Cysteine residues in the C-terminus of the neutral- and basic-amino-acid transporter heavy-chain subunit contribute to functional properties of the system $b^{0,+}$-type amino acid transporter

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The **n**eutral- and **b**asic-**a**mino-acid-**t**ransport glycoprotein NBAT (rBAT, D2) expressed in renal and jejunal brush-border membranes interacts with the $b^{0,+}$AT permease to produce a heteromeric transporter effecting amino acid and cystine absorption. NBAT mutations result in type I cystinuria. The $b^{0,+}$AT permease is presumed to be the catalytic subunit, but we have been investigating the possibility that cysteine residues within the C-terminus of NBAT are also important for expression of transport function. NBAT mutants were produced with combinations of $Cys^{664/671/683} \rightarrow$ Ala substitutions. Mutants with $Cys^{664} \rightarrow$ Ala show decreased arginine and cystine transport and specifically lose sensitivity to inhibition of transport by the thiol-group reagent *N*-ethylmaleimide (NEM). We suggest that the C-terminus of NBAT may have a direct role in the mechanism of System $b^{0,+}$ transport (the major transport activity defective in type I cystinuria) and that Cys^{664} of NBAT is the major target for NEM-induced inactivation of the transport mechanism.

Key words: cystine, cystinuria, exchanger, membrane transport, mutagenesis, rBAT.

INTRODUCTION

The **n**eutral- and **b**asic-**a**mino-acid-**t**ransport protein NBAT [1] (also known as rBAT [2] and D2 [3]), is an 85 kDa glycoprotein predominantly expressed in the brush-border membranes of epithelium lining the jejunum and renal proximal straight (S3) tubule (see [4] for a review). NBAT and the structurally related CD98hc (4F2hc) glycoprotein both associate covalently (via disulphide bridges) with members of a new family of amino acid permeases [5–8] termed 'glycoprotein-associated amino acid transporters' (gpaATs) [7]. NBAT has very recently been shown to interact with a gpaAT named BAT1 [5] or $b^{0,+}$AT [6] to form a hetero-oligomeric 'system $b^{0,+}$-type' amino acid transporter complex. This transporter is involved in renal reabsorption of amino acids (zwitterionic and cationic) and also cystine. Mutations of human NBAT and $b^{0,+}$AT are associated with the inherited diseases type I cystinuria [9] and non-type I cystinuria [10] respectively, both of which are characterized by urinary hyperexcretion of cationic amino acids and cystine, owing to a defect in their reabsorption.

NBAT or CD98hc are believed to be necessary for surface expression of their respective gpaAT subunits and may act as the 'regulatory' subunit of the heteromeric protein complex [4,7,11]. CD98hc, at least, also appears to regulate intracellular trafficking and membrane topology of the heteromers [11,12]. We [13] and others [14,15] have also produced evidence suggesting that NBAT has a direct role in the solute transport mechanism itself. In particular, recent studies [13–15] are suggestive of an important role for the NBAT C-terminus in functional transport expression, distinct from that of a more N-terminal region (including Cys^{111}) which is recognized to be directly involved in covalent -S–S- interactions between the transporter subunits [4,15]. We observed [13] that the extreme C-terminus of NBAT was required for expression of NEM-sensitive cystine transport and hypothesized that one or more cysteine residues found in this region were the target(s) for NEM action and also essential for normal NBAT-related transport function. In the present study we have investigated these possibilities by functional analysis of NBAT mutants in which C-terminal cysteine residues have been replaced conservatively by alanine residues. Transport function has been assessed using the *Xenopus* oocyte expression system, in which NBAT produces system $b^{0,+}$-type transport activity, presumably by association with endogenous *Xenopus* $b^{0,+}$AT-type subunits [6].

