Heterodimeric amino acid transporter glycoprotein domains determining functional subunit association

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The heteromeric amino acid transporter glycoprotein subunits rBAT and 4F2hc (heavy chains) form, with different catalytic subunits (light chains), functional heterodimers that are covalently stabilized by a disulphide bridge. Whereas rBAT associates with b\(^0\)+AT to form the cystine and cationic amino acid transporter defective in cystinuria, 4F2hc associates with other homologous light chains, for instance with LAT1 to form a system L neutral amino acid transporter. To identify within the heavy chains the domain(s) involved in recognition of and functional interaction with partner light chains, chimaeric and truncated forms of rBAT and 4F2hc were co-expressed in *Xenopus laevis* oocytes with b\(^0\)+AT or LAT1. Heavy chain–light chain association was analysed by co-immunoprecipitation, and transport function was tested by tracer uptake experiments. The results indicate that the cytoplasmic tail and transmembrane domain of rBAT together play a dominant role in selective functional interaction with b\(^0\)+AT, whereas the extracellular domain of rBAT appears to facilitate specifically L-cystine uptake. For 4F2hc, functional interaction with LAT1 was mediated by the N-terminal part, comprising cytoplasmic tail, transmembrane segment and neck, even in the absence of the extracellular domain. Alternatively, functional association with LAT1 was also supported by the extracellular part of 4F2hc comprising neck and glycosidase-like domain linked to the complementary part of rBAT. In conclusion, the cytoplasmic tail and the transmembrane segment together play a determinant role for the functional interaction of rBAT with b\(^0\)+AT, whereas either cytoplasmic or extracellular glycosidase-like domains are dispensable for the functional interaction of 4F2hc with LAT1.

Key words: amino acid influx, amino acid transporter, co-immunoprecipitation, glycoprotein, subunit association, *Xenopus laevis* oocyte.

INTRODUCTION

Heterodimeric amino acid transporters (for reviews, see [1–6]) are composed of two different subunits, a catalytic subunit called the light chain and a glycoprotein heavy chain. These heterodimers function in the plasma membrane as antiporters, exchanging amino acids.

The heavy-chain subunit is a type II glycoprotein (SLC3A), the extracellular C-terminal domain of which resembles \(\alpha\)-amylases and belongs to the glycosyl hydrolase family 13 [7]. This domain is thus expected to be composed of a TIM barrel with a protruding \(\beta\) strand structure and a C-terminal Greek key \(\beta\)-motif [2]. However, no glycosidase activity associated with this domain has been detected so far [8]. Two structurally related heavy chains have been identified, rBAT and 4F2hc, that share 30 % identity and 50 % similarity [9].

The light chains are non-glycosylated, approx. 500 amino acids long polypeptide proteins that resemble other amino acid transporters, have 12 predicted transmembrane segments and intracellular termini. They represent the catalytically active part of the heterodimers, as shown for b\(^0\)+AT by reconstitution in liposomes [10]. Correspondingly, the characteristics of the transport mediated by the heterodimers are determined by the light chain that alone, however, does not reach the plasma membrane [11,12]. The light chains identified so far display at least 40 % identity with each other, but only one (b\(^0\)+AT) is a physiological partner of rBAT, whereas six (LAT1, LAT2, \(y\)-LAT1, \(y\)-LAT2, asc-1, xCT) functionally associate with 4F2hc.

Under overexpression conditions, 4F2hc was shown to interact also with b\(^0\)+AT in mammalian cells and in *Xenopus laevis* oocytes [13,14] and, conversely, rBAT was co-precipitated with LAT2 when co-expressed in *Xenopus* oocytes [15]. The association of the two subunits is stabilized by a covalent bond between two highly conserved cysteine residues. In the heavy chains, this cysteine is located in the extracellular neck, a few amino acids away from the membrane. In the light chains, the interacting cysteine is located in the second putative extracellular loop. The role of this disulphide bond appears to be the stabilization of the subunit interaction, since it has been shown, in overexpression systems, that it is neither necessary for the formation of the heterodimer nor for the expression of its transport function [16].

To identify within the glycoprotein subunits the structural determinants that mediate the recognition of and the functional association with the corresponding light chains, chimaeric and truncated forms of rBAT and 4F2hc were generated and co-expressed with light chains in *X. laevis* oocytes. Association of these chimaeric and truncated heavy chains with co-expressed light chains and resulting amino acid transport at the cell surface were investigated.

