and membranes (four dishes of cells were used for each extraction) were prepared as described by Galli et al. [10]. The membranes were stored at a concentration of 3–4 mg/ml in 50 mM Tris/100 mM NaCl, pH 7.4, at −70 °C. Treatment with peptide *N*-glycosidase F was carried out by incubating membranes (30–40 μg of protein) in 15 μl containing 1 % 2-mercaptoethanol and 0.5 % SDS for 15 min at room temperature. Then 10 % Nonidet P40 and 1 M sodium phosphate, pH 7.0, were added to give a final concentration of 50 mM sodium phosphate (pH 7.0)/1 % Nonidet P40 in a final volume of 20 μl. Peptide *N*-glycosidase F (2 μl; Boehringer, 1365 185) or water (2 μl) in control experiments was added and the samples incubated for 2 h at 37 °C. The samples were then diluted with an equal volume of SDS/PAGE treatment buffer (0.125 M Tris/HCl, pH 6.8, 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol) before electrophoresis.

SDS/PAGE and Western blotting

Proteins were separated on a 10 % (8 cm × 10 cm) resolving gel using a discontinuous buffer system [11]. Membrane samples were treated with an equal volume of SDS/PAGE treatment buffer for 30 min at room temperature before electrophoresis. The gels were soaked in transfer buffer (0.192 M glycine, 25 mM Tris, pH 8.3, containing 20 % methanol and 0.01 % SDS) and proteins were electroblotted (50 V for 2 h) to a nitrocellulose membrane. The membrane was stained with Ponceau S to identify standards and locate sample lanes. For immunodetection the nitrocellulose membrane was blocked by incubation with 5 % non-fat milk powder in 20 mM Tris/0.137 M NaCl, pH 7.6, containing 0.05 % Tween 20 (TBST) and incubated for 2 h with the anti-NATM2 serum (1:3000 dilution) in TBST containing 1 % non-fat milk powder. After being washed, the membrane was incubated with a 1:3000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad; H and L chains). Immunoreactive bands were detected by enhanced chemiluminescence (Amersham).

Immunofluorescence

HEK293-PC, -NAT1 and -NAT2 cells were plated on to poly-lysine (Sigma; P-1399)-coated coverslips. After 24–48 h, cells were washed twice in PBS, fixed in 3 % paraformaldehyde for 10 min and permeabilized using 0.3 % Triton X-100/10 mM

sodium phosphate/0.5 M NaCl, pH 7.4, containing 10% fetal bovine serum. Cells were incubated for 1 h with the primary antibody (1:200 dilution of anti-NATM2 serum depleted of GST reactive antibodies) washed four times with PBS and then incubated with a 1:100 dilution of FITC-conjugated goat anti-rabbit IgG (Vector laboratories) for 1 h. After washes with PBS, coverslips were mounted using Vectashield (Vector laboratories) and viewed using an Olympus fluorescence microscope. All antibody dilutions were prepared in 10 mM sodium phosphate/0.5 M NaCl, pH 7.4, containing 10% fetal bovine serum, and incubations were carried out at room temperature.

Northern-blot analysis

Total RNA was extracted from 1 g of adrenal medullary tissue which had been collected and frozen in liquid nitrogen as described by Chomczynski and Sacchi [12]. Polyadenylated [poly(A)$^+$] RNA was purified using the PolyATtract system (Promega). Poly(A)$^+$ RNA (1.5 μg/lane) was separated on denaturing formaldehyde–agarose gels and transferred on to a Nytran (Schleicher and Schuell) membrane by electroblotting. DNA probes (as described in the text) were labelled by random priming using [α-^{32}P]dCTP (Amersham Life Sciences; Redivue; 5000 Ci/mmol). Hybridizations were carried out at 42 °C overnight in solution consistency of 50% formamide, 5 × SSC, 5 × Denhardt's 0.1% SDS, 250 μg/ml salmon sperm DNA, 250 μg/ml yeast tRNA and 50 mM sodium phosphate, pH 7.4, where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate and 1 × Denhardt's is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% BSA. Membranes were washed twice for 10 min in 2 × SSC/0.1% SDS followed by a wash in 0.1 × SSC/0.1% SDS for 1 h at 60 °C. Membranes were exposed to X-ray film (Amersham Hyperfilm-HP) at −70 °C using an intensifying screen.