EXPERIMENTAL

Materials

Chemicals were obtained from Sigma, with the exception of collagenase A (Boehringer), Ultraspec water (Ambion, Austin, TX, U.S.A.), 9E10 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and secondary antibodies (Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, Scotland, U.K.). Radiotracers were purchased from NEN (U.K.), except L-[^{35}S]cystine from Amersham. All cDNA clones for oocyte expression were ligated into pSG5 (a simian-virus-40-driven expression plasmid) modified by inclusion of a sequence encoding the c-*myc* epitope EQKLISEEDL (target of the 9E10 monoclonal antibody) distal to a unique *Xho*I site within the multiple cloning region [13]. Plasmid construction was

Abbreviations used: ECL® (Amersham), enhanced chemiluminescence; HRP, horseradish peroxidase; MBM, modified Barths' medium; NBAT, neutral- and basic-amino-acid transporter; NEM, *N*-ethylmaleimide; TFMH, 2-trifluoromethylhistidine; TMA(Cl), tetramethylammonium (chloride); gpaAT, glycoprotein-associated amino acid transporters; C664A mutant, $C^{664} \rightarrow$ Ala.

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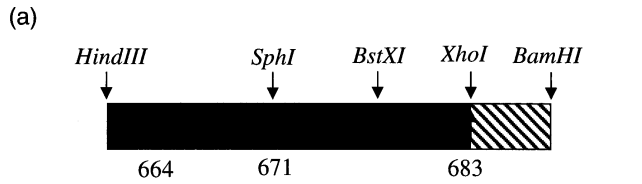
Table 1 Nomenclature of NBAT*myc* cysteine/alanine mutants

The numbers indicate the position of the cysteine residue(s) mutated to alanine in each of the mutant constructs

Mutant name	Cysteine residue mutated to alanine		
NBATmyc	–	–	–
NBAT-CCA*myc*	–	–	683
NBAT-CAC*myc*	–	671	–
NBAT-ACC*myc*	664	–	–
NBAT-AAC*myc*	664	671	–
NBAT-ACA*myc*	664	–	683
NBAT-CAA*myc*	–	671	683
NBAT-AAA*myc*	664	671	683



(b)

Human	FRDR**C**FVSNRA**C**YSSVLNILYTS**C** (R, P)	(685)
Rat	FRDK**C**FISNRA**C**YSSVLDLLYSS**C**	(683)
Rabbit	FRDR**C**FISSRA**C**YSSALDILYSS**C**	(677)
Mouse	FRDR**C**FVSSRA**C**YSSALDILYSS**C**	(685)

Figure 1 Diagrammatic representations of the mutated cysteine residues on NBAT*myc*

(**a**) The positions of Cys^{664}, Cys^{671} and Cys^{683} of rat NBAT*myc* which were mutated to alanine in the series of mutants constructed in the present study are shown, in relation to the restriction sites (in italics) used for oligocassette insertion. The *myc* tag is indicated by the hatched area. The diagram is not drawn to scale. (**b**) Comparison of C-terminal amino acid sequences of mammalian NBAT proteins (data from references [1–3,9,14,25]). Conserved cysteine residues corresponding to Cys^{664}, Cys^{671} and Cys^{683} of rat NBAT are shown in **bold**. Known type I cystinuria mutations are indicated below the human NBAT sequence. Values in parentheses indicate the number of amino acid residues in each NBAT sequence.

confirmed by DNA sequencing. Oligonucleotides were synthesized by the MRC Protein Phosphorylation Unit, University of Dundee, Dundee, Scotland.

Construction of NBAT*myc* mutants

Construction of the C terminal, c-*myc*-epitope-tagged NBAT (pSG5/NBAT*myc*) cDNA has been described previously [13]. A series of mutant NBAT cDNAs were constructed whereby the cysteine residues at positions 664, 671 or 683 of the encoded protein were mutated to alanine, either individually or in combination with each other (see Table 1). All DNA constructs were made by inserting a synthetic oligonucleotide cassette containing the desired mutation(s) between specific restriction sites within the pSG5/NBAT*myc* sequence (see Figure 1). Briefly, each cassette consisted of complementary single-stranded oligonucleotides (38–52 nucleotides in length) that were annealed together by heat denaturation. The double-stranded product was phosphorylated and ligated into specified restriction sites. Cys^{683} was mutated by cassette insertion between *Sph*I and *Xho*I restriction sites to generate NBAT-CCA*myc* (Figure 1). The mutation of residues 664 and 671 either individually or together (within the same mutant construct) involved inserting the appropriate oligocassettes between *Hin*dIII and *Bst*XI sites (Figure 1). Combination mutants, including the C683A ($Cys^{683} \rightarrow Ala$) point mutation, were constructed by cassette insertion between *Hin*dIII and *Bst*XI within NBAT-CCA*myc*. All mutant constructs were verified by DNA sequencing through this region.