EXPERIMENTAL
cDNAs and constructs

The cDNAs of wild-type 4F2hc, hBAT and of all chimaeric and truncated constructs were inserted in the pSPORT-1 vector (4F2hc [17], hBAT [18]) hLAT1 cDNA was in pcDNA1/Amp-pSP64T [19] and mb\(^0\)+AT in pSD5easy [20].

Chimaeric constructs were made using the recombination PCR method [21]. As a first step, the DNA segment that had to be inserted into a recipient construct and the recipient construct itself

Abbreviations used: 2-ME, 2-mercaptoethanol; ER, endoplasmic reticulum; TEA, triethanolamine.

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were amplified from the corresponding templates in two separate amplification reactions. PCR was performed with Pfu DNA polymerase (Stratagene, Cambridge, U.K.) with the following cycling parameters: 1 min at 94 °C followed by 24 cycles; 30 s at 94 °C; 30 s at optimized annealing temperature; 1 min (for the insert) or 12 min (for the recipient construct) at 72 °C. The final elongation was for 7 (insert) or 15 min (for the recipient construct) at 72 °C. Primers were designed such that the two blunt-end fragments (insert and recipient vector) had at least 30 identical homologous ends. Correct recombination was checked by DNA sequencing (Microsynth, Balgach, Switzerland).

To obtain the truncated forms of 4F2hc (117 and 133 amino acids) and of rBAT (117 amino acids), site-directed mutagenesis was performed to introduce stop codons at the desired positions of the cDNA. Complementary primers with mutated nucleotide(s) at their centre were used to amplify the complete template cDNA plasmid. PCR amplification was performed using Pfu DNA polymerase (Stratagene) and the following cycling parameters: 30 s at 95 °C; 16 cycles of 30 s at 95 °C; 1 min at 55.2 °C for 4F2hc Δ117, 59.7 °C for 4F2hc Δ133 and 57.5 °C for rBAT Δ117; 13 min at 68 °C. Selective DNA template digestion was made with DpnI. Plasmids isolated from transformed bacteria were sequenced to verify the presence of the desired point mutation.

The following sense primers were used (mutated bases underlined, antisense oligonucleotides complementary): 4F2hc Trp118→stop; 5'-GCAGAAGTGGTGACACACAGGCGGCCCTC-3'; 4F2hc Gly134→stop; 5'-AGCGCTTTCCAGTGACACAGGCGGCCCTAC-3'; rBAT Trp118→stop; 5'-AACGTGCTAGACTGTAGCCAGAGGCGCCCTAC-3'.

cRNA synthesis

Constructs made in pSPORT-1 and pcDNA1/Amp-pSP64T vectors were linearized with HindIII and EcoRV respectively. T7 RNA polymerase, reagents and method for the RNA synthesis reaction were taken from the MEGAscript kit (Ambion, Austin, TX, U.S.A.). Linearization of plasmid containing the vector pSD5easy was done with PvuII, and Sp6 RNA polymerase (MEGAscript kit; Ambion) was used for cRNA synthesis. cRNAs were purified using the RNeasy mini kit (Qiagen). RNA integrity was checked by agarose gel electrophoresis and concentration determined by the measurement of absorbance at 260 nm.

Immunoprecipitation, SDS/PAGE and fluorography

cRNAs (10 ng each) of the heavy-chain constructs and/or of the light chain were injected into oocytes that were then incubated at 16 °C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2 and 5 mM Heps, pH 7.4) supplemented with 1 mCi/ml L-[35S]methionine for 2–3 days of expression. After

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### Table 1 Primers used for recombination PCR

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### Table 2 PCR products used for each heavy-chain construct

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<td>7 + 8 on hF2hc-pSPORT</td>
<td>BBB4</td>
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<td>BBB4</td>
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<td>4 + 18 on 444B-pSPORT</td>
<td>BBB</td>
</tr>
</tbody>
</table>

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s, sense; as, antisense. In longer primers, a hyphen indicates the limit between the part of the primer complementary with the template and the over-hang (in italic). The overhang sequence is complimentary to one shorter primer.
extensive washing with ND96, oocytes were lysed by using 20–30 µl of lysis buffer [120 mM NaCl, 50 mM Tris/HCl (pH 8) and 0.5% Triton X-100 (Sigma, St. Louis, MO, U.S.A.)], supplemented with 0.1% protease inhibitor cocktail (Sigma) and 200 mM PMSF per oocyte, vortexed for 20 s, incubated briefly on ice and then centrifuged at 16 000 g for 10 min at 4°C. Incorporated radioactivity was determined in trichloroacetic acid precipitates.

