The Structural and Functional Units of Heteromeric Amino Acid Transporters

THE HEAVY SUBUNIT rBAT DICTATES OLIGOMERIZATION OF THE HETEROMERIC AMINO ACID TRANSPORTERS

Esperanza Fernández1,2, Maite Jiménez-Vidal1,3, María Calvo4, Antonio Zorzano5, Francesc Tebar5, Manuel Palacín4,5, and Josep Chillarón4,6

From the 1Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona and Institute for Research in Biomedicine, Barcelona Science Park, E-08028 Barcelona, Spain and 2Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, Av. Diagonal, 645, E-08036 Barcelona, Spain.

Heteromeric amino acid transporters are composed of a catalytic light subunit and a heavy subunit linked by a disulfide bridge. We analyzed the structural and functional units of systems b0,+ and xCT, formed by the heterodimers b0,+rBAT and xCT-4F2hc, respectively. Blue Native gel electrophoresis, cross-linking, and fluorescence resonance energy transfer in vivo indicate that system b0,+ is a heterotetramer (b0,+rBAT)2, whereas xCT-4F2hc seems not to stably or efficiently oligomerize. However, substitution of the heavy subunit 4F2hc for rBAT was sufficient to form a heterotetrameric [xCT-rBAT]2 structure. The functional expression of concatamers of two light subunits (which differ only in their sensitivity to inactivation by a sulphydryl reagent) suggests that a single heterodimer is the functional unit of systems b0,+ and xCT.

The abbreviations used are: HAT, heteromeric amino acid transporters; LSHAT, light subunits of HAT; YFP, yellow fusion protein; Wt, wild-type; FRET, fluorescence resonance energy transfer; FRETN, normalized sensitized FRET; NTA, nitrilotriacetic acid; DTT, dithiothreitol; EGFR, epidermal growth factor receptor; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate.

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5 These authors contributed equally to this study and share last authorship.

6 A senior researcher from the Programa Ramón y Cajal of the Spanish Ministry of Science and Technology. To whom correspondence may be addressed: IRB-PCB, Barcelona Science Park, Josep Samitier 1-5, 08028, and Dept. of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, Av. Diagonal, 645, E-08028, Barcelona, Spain. Tel.: 34-934034617 and 34-934034700; Fax: 34-934034717; E-mail: mpalacin@pcb.ub.es.

7 The abbreviations used are: HAT, heteromeric amino acid transporters; DMS, dimethyl suberimidate; HSHAT, heavy subunits of HAT; LSHAT, light subunits of HAT; pCMB, p-chloromercuribenzoate; CFP, cyan fusion protein; YFP, yellow fusion protein; Wt, wild-type; FRET, fluorescence resonance energy transfer; FRETN, normalized sensitized FRET; NTA, nitrilotriacetic acid; DTT, dithiothreitol; EGFR, epidermal growth factor receptor; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate.
band of 250 kDa (4). However, neither the native structure nor the functional unit of the HAT is known. Here we present evidence indicating that the single heterodimer is the functional unit of systems β0,+ and xc−, 4F2hc-associated heterodimers seem not to form stable oligomers, whereas β0,+AT-rBAT is a heterotetramer in vivo. Finally, we demonstrate that rBAT promotes the oligomerization of HAT.

**EXPERIMENTAL PROCEDURES**

The construction of the distinct cDNAs is described under supplemental “Experimental Procedures.”

Reagents and Antibodies—Reagents were obtained from Sigma if not indicated otherwise. Antibodies against human and mouse β0,AT and rBAT are described elsewhere (4, 24, 25). The anti-Xpress antibody was purchased from Invitrogen, and the anti-Myc 9E10 hybridoma was from ATCC. The rabbit polyclonal antibody against mouse LAT2 was produced at Research Genetics. The antigenic peptide was PIFKPTVPKD-PDSEEQP (the C-terminal 17 residues). Specificity of this antibody was tested by comparing the signal in Western blot with the preimmune antiserum obtained from the same rabbit (data not shown).

cRNA Synthesis, Injection, and Maintenance of Xenopus Oocytes—The synthesis of human 4F2hc cRNA has been described elsewhere (26). In vitro synthesis of human xCT wild type, xCT C327S, and xCT concatamers was conducted with the NotI-linearized plasmid template using the in vitro transcription protocol from AMBION (mMESSAGE mMACHINE, Ambion, Austin, TX). Mixtures of cRNAs were prepared immediately before injection with a calibrated pipette. The amount of transcribed RNA was calculated by 260-nm absorbance measurement before microinjection into Xenopus oocytes. Each of the cRNA species was synthesized at least on two occasions. In mixing experiments, oocytes were injected with 4F2hc cRNA (5 ng) together with xCT Wt (5 ng), xCT C327S (5 ng), or with a mixture of xCT Wt and xCT C327S (total amount of 5 ng of xCT cRNAs). For the concatamers, 25 ng of the corresponding cRNA together with 5 ng of 4F2hc cRNA were injected per oocyte.