Preparation of NBAT*myc*-expressing *Xenopus* oocytes

Oocytes were isolated by collagenase treatment of ovarian tissue obtained from mature female *Xenopus laevis* toads (South African *Xenopus* facility) using methods described previously [16]. Defolliculated, stage V–VI (prophase-arrested) oocytes were selected and maintained at 18 °C in modified Barths' medium (MBM) containing (in mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4 \cdot 7H_2O$, 0.66 $NaNO_3$, 0.75 $CaCl_2 \cdot 2H_2O$ and 5.0 Hepes, pH 7.6 (adjusted with Tris base) and 10 mg/l gentamycin sulphate. The nucleus of individual oocytes was injected with 2 ng of NBAT-construct DNA in 15 nl of Ultraspec water using the pneumatic delivery system described previously [16]. Injected oocytes were incubated in MBM at 18 °C for 3–4 days to allow expression of injected DNA before experimentation.

Measurement of amino acid uptake

Amino acid transport in oocytes at 3–4 days post-injection was measured at 22 °C as influx of radiolabelled amino acid tracer. Experiments were carried out using an Na^+-free transport buffer containing 100 mM tetramethylammonium chloride (TMACl), 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes (adjusted to pH 7.5 with Tris base), except where Na^+-containing buffer was specified, in which case 100 mM NaCl replaced TMACl in the buffer. Radiolabelled amino acid and cystine uptake was measured as described previously [13,16]. In experiments using the thiol-group reagent *N*-ethylmaleimide (NEM), oocytes were pre-incubated in MBM containing 2 mM NEM for 10 min [13], rinsed in transport buffer and then uptake experiments were carried out in the absence of NEM. NEM pretreatment has no significant effect on amino acid concentrations of oocytes [13]. Experiments involving cystine were performed in the presence of 0.5 mM diamide [13].

Membrane isolation and Western blotting

Oocyte membranes were isolated using the method of Wang and Tate [17]. Proteins were resolved by PAGE (with or without 5% 2-mercaptoethanol as a reducing agent) and electroblotted on to nitrocellulose membranes. NBAT*myc* mutants were detected by enhanced chemiluminescence (ECL®; Amersham) using 9E10 monoclonal antibody (1 μg/ml) and HRP-labelled rabbit anti-mouse secondary antibody (1:300 dilution). Immunoblot quantification was performed using a Bio-Rad GS-670 imaging densitometer.

Data analysis and presentation

Data are expressed as mean values $\pm$ S.E.M., n being the number of observations. Experimental measurements in each batch of oocytes were made on nine to eleven individual oocytes.

Differences between mean values were assessed using Student's unpaired *t*-test, with significance assigned at $P < 0.05$.

RESULTS

Expression of NBAT*myc* mutants in *Xenopus* oocyte membranes

NBAT*myc* expressed in oocyte membranes was detected as a protein band of approx. 85 kDa by Western blot under reducing conditions (Figure 2) and also in higher-molecular-mass protein complexes (of approx. 130, 170 and > 200 kDa) under non-reducing conditions in which intermolecular disulphide linkages are likely to remain intact (see Figure 2). All NBAT mutants were expressed in the oocyte membrane at readily detectable levels and exhibited some high-molecular-mass complex formation (Figure 2). The NBAT-CCA*myc* and NBAT-CAC*myc* mutants in particular appeared at an abundance at least equivalent to that of NBAT*myc* and both showed detectable bands of a 130 kDa protein complex (molecular mass consistent with that of an NBAT-gpaAT heterodimer [6]) which was not observed for the other mutants (Figure 2).