For immunoprecipitation, rabbit serum anti-human rBAT number 564 (N-terminal epitope MAEDKSKRDSIEMSMKGC) [22] and rabbit serum anti-human LAT1 number 555 (C-terminal TVLCQKLMQVVPQET; amino acids 493–507) were used.

Lysates containing an equal quantity of incorporated labelled L-[35S]methionine were made up to a final volume of 200 µl with lysis buffer, 50 µl of immunoprecipitation buffer [20 mM sodium phosphate (pH 7.5), 500 mM NaCl, 0.1% SDS, 1% Triton X-100 (Sigma), 0.5% sodium deoxycholate and 0.02% sodium azide] and 15 µl of serum. Samples were rotated overnight at 4°C. UltraLink™ Immobilized Protein A (50 µl; Pierce, Rockford, IL, U.S.A.) was then added and samples were rotated for two additional hours. Beads were washed six times with 0.5 ml of immunoprecipitation buffer and the bound antigen–antibody complex was eluted in 40 µl of SDS/PAGE loading buffer, by heating at 65°C for 15 min. Samples were reduced by adding 2.5% (v/v) 2-ME (2-mercaptoethanol) and heating again as above. After migration, gels were stained with Coomassie Blue, treated with Amplify Fluorographic Reagent (Amersham Biosciences, Little Chalfont, Bucks, U.K.) for 20 min, dried and exposed to Kodak X-OmatAR Film XAR-5 at −80°C.

### Cell-surface biotinylation and streptavidin precipitation

For immunoblotting, proteins were transferred electrophoretically from unstained gels to PVDF membranes (Immobilon-P, Millipore, Bedford, MA, U.S.A.). After blocking with 5% (w/v) milk powder in Tris-buffered saline and 0.1% Tween 20 for 60 min, the blots were incubated with rabbit anti-mouse b^2^−AT (number 400, 1: 2000) overnight at 4°C [22]. After washing and subsequent blocking, blots were incubated with secondary antibody conjugated with alkaline phosphatase (goat anti-rabbit; 1:5000) for 1 h at room temperature (~22°C). Antibody binding was detected with the CDP Star kit (Roche Diagnostics, Indianapolis, IN, U.S.A.) and exposure to an X-ray film (Kodak, Rochester, NY, U.S.A.). One out of three similar blots are shown.

### Deglycosylation

Lysates of oocytes labelled with L-[35S]methionine were prepared as described above. Lysates containing an equal quantity of incorporated labelled L-[35S]methionine were heated at 65°C for 15 min in a buffer containing 20 mM sodium phosphate, pH 7.5, 0.1% SDS, 0.5% Triton X-100 (Sigma), 2.5% 2-ME and 0.3% protease inhibitors cocktail (Sigma). Subsequently, each sample was divided into three equal aliquots and 1 m-unit of endoglycosidase H (Boehringer Mannheim, Mannheim, Germany), 1 unit of glycopeptidase F (Sigma) or no enzyme was added. The aliquots were incubated overnight at 37°C, before proceeding with the immunoprecipitation.

### Uptake rate of labelled amino acids

cRNAs (10 ng) of the glycoprotein constructs and/or of the light chain were injected into oocytes that were then kept at 16°C in ND96 buffer for the indicated expression time. Oocytes were washed six times in a Na^+^-free solution (100 mM choline chloride, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> and 10 mM HEPES, pH 7.5) and incubated for 2 min at 25°C, before performing uptakes of 10 min in 100 µl of Na^+^-free solution containing the amino acid at the indicated concentration and radioactive tracer. For L-[14C]cystine, an excess of diamide (10 mM) was added at the uptake solution to prevent its reduction. When measuring LAT1 function, an excess of unlabelled L-arginine (10 mM) was added to the L-[14C]cystine uptake solution to inhibit the potential uptake of L-cystine by the endogenous Xenopus b^2^− light chain. Trans-stimulation of L-[14C]cystine influx was achieved by the injection of 1 nmol of unlabelled L-phenylalanine (in 33 nl of ND96-TEA solution) into the L-[14C]cystine uptake solution. The uptake rate of labelled amino acids was calculated as pmol·h·oocyte⁻¹·L⁻¹. L-[14C]Arginine and L-[14C]cystine transport rates measured with heavy-chain constructs (see Figure 4) were normalized in each experiment to the mean obtained for rBAT (after subtracting the mean of non-injected oocytes). The normalized data from different experiments were pooled. Data given in Figure 6 represent the function of the heavy-chain constructs together with exogenous LAT1. Transport activity of putative endogenous light chains associated with the same heavy-chain construct expressed alone, or background activity of non-injected oocytes for LAT1 (see Figures 6A and 6B) and rBAT-LAT1 (Figure 6A), were subtracted before normalization to the transport rate observed for 4F2hc expressed with LAT1.