The procedures for injection and maintenance of oocytes have been described in detail elsewhere (27). Oocytes were injected with either 50 nl of water or 50 nl of water containing the cRNAs. Oocytes were incubated in modified Barth’s medium. The procedures for injection and maintenance of oocytes were performed 2–4 days after injection.

Cell Culture and Transfection—Growth, maintenance, and calcium phosphate transient transfection were performed as described (25). The efficiency of transfection was above 70% in all experiments. For fluorescence resonance energy transfer (FRET) analysis, the cells were plated on a 6-well plate and transiently transfected using FuGENE-6 (Roche Applied Science). This reagent (6 μl) was added to serum-free medium (100 μl) at room temperature for 5 min. This medium was incubated with plasmids encoding the CFP and YFP fusion proteins (1 μg each) for 30 min, and the mixture was added to the cells grown in culture medium. After 6 h of incubation, the cells were washed twice with phosphate-buffered saline, trypsinized, and reseeded on a 6-well plate containing one coverslip (22 mm, Electron Microscopy Science) per well.

Transport Assays and Transport Reconstitution—Influx rates of 100 μM L-[3H]glutamate (ARC) or 20 μM L-[35S]cystine (Amersham Biosciences) in Xenopus oocytes or transfected HeLa cells were performed as described (22, 25). The effect of pCMB and MTS reagents (Toronto Research Chemicals, Inc.) was assayed as described (3, 22). 1 mM pCMB and 2.5 mM MTSEA inhibited the activity of wild-type xCT (22) and wild-type β0,AT, respectively. Reconstitution of wild type β0,AT and the C321S mutant into liposomes and uptake measurements in the reconstituted system were performed as described (21).

Membrane Preparation and Protein Purification by Ni2+-NTA Chromatography—Kidney brush border membranes were obtained as described (4). For the preparation of kidney total membranes, the kidney was homogenized in 25 mM Hepes, pH 7.4, 4 mM EDTA, 250 mM sucrose, and 20 mM N-ethylmaleimide with the protease inhibitors aprotinin, leupeptin, phenylmethylsulfonyl fluoride, and pepstatin on a CPCU Polytron. The homogenate was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was further centrifuged at 200,000 × g for 90 min at 4 °C. A similar procedure was used to obtain total membranes from HeLa cells, but the cells (~107 cells/1 ml homogenization buffer) were homogenized by 15 passages through a 25-gauge needle. For Blue Native PAGE or cross-linking, the membranes were resuspended directly in the appropriate buffers (see below).

For Ni2+-NTA chromatography, total membranes from HeLa cells were resuspended in 25 mM Tris-HCl, pH 7, 50 mM NaCl, 1% digitonin (final detergent/protein (w/w) ratio of 3.3), and solubilization proceeded for 30 min. Insoluble material was discarded by a 10,000 × g centrifugation at 4 °C for 10 min. The supernatant was diluted in the above buffer containing 15 mM imidazole and applied to the Ni2+-NTA beads (Qiagen). After 30 min of end-over-end mixing at room temperature, the beads were washed 4 times in a similar buffer containing 0.1% digitonin and 10 mM imidazole. Elution was performed by raising the imidazole concentration to 100 mM for 10 min at room temperature. The eluted material was then processed for Blue Native PAGE (see below).

Blue Native and SDS-PAGE—In preliminary experiments we tested a range of detergent/protein (w/w) ratios with a fixed Coomassie Blue G/detergent ratio (w/w) of 1/2.5 (28). With 0.5–1% digitonin extracts and a 3.3/1 detergent/protein ratio, we observed that rBAT and β0,AT appeared as a single band of 535 ± 18 kDa (Fig. 1A). Similar results were obtained with 0.25–0.5% dodecyl-β-D-maltoside, although a smear appeared above the ~535-kDa band. N-Octylglucoside did not resolve any band, and Triton-X-100 increased the smearing until no distinct band could be seen (data not shown). We did not observe any additional bands. Digitonin/protein ratios below 3.3/1 increased smearing and decreased the intensity of the ~535-kDa band, and at a ratio of 0.25/1 no band was detected; ratios up to 10/1 were similar to the 3.3/1 ratio (data not shown).