Functional properties of NBAT*myc* Cys-Ala mutants

We have shown previously [13] that addition of a C-terminal *myc* epitope tag does not appear to significantly affect transport functions of NBAT. Uptakes of L-[^{3}H]arginine and L-[^{35}S]cystine in NBAT*myc* expressing oocytes were markedly (15–40 times) greater than in control (uninjected) oocytes at days 3 and 4 post-injection (Figures 3 and 4). NBAT*myc* mutants in which Cys^{671} and/or Cys^{683} only were mutated to Ala (NBAT-CCA*myc*, NBAT-CAC*myc* and NBAT-CAA*myc*) showed amino acid and cystine uptakes of similar magnitude to those of NBAT*myc*. In contrast, mutants with C664A mutations (NBAT-ACC*myc*, NBAT-AAC*myc*, NBAT-ACA*myc* and NBAT-AAA*myc*) showed substantial decreases in arginine, cystine and Na^+-independent leucine transport (Figures 3–5). Effects on uptake of L-[^{3}H]phenylalanine (not shown) were qualitatively similar to those observed with L-[^{3}H]arginine. The C664A mutants also exhibited a pronounced resistance to inactivation of transport activity by the thiol-group reagent NEM compared with NBAT*myc* and other mutants (Figures 3 and 4), such that no significant NEM-sensitive transport activity was detectable in oocytes expressing them. The transport properties of C664A NBAT mutants included Na^+-independent uptake of the cationic amino acid arginine (Figure 5a; note increased resistance to inhibition by alanine in the mutants), but predominantly Na^+-dependent uptake of leucine (Figure 5b). There was no clear association between expressed transport activity and the abundance of expressed protein, as exemplified by the NBAT-CAA*myc* mutant protein, which displayed among the highest transport activities in combination with one of the lowest membrane abundances.

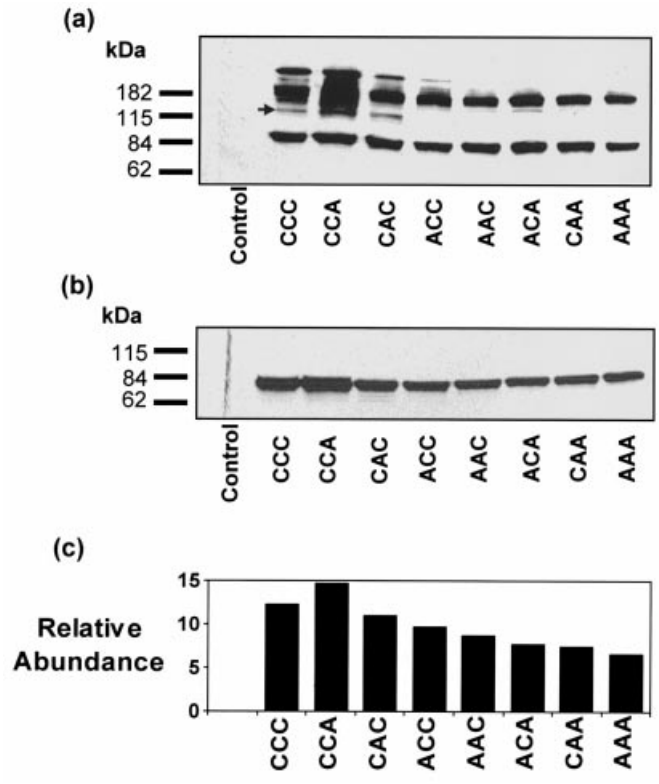


Figure 2 Expression of NBAT*myc* mutants in cell membranes of *Xenopus* oocytes

Western blot of oocyte membranes following electrophoresis under non-reducing (**a**) or reducing (**b**) conditions. Each lane contained 40 μg of protein probed with 9E10 anti-*myc* antibody. Oocytes were injected with NBAT*myc* (CCC) or mutant DNA as indicated (see Table 1 for mutant nomenclature) and uninjected oocytes were used as a control. (**c**) Shows the results of a densitometric scan of the blot shown in (**b**); absorbance is presented as relative protein abundance on an arbitrary scale. The arrow in (**a**) indicates the position of a 130 kDa protein complex including NBAT*myc*, corresponding to an NBAT–gpaAT heterodimer.