RESULTS

A 3.3 kb cDNA was isolated from a bovine adrenal medullary cDNA library which encoded a protein identical with that reported for the bovine noradrenaline transporter [6] except for part of the predicted C-terminal cytoplasmic-facing region. We have used bNAT2 to denote this C-terminal variant and bNAT1 to refer to the cDNA encoding the transporter protein with a C-terminal region almost identical with that of the human noradrenaline transporter [4,6]. A cDNA identical with bNAT2 has also been isolated by Nelson and co-workers [13] (GenBank accession no. U09198). A comparison of the amino acid sequence of the C-terminal predicted cytoplasmic-facing regions of bNAT1 and bNAT2 is shown in Figure 1(A). The sequence of the 3′-untranslated region of bNAT2 also differs from that of bNAT1 (Figure 1B).

A

bNAT1(571-615) IVYKFFSTRGSIRE**RLAYGITPASEHHLVAQRDIRQFQLQHWLAI**-COOH
bNAT2(571-602) IVYKFFSTRGSIRE**MQMRQRRRGPANSCQISC**-COOH

B

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                                                    ATCGTCTACAAGTTCTTCAGCACCCGGGGCTCCATTCG 1800
AGAGATGCAAATGAGACAGAGGAGAAGGGGACCTGCCAACTCTTGCCAAATATCCTGCTA 1860
AATAAAGGTCCTCAAAGCTGCCTTAATTCTCAGAGGGGTTCCAGCCCAGGGGGGAGCCTG 1920
TCTCTTGCCAAAGGATCCTGGTAATGTGAGTGAGACTGCACATGCTTACCAAAGATGGCA 1980
TTTCTTAGTAAGAAGTTCACTGAGCTCGTTTTGTTTATATCCTTTGAAAGCCATGGATGG 2040
GAGAACAGAGAGGGAGTGTGGCGTGGGGGAAACAGCACAGCATTTAGAGTTGTGCCAGGC 2100
TGCATCTGTGACTATCTTACTGGGTAACCTGGGGGACGGGCTCCACTTCTGTGGACTGCA 2160
GAGTTCTCTGTCTGTAAAAGCAAGGGTTGAATCAGAGAATTGAAGGTCTCACCCAGTAAA 2220
AAAAAAAAAAAAAAA
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Figure 1 Alignment of the C-terminal cytoplasmic tail regions of the bovine noradrenaline transporter encoded by the bNAT1 and bNAT2 cDNA clones and 3′ sequence of the bNAT2 cDNA

(**A**) A comparison of the C-terminal regions of the bNAT1 and bNAT2 proteins. The bNAT1 sequence was obtained from [5] and bNAT2 from our own data and [13] (GenBank accession no. U09198). The bold type shows amino acid residues that are different in bNAT1 and bNAT2. (**B**) The 3′ region of the bNAT2 cDNA. The sequence encoding the unique C-terminal region of bNAT2 is underlined and the stop codon is in bold type.

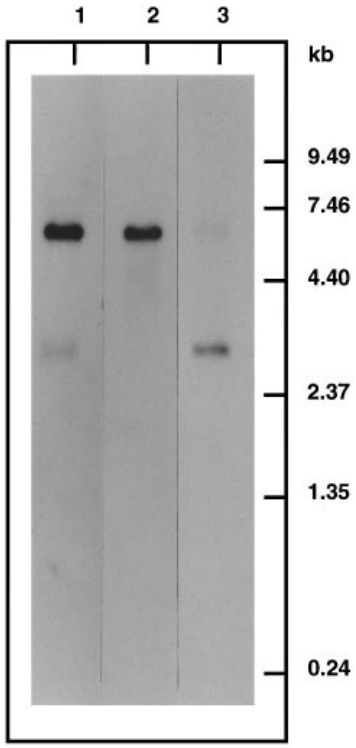

Figure 2 Northern-blot analysis of poly(A)$^+$ mRNA isolated from bovine adrenal medullary tissue

Poly(A)$^+$ mRNA (1.5 μg/lane) was run on denaturing formaldehyde–agarose gels and transferred to a Nytran membrane. Each lane shows the results of hybridization with a different probe: lane 1, a 1400 bp 5′ *Eco*RI–*Xmn*I fragment obtained from the bNAT1 cDNA that is also present in the bNAT2 cDNA; lane 2, an 800 bp *Bgl*II–*Eco*RI fragment derived from the 3′-untranslated region of the bNAT1 cDNA; lane 3, a 373 bp *Sma*I–*Pst*I fragment from pCDSP6T7 which contains 71 bp encoding the variant C-terminal region of bNAT2 and 302 residues of 3′-untranslated sequence from the bNAT2 cDNA clone.