### Statistics

Error bars indicate the S.E.M. Significance was evaluated using ANOVA with Bonferroni post-test and Student’s t-test (two-tailed, unpaired).
The glycoprotein heavy-chain subunits 4F2hc and rBAT selectively interact with related catalytic light-chain subunits that differ in their amino acid transport selectivity. We have divided the glycoprotein subunits, 4F2hc (529 amino acids) and rBAT (685 amino acids), into four domains, (1) the cytoplasmic N-terminal tail (1–81 in 4F2hc, 1–88 in rBAT), (2) the transmembrane segment (82–104 and 89–110 respectively), (3) the neck (105–117 and 111–117 respectively) and (4) the C-terminal glycosidase-like ectodomain (118–529 and 118–685 respectively) [2,8]. A conserved pair of tryptophan residues at positions 117 and 118 marks, in both glycoproteins, the transition from the neck to the glycosidase-like domain. It is at the level of this short neck that the light chain is covalently attached to the heavy chain by a disulphide bridge [16]. The structure of the heavy-chain chimaeras and deletion constructs used in the present study is schematically represented in Figure 1.

To clarify the nature of the glycosylation pattern of the heavy-chain constructs, we performed deglycosylation experiments using endoglycosidase H (inactive on terminally processed N-glycans) and N-glycosidase F (Figure 2). Untreated rBAT (co-expressed with b0+AT; lanes 1–3) appeared on SDS/PAGE fluorography as a doublet (lane 1), the lower band of which was shifted to a lower molecular mass by endoglycosidase H treatment (lane 2) and thus corresponds to a core-glycosylated but not terminally glycosylated form. For B44B (co-expressed with b0+AT; lanes 4–6), the endoglycosidase H-resistant band was quite weak, suggesting that, although B44B has the same glycosidase-like domain as rBAT, the maturation of the construct was somewhat compromised. Lanes 7–9 show a construct (BBB4) that contains the extracellular domain of 4F2hc (co-expressed with b0+AT). As already known for 4F2hc, the band corresponding to the terminally processed form (endoglycosidase H-resistant) migrates at the same rate as the corresponding band of rBAT, whereas the other forms (core-glycosylated and deglycosylated) migrate relatively faster, as expected from their lower molecular mass. For BBB4, only a relatively small fraction was endoglycosidase H-resistant (lane 8) and thus fully mature.
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Figure 3  Co-immunoprecipitation and cell-surface expression of light-chain subunit b0+AT and glycoprotein subunit constructs

Oocytes were injected with the indicated cRNAs and, for (A, C, D), the newly synthesized proteins biosynthetically labelled with L-[35S]methionine. (A, C) Immunoprecipitations of the biosynthetically labelled material using an antibody raised against the N-terminus of rBAT. (B) Western blot of cell-surface proteins with an antibody raised against the light chain b0+AT. (D) Immunoprecipitation of biosynthetically labelled material using the anti-b0+AT antibody. SDS/PAGE was performed under reducing conditions where indicated (βME; = 2-ME). hc, heavy chain; Bt, rBAT terminally glycosylated; 4t, 4F2hc terminally glycosylated; Bc, rBAT core glycosylated; 4c, 4F2hc core glycosylated; B, rBAT; hd,117, heterodimer of rBATΔ117 and b0+AT; BΔ117, rBATΔ117.

band corresponding to co-precipitated b0+AT indicates that an interaction with the light chain took place (Figure 3A, lane 4).

Co-precipitation of b0+AT was also observed with both chimaeras that contain the extracellular domain of 4F2hc, BBB4 and BB44 (Figure 3A, lane 2 and 5). The two constructs have a predicted molecular mass that is similar to that of 4F2hc and thus the terminally glycosylated form is expected to migrate at approximately the same level as mature rBAT, whereas the stronger bands of approx. 70 kDa represent a core-glycosylated form (see Figure 2). A confusing factor for the interpretation of the bands is that b0+AT forms, probably by dimerization, a second weaker band that migrates just below the terminally glycosylated form of the heavy chains.