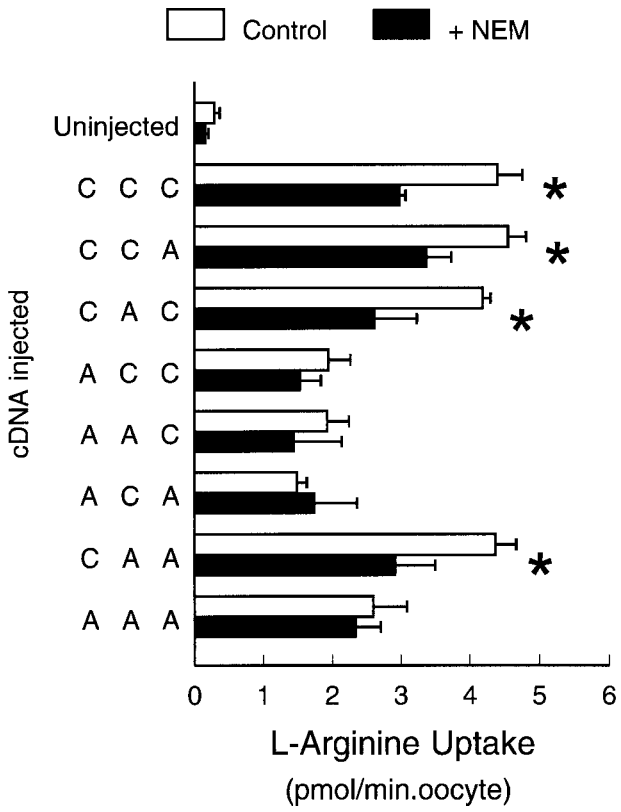


Figure 3 Arginine transport in mutant NBAT*myc*-expressing oocytes

Effect of NEM (2 mM; 10 min) on [^{3}H]arginine tracer (50 μM) uptake into oocytes. Values are presented as the mean uptake ± S.E.M. of data obtained from three different batches of oocytes. *, Signifies a significant decrease in transport activity after NEM treatment ($P < 0.05$).

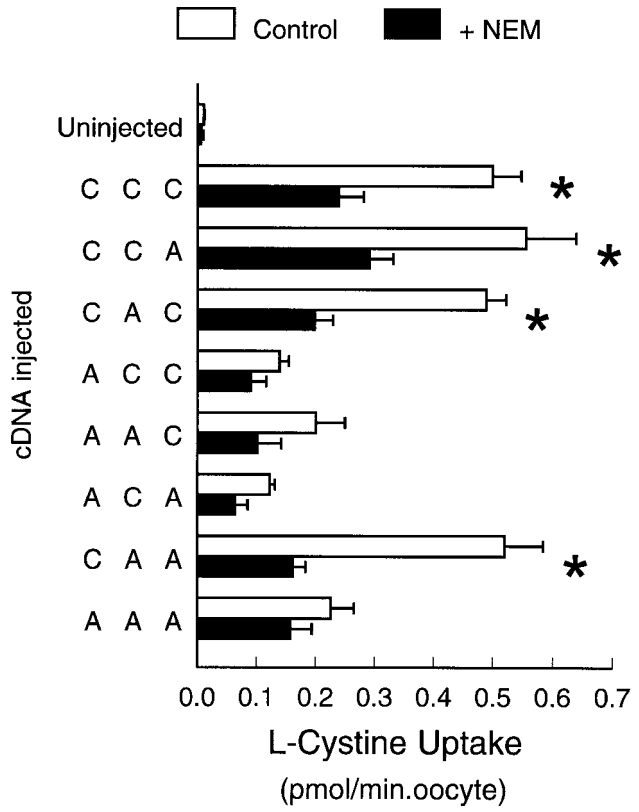


Figure 4 Cystine transport in mutant NBAT*myc*-expressing oocytes

Effect of NEM (2 mM; 10 min) on [^{35}S]cystine tracer (10 μM) uptake into oocytes. Data are mean $\pm$ S.E.M. for 11 oocytes from a single batch. *, Signifies a significant decrease in transport activity after NEM treatment ($P < 0.05$).