To determine whether bNAT1 and bNAT2 are encoded by distinct mRNA species, poly(A)$^+$ RNA from the bovine adrenal medulla was hybridized to probes corresponding to a common region of both cDNAs and to probes of the 3′-untranslated regions specific for the bNAT1 and bNAT2 cDNAs (Figure 2). A DNA fragment which is part of the coding sequence present in both cDNA clones hybridized to a major 5.8 kb and a less abundant 3.6 kb species. The 5.8 kb species was detected with a probe from the 3′-untranslated region of bNAT1 cDNA (Figure 2, lanes 1 and 2). A probe consisting of a 373 bp fragment encoding the variant C-terminal portion of the bNAT2 transporter and approx. 300 bp of the adjacent 3′-untranslated region recognized the 3.6 kb species (Figure 2, lane 3). Thus the two

Table 1 Noradrenaline transporting activity of stably transfected HEK293 cells

HEK293 cells resistant to G418 after transfection with empty pcDNA3.1+ (HEK293-PC) or clonal cells expressing bNAT1 (HEK293-bNAT1) or bNAT2 (HEK293-bNAT2) were plated on to 12-well culture dishes and grown to confluence. The uptake of [^{3}H]noradrenaline was determined in the absence or presence of 10 μM desipramine. The results represent the mean ± S.E.M. from quadruplicate determinations.

	[^{3}H]Noradrenaline uptake (pmol/10 min per mg of protein)	
	No treatment	Desipramine (10 μM)
HEK293-PC	0.032 ± 0.002	0.034 ± 0.003
HEK293-bNAT1	2.044 ± 0.074	0.051 ± 0.004
HEK293-bNAT2	0.042 ± 0.001	0.047 ± 0.002

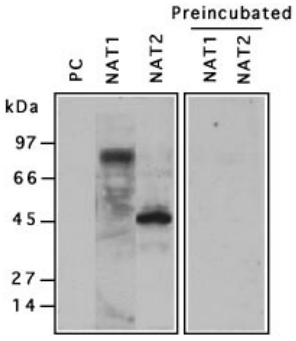

Figure 3 Characterization of bNAT1 and bNAT2 expressed in HEK293 cells by Western blotting

Samples of membranes prepared from HEK293-PC (11 μg), HEK293-bNAT1 (14 μg) and HEK293-bNAT2 (9 μg) were run on a SDS/10% polyacrylamide gel and electroblotted to a nitrocellulose membrane. The membrane was incubated with a 1:3000 dilution of anti-NATM2 serum (depleted of GST-reactive antibodies) followed by a 1:3000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG. Bands were visualized by enhanced chemiluminescence. The right-hand portion indicates the results obtained with the anti-NATM2 serum after preincubation with 100 μg/ml GST–NATM2 fusion protein for 30 min at room temperature. The positions of molecular-mass standards detected by Ponceau S staining are indicated on the left.

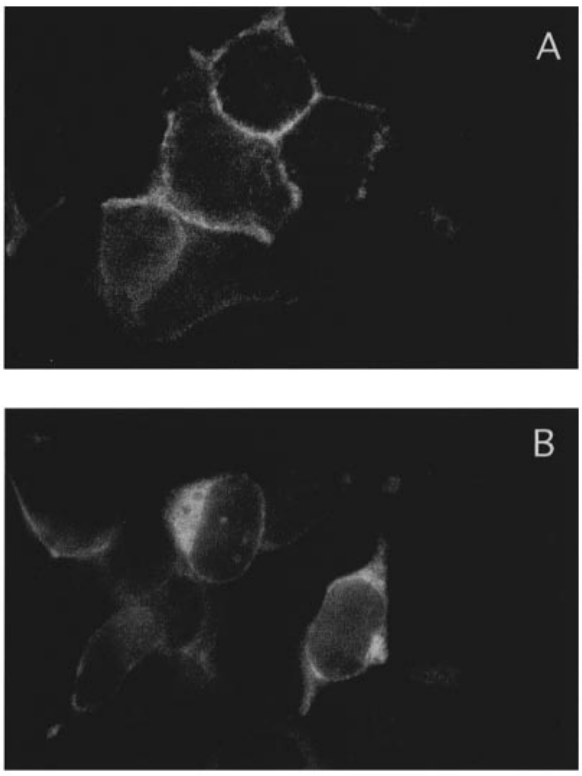

Figure 4 Immunofluorescence analysis of bNAT1 and bNAT2 stably expressed in HEK293 cells