Membranes were solubilized for 30 min at room temperature in 25 mM Tris-HCl, pH 7, 50 mM NaCl, 1% digitonin at a deter-
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gent/protein ratio (w/w) of 3.3 (see above). Further treatment with a range of urea concentrations, 100 mM DTT, or 2% SDS with or without 100 mM DTT was carried out at 37 °C for 30 min. After solubilization, Blue Native buffer was added, and Blue Native PAGE was performed as described (29, 30). Native molecular weight markers (Amersham Biosciences) were visualized by Coomassie staining. For control SDS-PAGE, SDS sample buffer (without DTT) was added to the solubilized samples, which were immediately loaded (without heating) in SDS-PAGE gels. Western blots were performed as described (31). After transference of Blue Native gels, the membrane was destained in methanol 50% and acetic acid 10% to eliminate excess Coomassie Blue G (Serva) and washed with bi-distilled water before blocking.

Cross-linking—Total membrane proteins from transfected HeLa cells were resuspended in phosphate-buffered saline and incubated at 2 mg/ml with the cross-linker dimethyl suberimidate (DMS, from Pierce) for 30 min at room temperature. Cross-linking was terminated by the addition of 100 mM Tris-HCl, pH 8.0, for 15 min at room temperature.

FRET—For FRET analysis the concatamers CFP-b0, + AT/rBAT and YFP-b0, + AT/rBAT, CFP-xCT/4F2hc and YFP-xCT/4F2hc, and the CFP-EGFR and YFP-EGFR fusion proteins were transiently expressed in HeLa cells.

A Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg GmbH, Manheim, Germany) equipped with an argon laser, 63× oil immersion objective lens (458/514 nm) was used. We used CFP as the donor fluorochrome paired with YFP as the acceptor fluorochrome.

FRET measurements were based on the sensitized emission method previously described (32, 33) with minor modifications for the confocal microscope. In some experiments in which the sensitized emission method was performed, FRET efficiency was also calculated using the acceptor photobleaching method (34, 35). The same set of transfected cells (but distinct dishes) was used for both methods.

Sensitized Emission Method—The sensitized emission method is based on the increase of acceptor fluorescence caused by FRET during excitation. To measure FRET, three images were acquired in the same order in all experiments through 1) the CFP channel (absorbance 458 nm, emission 465–510 nm), 2) the FRET channel (absorbance 458 nm, emission 525–600 nm) 3) the YFP channel (absorbance 514 nm, emission 525–600 nm). Background was subtracted from images before performing FRET calculations. Control and experiment images were taken under the same conditions of photomultiplier gain, offset, and pinhole aperture.

The FRET image must be corrected for the cross-talk of the donor emission and the direct excitation of the acceptor by the donor excitation wavelength. The crossover of the donor and acceptor emission and the direct excitation of the acceptor by the photomultiplier gain, offset, and pinhole aperture.

The negative FRETC values obtained in some experiments are due to slight over-estimation of the spectral bleed-through coefficients. Areas with unusually high or low CFP/YFP ratios (i.e. outside the 1:1 to 1:4 stoichiometric range) were excluded from analysis. All calculations were performed using the Image Processing Leica Confocal Software and Microsoft Excel.

Acceptor Photobleaching Method—In the presence of FRET, bleaching of the acceptor (YFP) results in a significant increase in fluorescence of the donor (CFP). Half the cell was bleached in the YFP channel using the 514 argon laser line at 100% intensity. Before and after YFP bleaching, CFP and YFP images were collected to assess changes in donor and acceptor fluorescence. To minimize the effect of photobleaching caused by imaging, images were collected at low laser intensity. The gain of the photomultiplier tubes was adjusted to obtain the best possible dynamic range. FRET efficiency was calculated as,

$$E = \frac{(I_{\text{post}} - I_{\text{pre}}) \times 100}{I_{\text{pre}}}$$

where $I_{\text{pre}}$ is the pre-bleach CFP intensity, and $I_{\text{post}}$ is the post-bleach CFP intensity in the bleached region. An internal negative control, an unbleached region in the same cell was measured.