DISCUSSION

The present results demonstrate the importance of the conserved cysteine residue, Cys^{664}, near the C-terminus of NBAT for full expression of functional transport activity (at least for transport activity induced by NBAT expressed in *Xenopus* oocytes). This importance is highlighted by comparison of the different transport properties obtained with the NBAT-CAA*myc* and NBAT-AAA*myc* mutants (see Figures 3 and 4), which were both expressed at similar abundances in oocyte membranes. NBAT mutants with C664A show decreased arginine, phenylalanine, leucine and cystine transport and specifically lose sensitivity to inhibition of transport by the thiol-group reagent NEM. This NEM-sensitive transport activity defective in the C664A NBAT mutants exhibits Na^+-independent uptake of arginine and leucine (and of cystine; results not shown) and is also substantially inhibited by alanine in Na^+-free medium (see Figure 5). The above are all characteristic properties of the transport system $b^{0,+}$ normally associated with NBAT function [5,6,13,16]. In contrast, the NEM-resistant transport activity remaining in the C664A mutants displays Na^+-independent uptake of the cationic amino acid arginine, but predominantly Na^+-dependent uptake of the large neutral amino acid leucine. In fact the Na^+-dependent component of expressed leucine uptake (≈ 1 pmol/min per oocyte at 0.05 mM leucine) appears to be unaffected by C664A mutation in NBAT (Figure 5b). In addition, the residual arginine uptake expressed by C664A mutants is not substantially inhibited by the small neutral amino acid alanine under Na^+-free conditions (indeed alanine-resistant uptake of 0.05 mM arginine is not decreased from the wild-type value of ≈ 2 pmol/min per oocyte; Figure 5a). These latter characteristics are all consistent with continued expression of a system y^+L-like transport activity [7,8,16] by the C664A mutants.

Expression of transport system $b^{0,+}$ activity results from insertion of a heteromeric protein complex between NBAT glycoprotein and $b^{0,+}$AT permease into the cell membrane [5,6]. Cystine$\leftrightarrow$neutral amino acid exchanges by this transporter complex at the renal brush border are believed to form the basis of a tertiary active transport mechanism for renal cystine reabsorption [4,18], which is defective in cystinuria [9,10]. The additional system y^+L-like transport component identified in this study is presumably produced by association of overexpressed NBAT protein with an endogenous y^+LAT type permease ([7,8] for review) in the oocyte. Mammalian y^+LAT permeases preferentially associate with CD98hc rather than NBAT (see [7] for a review), but there is very recent evidence that certain light-chain permeases may form complexes with both CD98hc and NBAT [19], and we speculate that the putative y^+LAT type permease of *Xenopus* oocytes falls into this latter category.

The fact that transport activity produced by the C664A NBAT mutation is qualitatively as well as quantitatively distinct from that produced by wild-type NBAT indicates that the mutation has a specific effect on an NBAT-dependent functional property of the transporter, rather than merely affecting the overall extent of recruitment of endogenous gpaAT subunit to the oocyte plasma membrane. Our data are consistent with the possibility that Cys^{664} is the major target for NEM-induced functional inactivation of system $b^{0,+}$ transporter complexes already recruited to the cell membrane, in which case Cys^{664} may have a direct role in substrate binding or in production of a conformational change essential to the system $b^{0,+}$-type transport cycle. Alternatively it is possible that the C664A mutation selectively abolishes the ability of NBAT to interact with (and recruit to the cell membrane) the oocyte $b^{0,+}$AT permease, but not other functionally distinct endogenous gpaAT subunits capable of interacting with the glycoprotein when it is expressed in oocytes. This latter possibility can only be examined in detail when antibodies to appropriate gpaAT subunits become available, but nevertheless both possibilities are consistent with our current and previous [13,16] observations that multiple transport activities are induced following expression of NBAT in oocytes.