HEK293 cells stably transfected with bNAT1 (**A**) and bNAT2 (**B**) were plated on to polylysine-coated coverslips and grown for several days. The cells were processed for immunofluorescense by fixation with paraformaldehyde and permeabilization with Triton X-100 and stained with the anti-NATM2 serum (1:200 dilution of the serum depleted of GST-reactive antibodies) followed by a 1:100 dilution of FITC-conjugated goat anti-rabbit IgG.

forms of the bovine noradrenaline transporter are encoded by separate RNA species present in poly(A)$^+$-enriched RNA.

To compare the functional properties of bNAT1 and bNAT2, each of the transporter cDNAs was stably expressed in HEK293 cells. Expression of bNAT1 in HEK293 cells (HEK293-bNAT1) resulted in a rate of [^{3}H]noradrenaline uptake approx. 60-fold higher than in G418-resistant control cells (HEK293-PC) stably transfected with the pcDNA3.1 alone (Table 1). Furthermore, treatment with desipramine, a specific inhibitor of the noradrenaline transporter, reduced the uptake activity of HEK293-bNAT1 cells to that of the control cells. In contrast, cells stably transfected with the bNAT2 cDNA showed a similar level of [^{3}H]noradrenaline uptake to the HEK293-PC cells. Desipramine treatment had no effect on [^{3}H]noradrenaline uptake for either the HEK293-bNAT2 or HEK293-PC cells.

To investigate reasons for the differences seen in functional expression, noradrenaline transporter immunoreactivity was analysed in crude membranes from HEK293-bNAT1 and -bNAT2 cells by Western blotting (Figure 3). The intensity of the signal indicated that similar levels of the transporter species were expressed by the HEK293-bNAT1 and -bNAT2 cells. However, the apparent molecular mass of the transporters was different. Major 80 and 50 kDa species were detected in membranes prepared from HEK293-bNAT1 and -bNAT2 cells respectively. No immunoreactive bands were seen in membranes prepared from control cells. The specificity of the anti-NATM2 serum was also confirmed by preincubation with the GST–NATM2 fusion protein which abolished immunostaining of both bNAT1 and bNAT2 (Figure 3).

The differences seen in the molecular mass of bNAT1 and bNAT2 suggested that altered intracellular trafficking may account for the differences seen in functional expression. We examined the subcellular distribution of bNAT1 and bNAT2 in HEK293 cells by immunofluorescence (Figure 4). In HEK293-bNAT1 cells very strong staining of the plasma membrane was observed (Figure 4A). In contrast, cells expressing bNAT2 showed bright intracellular staining typical of the endoplasmic reticulum/Golgi region (Figure 4B). A similar staining pattern was observed for bNAT2 expressed in a number of HEK293 clones (results not shown). The antisera showed no cross-reactivity with 293-PC cells (results not shown).

Thus it appeared that the bNAT2 protein accumulated in an intracellular compartment and was of a lower molecular mass than bNAT1. It is possible that the extent of glycosylation of bNAT2 was different from that of bNAT1. Membrane samples from bNAT1 and bNAT2 were treated with peptide *N*-glyco-

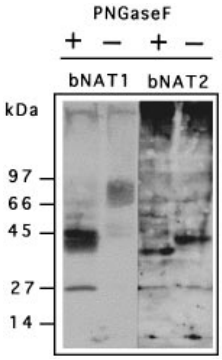

Figure 5 Effect of treatment of bNAT1 and bNAT2 with peptide *N*-glycosidase F

Samples of membranes from HEK293-bNAT1 (10 μg) and HEK293-bNAT2 (14 μg) cells were run on SDS/polyacrylamide gels before and after treatment with peptide *N*-glycosidase F (PNGaseF) and characterized by immunoblotting with the anti-NATM2 serum.

sidase F to remove carbohydrate attached to asparagine residues. This resulted in the conversion of bNAT1 immunoreactivity to a predominantly 50 kDa species (Figure 5). Similar treatment made only minor changes to the apparent molecular mass of the bNAT2 band indicating that the glycosylation of this variant is different from that of bNAT1. This supports the view that bNAT2 is retained in the endoplasmic reticulum and contains an immature carbohydrate structure.