To address the question of how much b0+AT reaches the surface when associated with these different glycoprotein constructs, we performed cell-surface biotinylations followed by precipitation of the surface labelled proteins using streptavidin beads. The Western blot performed on the total oocyte lysates revealed that there were similar total amounts of b0+AT in the different samples (results not shown). Interestingly, the Western blots on streptavidin-precipitated cell-surface proteins revealed the presence of roughly similar amounts of cell-surface-expressed b0+AT, whichever construct containing the intracellular part of rBAT was co-expressed (Figure 3B).

For oocytes co-expressing the truncation rBATΔ117 and b0+AT, the truncated protein alone and a heterodimer with b0+AT (60 kDa) were visible under non-reducing conditions (Figure 3C, lane 5). The reducing treatment disrupted the heterodimer, leading to the appearance of b0+AT and to an increase in the amount of non-bound rBATΔ117 (Figure 3C, lane 2). None of the chimaeric constructs containing the intracellular tail of 4F2hc associated with b0+AT, as shown by co-immunoprecipitations made with anti b0+AT antibody (Figure 3D).

In conclusion, all constructs containing the intracellular domain of rBAT interacted at least to some extent with b0+AT. However, only the continuity of the intracellular tail and the transmembrane segment of rBAT were compatible with an efficient terminal glycosylation, irrespective of the combination of neck and glycosidase-like ectodomain.

Functional association of heavy-chain constructs with b0+AT

The b0+AT catalytic subunit that is normally associated with rBAT mediates the high-affinity uptake of cationic amino acids and L-cystine in exchange for neutral amino acids, whereas the catalytic subunit y-LAT1 that is normally associated with 4F2hc exchanges preferentially intracellular cationic amino acids against extracellular neutral amino acids and Na+. Xenopus oocytes
express endogenously a \(b^0\)-\(\Delta\text{LAT}\)-like and, to a lesser extent, a \(y^\Delta\text{LAT}\)-like light chain that might both interact with heavy-chain chimaeras [14]. First, the induction of \(L-[\text{H}]\)arginine uptake by the chimaeras was compared with that induced by \(r\text{BAT}\) (associating with endogenous \(b^0\)-\(\Delta\text{LAT}\)) and \(4F2\text{hc}\) (associating with endogenous \(y^\Delta\text{LAT}\)). As shown in Figure 4(A), three constructs induced an \(L-[\text{H}]\)arginine influx rate that appeared to be clearly higher than that induced by \(4F2\text{hc}\) alone. A small \(L\)-arginine uptake above background (non-injected oocytes) was detected also for \(44BB\) (only glycosidase-like domain from \(r\text{BAT}\)) and \(44BB\) (only neck from \(r\text{BAT}\)), but the transport activity was of the same order of magnitude as that induced by \(4F2\text{hc}\) that is known to associate with endogenous \(y^\Delta\text{LAT}\). However, no transport was induced by \(44BB\), \(4BB\) and \(44BB\) construct that is associated with \(b^0\)-\(\Delta\text{LAT}\). Therefore, the uptake of \(L-[\text{H}]\)arginine, \(L-[\text{C}]\)cysteine or \(L-[\text{H}]\)leucine could be observed.

**Figure 4** \(b^0\)-\(\Delta\text{LAT}\)-like transport activity induced by heavy-chain constructs with endogenous oocyte light chain

Oocytes were injected with 10 ng of each \(c\text{RNA}\), with the exception of \(r\text{BAT}\Delta\text{LAT}\) in (A) where 30 ng was used. After 2–3 days of expression, uptake (10 min in Na\(^+\)-free solution) of (A) \(L-[\text{H}]\)arginine (100 \(\mu\text{M}\)) or (B) \(L-[\text{C}]\)cysteine (50 \(\mu\text{M}\), in the presence of 10 mM diamide) was measured. When compared with the \(L\)-arginine transport rate induced by \(4F2\text{hc}\), that induced by \(BBB4\) and \(BB4B\) were significantly higher than that induced by \(r\text{BAT}\). For \(L\)-cysteine, the uptake by none of the chimaeras was statistically different from that with \(4F2\text{hc}\) according to ANOVA post-test. A fair amount of \(BB4B\) uptake was induced by \(BB4B\) in both transport mediated by endogenous \(b^0\)-\(\Delta\text{LAT}\) and \(4F2\text{hc}\) (associating with endogenous \(y^\Delta\text{LAT}\)). However, no transport was induced by \(BBB4\), \(BB4B\) and \(BB44\) construct that is associated with \(b^0\)-\(\Delta\text{LAT}\).