Heteromeric complexes of wild-type NBAT and light-chain permeases may be visualized as ≈ 130 kDa bands under non-reducing SDS/PAGE [5,6], which also shows NBAT as a component of higher-molecular-mass bands corresponding to higher-order complexes (possibly including NBAT homodimers) [6,15,18]. Several of our NBAT mutants do not form ≈ 130 kDa heterodimers visible on Western blots under non-reducing conditions, although at least one of these (NBAT-CAA*myc*) expresses functional system $b^{0,+}$-type transport activity. It is possible that heterodimer abundances below the threshold of detection by our Western-blot procedure are sufficient for functional expression of transport. Nevertheless, mutation of NBAT cysteine residues, including Cys^{664}, will decrease the possibilities for intra- or inter-molecular -S–S- bond formation, and this may affect the behaviour of NBAT on SDS/PAGE as well as the functional conformation of the glycoprotein–permease complex in the cell membrane. Formation of a covalent -S–S- linkage between transporter subunits is not absolutely essential for surface expression and transport function of the glycoprotein-permease complex [20,21] and indeed NBAT mutants lacking the cysteine residue (Cys^{111}) involved in disulphide-bridge formation only show decreased amino acid transport when experimental

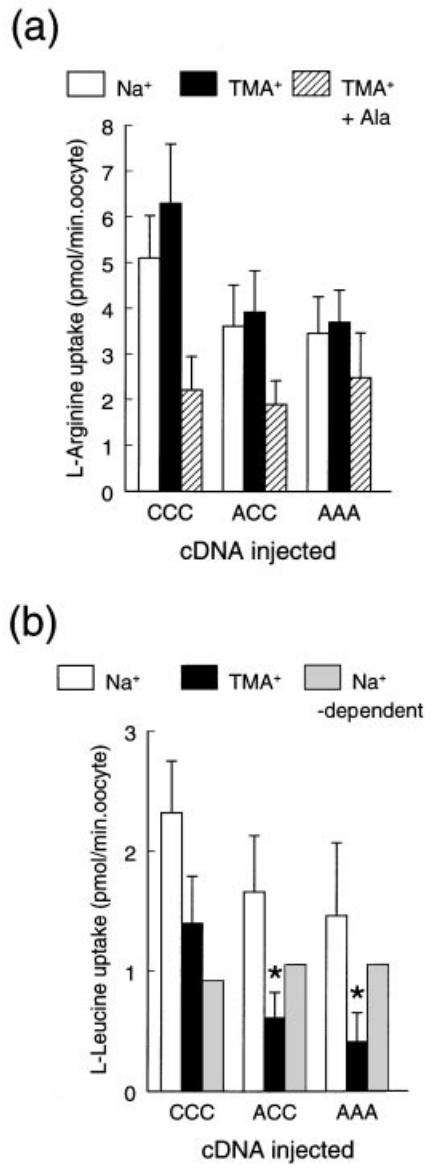


Figure 5 Ionic dependence of arginine and leucine transport in mutant NBAT*myc*-expressing oocytes

Effect of including Na^+ or TMA^+ as the major cation in transport buffer on uptake of (**a**) [^{3}H]arginine and (**b**) [^{3}H]leucine tracers (both at 50 μM) into oocytes expressing wild-type NBAT*myc* (CCC) and the representative C664A mutants NBAT-ACC*myc* (ACC) and NBAT-AAA*myc* (AAA). Uptake values have been corrected by subtraction of amino acid uptake in uninjected oocytes (results not shown). (**a**) also shows effect of 5 mM L-alanine on arginine uptake, and (**b**) also shows Na^+-dependent leucine uptake (calculated as the difference between uptakes in NaCl and TMACl transport buffers). Data are means $\pm$ S.E.M. for nine to eleven oocytes. *, Indicates that leucine uptake by NBAT mutants is significantly ($P < 0.05$) lower than that by wild-type NBAT.