DISCUSSION

We have characterized a variant (bNAT2) of the recently described bovine noradrenaline transporter, bNAT1 [6]. In the bovine adrenal medulla these are encoded by 3.6 and 5.8 kb mRNA species respectively. The two bNAT cDNA species are very likely to be the result of different mRNA splicing, since Pörzgen et al. [14] have shown that the human NAT gene contains an intron that interrupts the sequence encoding the C-terminal region of the transporter. The exon/intron boundary coincides exactly with the point at which the deduced protein sequence of human NAT and the highly homologous bNAT1 start to differ from that of the bNAT2 sequence (shown in bold type in Figure 1A).

The stable expression of both bNAT1 and bNAT2 in HEK293 cells revealed major differences in noradrenaline-transport activity, with bNAT2 cells having no significant desipramine-sensitive [^{3}H]noradrenaline-uptake activity. This result was surprising as bNAT2 is identical with bNAT1 apart from the C-terminal 18 amino acid residues. We first noticed the lack of functional expression of bNAT2 in preliminary experiments using transient expression in COS-7 cells (results not shown). To investigate this we chose to compare bNAT1 and bNAT2 in stably transfected HEK293 cells. This system offered advantages over transient expression in COS-7 cells where the very high levels of expression typically leads to retention of protein in intracellular compartments, making studies of the subcellular distribution difficult. Expression of bNAT1 in HEK293 cells gave high levels of [^{3}H]noradrenaline-uptake activity which correlated with strong plasma-membrane staining as seen by immunofluorescence microscopy. The major 80 kDa immunoreactive band revealed by Western blotting is similar to that seen by other workers [10].

Our study indicates that changes in the C-terminal region of the noradrenaline transporter affect functional expression. Experiments using proteolysis and C-terminally truncated mutants have indicated that neither the N- nor the C-terminal portions of the GABA transporter (GAT-1) are required for function [15,16]. However, the C-terminus has been shown to be required for the correct insertion of the glycine transporter into the plasma membrane of COS cells [17].

Addition of N-linked oligosaccharides has also been shown to be important for the cell-surface expression of both the noradrenaline transporter and the glycine transporter [18–20]. In our study, Western blotting and immunofluorescence microscopy indicated that the bNAT2 protein was expressed at high levels but that it was found predominantly intracellularly and was of an apparently lower molecular mass. Peptide *N*-glycosidase F treatment indicated that the transporter contained only minor amounts of N-linked carbohydrate.

It is possible that the unique C-terminus of bNAT2 interferes with protein folding or intracellular trafficking. Alternatively, the C-terminal 31 residues of bNAT1 that are conserved in the human NAT may be required for the folding, oligomerization and exit of the transporter from the endoplasmic reticulum. A recent paper gives some support for the second possibility. The cytosolic tail of the betaine transporter was shown to be important for its basolateral targeting in MDCK cells [21]. However, deletion of a short segment of the tail (residues 565–572) resulted in the retention of the transporter in the endoplasmic reticulum. It was concluded that this highly basic sequence (FKKRLRQL) is required for both basolateral targeting and folding/oligomerization of the betaine transporter. Although bNAT1 does not contain a similar basic sequence, part of the C-terminal 31 resides may provide a similar function. The absence of such a region in the bNAT2 splice variant would account for its accumulation intracellularly and lack of N-linked glycosylation.

The biological significance of the bNAT2 variant is uncertain. It is encoded by a specific mRNA species and has been isolated independently by two separate laboratories (this study and [13]). Despite the lack of activity of bNAT2 in a range of cell types, we cannot exclude the possibility that this variant may have biological activity in specialized tissues such as placenta. The characterization of this naturally occurring variant has, however, been valuable in highlighting the importance of the cytoplasmic-facing C-terminal region of neurotransmitter transporters for their intracellular trafficking.

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