Attempts with various amounts of \(c\text{RNA}\) and expression times, no significant influx of \(L-[\text{H}]\)arginine, \(L-[\text{C}]\)cysteine or \(L-[\text{H}]\)leucine could be observed.

**Covalent interaction of heavy-chain constructs with \(\text{LAT}\)**

Figure 5 shows the SDS/PAGE (reducing conditions) fluorographs of immunoprecipitations of \(\text{LAT}1\) co-expressed with different heavy-chain constructs. \(\text{Human LAT1}\) was detected as an approx. 40 kDa band. Co-precipitated 4F2hc (Figure 5A, lane 11) appeared as two bands that correspond to its mature form (\(~90\text{ kDa}\) ) and its core-glycosylated form (\(~70\text{ kDa}\) ) respectively. It has to be mentioned that the relative intensity of these two bands was quite variable between experiments. The additional band visible around 130 kDa corresponds probably to a non-separated heterodimer. Human \(\text{BAT}\) is not a physiological partner of \(\text{LAT1}\), but it was co-precipitated from oocytes over-expressing both proteins (Figure 5A, lane 12). \(\text{rBAT}\) showed the typical doublet band, corresponding to the core- and terminally glycosylated forms, as previously seen in expression experiments also with \(\text{LAT2}\) [15]. The co-precipitation of terminally glycosylated \(\text{rBAT}\) with \(\text{LAT1}\) indicates that this ‘non-physiological’ heterodimer passes, in \(\text{Xenopus}\) oocytes, at least in part through the Golgi apparatus and might thus reach the oocyte cell surface.
Considering that both wild-type heavy chains can associate with LAT1, it is not surprising that mostly all chimaeras were co-precipitated to some extent with this exogenous light chain. Chimaeras 444B and 444B that contain the entire glycosidase-like extracellular domain of rBAT appeared on gels as rBAT in two bands, a band corresponding to a core-glycosylated form and a second band corresponding to the terminally glycosylated form (Figure 5A, lanes 3 and 9). In contrast, BB4B and B44B, which beside the extracellular part also have the cytoplasmic domain of rBAT, appeared mostly as relatively weak bands at the level of the core-glycosylated form of rBAT (Figure 5A, lanes 7 and 8). Chimaeras BBB4, 444B and BB44 contain the extracellular glycosidase-like part of 4F2hc and showed the same bands as wild-type 4F2hc (Figure 5A, lanes 4, 5 and 10). However, for BBB4 and BB44, the 70 kDa band of the core-glycosylated form was stronger than the approx. 90 kDa band (terminally glycosylated form), suggesting that a substantial part of the heterodimer did not exit the ER.

Immunoprecipitation using antibodies directed against the light chain was also performed on oocytes expressing the truncated 4F2hc together with LAT1 (Figure 5B). 4F2hcΔ117 and 4F2hcΔ133 have a predicted molecular mass of 12 and 14 kDa respectively. Complexes of the truncated 4F2hc and LAT1 were visible as bands of approx. 60 kDa, under non-reducing conditions (Figure 5B, lanes 3 and 4). These bands disappeared after sample reduction (results not shown), indicating the presence of a disulphide bond linking the truncated heavy-chain to the light-chain subunit.

To test whether rBAT when associated with LAT1 reaches the cell surface, we performed cell-surface biotinylation reactions on oocytes expressing LAT1 alone or together with either 4F2hc or rBAT (Figure 5C). Western blotting of the total oocyte lysate showed that similar amounts of LAT1 were expressed in the three cases (Figure 5C, lanes 2–4). Western blotting of the streptavidin-precipitated proteins revealed that LAT1 reached the surface together with core- and terminally glycosylated 4F2hc and, surprisingly, also with rBAT (core- and terminally glycosylated forms) (Figure 5C, lanes 7 and 8).