mutation is accompanied by a C-terminal truncation [15]. The C-terminus of NBAT may therefore have important functions in stabilizing the transporter complex (possibly via non-covalent intermolecular interactions) and Cys^{111} and Cys^{664} of rat NBAT may both be required for full expression of transport. Heterodimers stabilized largely by groupings other than -S–S- bridges may be disrupted by detergent solubilization prior to gel electrophoresis and thus not be detected even under non-reducing SDS/PAGE conditions [15,20,21].

The present study complements other recent work [19] which also indicates that the molecular structure of the glycoprotein subunit may influence the catalytic properties of the system $b^{0,+}$-type transporter, although it is clear that the ability of NBAT/CD98hc-containing transporters to recognize cystine as a substrate is determined at least partly by properties of the putative catalytic gpaAT subunit. Only three of the growing family of cloned gpaAT permeases transport cystine, although two of these [22,23] are reported to associate with CD98hc in preference to NBAT. Functional expression of NBAT-related transport activity in *Xenopus* oocytes is believed to be limited by the availability of pre-existing endogenous gpaAT light subunits [6,18]. This may account partly for the observation that expressed transport is poorly correlated to membrane abundance of NBAT mutants, although it does not exclude the possibility that some mutants recruit less gpaAT to the membrane than others on a mol/mol basis.

The importance of Cys^{664} to NBAT function revealed in the present study was initially surprising to us, given that this residue was not deleted in the NBAT-*Sph*I truncation mutant with similar phenotype which we characterized previously [13]. We speculate that the similarity arises because Cys^{664} is unable to perform its normal function in the truncation mutant NBAT-*Sph*I, possibly due to a conformational change induced by deletion of the last 13 amino acid residues which does not occur with the substitution mutants of the present study. The two cysteine residues (671 and 683) deleted in the NBAT-*Sph*I mutant therefore do not in themselves appear to be the targets for NEM action, rather their absence renders Cys^{664} resistant to the effects of NEM. The cysteine residues 664, 671 and 683 are highly conserved within NBAT proteins of different species (Figure 1b), but are not conserved in CD98hc, which does not express the system-$b^{0,+}$-like transport component displayed by NBAT [4,7]. These features are all consistent with our results, indicating an important functional role for Cys^{664} of NBAT in the expression of system-$b^{0,+}$-type transport activity.

Two natural type-I-cystinuria mutations ($Cys^{673} \rightarrow$ Arg, $Leu^{678} \rightarrow$ Pro; see Figure 1b) [4,9,24] have been identified in the extreme C-terminus of human NBAT. Cys^{673} of human NBAT corresponds to Cys^{671} in rat NBAT, which in the conservative cysteine-to-alanine mutation reported here appears not to alter transport phenotype. Nevertheless, a cysteine-to-arginine substitution at this residue would be predicted to have a more significant localized effect on protein conformation and positioning of the critical Cys^{664} residue than Cys to Ala (as would the neighbouring $Leu^{678} \rightarrow$ Pro cystinuria mutation), thus leading to loss of system $b^{0,+}$-type activity and development of the cystinuria phenotype. It should be noted that the exact orientation of the NBAT C-terminus within the plasma membrane is at present uncertain; both one- and four-membrane-spanning domain models have been proposed, which would result in extracellular or intracellular positioning of the C-terminus respectively (see [4] for review).

In the intact kidney NBAT expression is largely restricted to the S3 portion of the renal proximal tubule, whereas the $b^{0,+}$AT gpaAT is expressed throughout the proximal tubule [6,19]. Functional properties of renal cystine reabsorption differ between early- and late-proximal-tubule segments (see [4] for review) so, assuming $b^{0,+}$AT gpaAT is involved in cystine transport throughout the tubule, differences in these properties may be related to the presence or absence of NBAT in the transporter complex.

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