**Functional association of heavy-chain constructs with LAT1**

To examine the function of heterodimers of LAT1 formed with chimaeric or truncated heavy chains, transport of L-[14C]isoleucine was measured. To increase this uptake by trans-stimulation, unlabelled L-phenylalanine (1 nmol) was injected into oocytes 4 h before the influx measurement [23]. An excess of unlabelled L-phenylalanine for trans-stimulation. Transport rates are given as described in the Experimental section. When compared with the L-isoleucine transport rate induced by LAT1 alone, the rate induced by 444B and by both truncations, together with LAT1, was significantly higher according to the ANOVA post-test (Bonferroni), whereas that induced by 444B and BB44 reached a level of significance only according to t test analysis.

![Figure 6](image.png)

**Figure 6** L-type transport activity of LAT1 co-expressed with heavy chain constructs

Oocytes were injected with 10 ng of the indicated cRNAs. After 1 day of expression (4 days for truncations), uptakes of L-[14C]isoleucine (100 μM) were measured (10 min, in Na+-free buffer supplemented with 10 mM L-arginine to block b0−AT) after injecting 1 nmol of unlabelled L-phenylalanine for trans-stimulation. Transport rates are given as described in the Experimental section. When compared with the L-isoleucine transport rate induced by LAT1 alone, the rate induced by 444B and by both truncations, together with LAT1, was significantly higher according to the ANOVA post-test (Bonferroni), whereas that induced by 444B and BB44 reached a level of significance only according to t test analysis.

**DISCUSSION**

**Intracellular and transmembrane domains of rBAT determine functional interaction with b0−AT**

The co-immunoprecipitation experiments show that association with b0−AT requires the presence of the intracellular domain of rBAT and that, in contrast, chimaeras lacking the intracellular rBAT tail do not associate with b0−AT (Figure 3). This shows that the three other domains (transmembrane domain, neck and glycosidase-like domain) are neither necessary nor sufficient for association with b0−AT. For a surface-expression of functional heteromeric transporter containing b0−AT, the transmembrane domain of rBAT is necessary in association with its cytoplasmic tail, whereas the construct BB44B that lacks this transmembrane domain appears to reach the cell surface with b0−AT but does not support its function. This suggests that the continuity between rBAT intracellular tail and TM domain is important for permitting the transporting function of the b0−AT heterodimer. The truncated rBAT that associates with b0−AT did not induce any detectable b0−AT-type transport but substitution of the extracellular domain with that of 4F2hc prevented this loss of function (Figure 4).

Taken together, these results suggest that the cytoplasmic tail of rBAT is required for the association of this glycoprotein subunit to its catalytic subunit b0−AT, whereas the continuity of the rBAT cytoplasmic tail and transmembrane domains, but not the
rBAT extracellular domain that can be replaced by the equivalent domain of 4F2hc, is required for the cell surface transport function of the heterodimers containing b0,+.AT.

Other studies on progressive C-terminal truncations of rBAT have suggested a possible role of the C-terminal extracellular domain for the recognition of the light chain b0,+.AT [25,26]. However, these contrasting conclusions were based only on transport measurements and did not directly address the question of subunit association and cell-surface expression.

It is interesting that all functional b0,+.AT heterodimers containing a chimaeric heavy-chain construct supported the transport of L-cystine proportionally less efficiently compared with that of L-arginine. We do not know why substituting at the level of heavy-chain domains has a selective effect on L-cystine transport. It suggests, however, that the possibility that efficient L-cystine transport requires some specific functional co-operation of rBAT with the light chain or between heterodimers. This co-operation would be disrupted by the substitution and would be less important with the light chain or between heterodimers. This co-operation requires some specific functional co-operation of rBAT and b0,+.AT can take place in overexpressing systems, but at a quantitative level below our detection limit in coinmunoprecipitation and transport studies [22]. Whether this led to the surface expression of functional heterodimer or just allowed b0,+.AT monomers to escape the ER was not investigated.

**Functional interaction of 4F2hc with LAT1 is not limited to a specific domain of 4F2hc**

Unlike b0,+.AT, the 4F2 light chain LAT1 formed a disulphide bond with either co-expressed wild-type heavy chain in Xenopus oocytes [14] or 4F2hc and b0,+.AT in human retinal pigment epithelial cells [13], some b0,+.AT activity. It is thus probable that some physical and functional interaction between 4F2hc and b0,+.AT can take place in overexpressing systems, but at a quantitative level below our detection limit in coinmunoprecipitation and transport studies [22]. Whether this led to the surface expression of functional heterodimer or just allowed b0,+.AT monomers to escape the ER was not investigated.

**REFERENCES**