RESULTS

In Vitro Analysis of the Quaternary Structure of HAT—To analyze oligomerization of HAT, we used Blue Native PAGE (29, 30) followed by Western blot. We initially tested different detergents, detergent to protein ratios, and Coomassie Blue G detergent ratios to find good experimental conditions for...
Blue Native PAGE allows the determination of oligomerization stoichiometry with agents that partially dissociate complexes (28, 38). Up to 5 M urea did not alter the mobility of the \( b^{0+} \) -AT-rBAT complex (data not shown). In contrast, 2% SDS partially dissociated the complex in bands I and II, both in HeLa cells (Figs. 1, B and C) and in brush border membranes (data not shown). The relative amounts of the two bands varied slightly between experiments, but \( b^{0+} \) -AT-rBAT never shifted completely to band II. The slower mobility of band I compared with the untreated sample could be due to partial unfolding of \( b^{0+} \) -AT-rBAT. On the \( b^{0+} \) -AT/rBAT concatamer, SDS may cause further unfolding that results in even a slower mobility of bands I and II (Fig. 1C). The addition of the reducing agent DTT in the presence of SDS dissociated the complex into its subunits (Fig. 1B). rBAT appeared as a single band of 162 ± 11 kDa, and \( b^{0+} \) AT appeared as two bands of 180 ± 14 and 84 ± 9 kDa (\( n = 7 \)). SDS-PAGE followed by Western blot confirmed that DTT completely reduced the samples (data not shown). No dissociated subunits appeared with the \( b^{0+} \) AT/rBAT concatamer (Fig. 1C). According to a recently proposed empirical rule, the molecular weight of a polytopic transporter multiplied by a factor of 1.8 fits with its mobility in Blue Native gels (39). This applied to \( b^{0+} \) AT, confirming that the 84- and the 180-kDa bands were \( b^{0+} \) AT monomers and dimers, respectively (Fig. 1B). rBAT mobility was slower than expected (compare 162 ± 11 kDa in Fig. 1B (Blue Native PAGE) with ~94 kDa (SDS-PAGE) on Fig. 3B), which could be due to partial unfolding. In conclusion, Blue Native PAGE strongly suggested that system \( b^{0+} \) has a [\( b^{0+} \) AT-rBAT]_2 structure since (i) only two bands (band I and band II, see Figs. 1, B and C, lanes 2 of the gels) were observed after treatment of the dig ditagonin-solubilized sample with SDS, suggesting that \( b^{0+} \) -AT-rBAT is a heterotetramer (two heterodimers; band I) that can be partially dissociated by SDS to the single heterodimer (band II), (ii) after DTT reduction, \( b^{0+} \) -AT appeared as two bands compatible with a monomer and a dimer, and (iii) the size of the untreated sample (~535 kDa) was double that of band II (260 ± 19 kDa; \( n = 7 \)). In addition, the sum of the sizes of the monomeric rBAT (162 ± 11 kDa) and the monomeric \( b^{0+} \) AT (84 ± 9 kDa) matched the size of band II (260 ± 19 kDa).

The His-xCT-4F2hc and Myc-LAT2-4F2hc heterodimers expressed in HeLa cells were detected on Blue Native gels as a single band of ~250 kDa (Fig. 2, A and B, respectively), similar to band II of \( b^{0+} \) AT-rBAT. No dissociation occurred even with 2% SDS. Further addition of DTT resulted in the detection of the His-xCT and myc-LAT2 subunits in a monomeric form (~80 kDa). LAT2-4F2hc from kidney basolateral membranes behaved similarly (data not shown). A functional His-xCT/4F2hc concatamer (see supplemental Fig. C) ran with the same mobility as His-xCT-4F2hc (Fig. 2A, lanes 4–6) that was not modified by SDS or DTT, and a Myc-xCT/xCT dimeric concatamer co-transfected with 4F2hc (see below for functional data) ran at ~540 kDa, about twice the size of His-xCT-4F2hc heterodimer and the His-xCT/4F2hc concatamer (Fig. 2A, lanes 7–8). As expected, SDS did not dissociate this complex. It is worth mentioning that a faint band of similar size was observed with the His-xCT/4F2hc concatamer after long exposure (Fig. 2A, asterisk). In summary, our results suggest that detection of the transporters (see “Experimental Procedures” and Refs. 29 and 30). rBAT and \( b^{0+} \) AT were found as a single band of 535 ± 18 kDa (\( n = 12 \)) (Fig. 1A). This band did not correspond to isolated rBAT or \( b^{0+} \) AT subunits because (i) it was detected in brush border membranes, where only the disulfide-linked heterodimer is found (Fig. 1A, lanes 1) and (ii) a functional, purified, His-tagged \( b^{0+} \) AT/rBAT concatamer (36) (where “/” indicates fusion of the two subunits) had the same mobility (Fig. 1A, lanes 4). As expected, no \( b^{0+} \) AT appeared in membranes from the \( b^{0+} \) AT KO mice (37) (Fig. 1A, \( b^{0+} \) AT panel, lane 2). In contrast, rBAT was still detected as the 535-kDa band because of its binding to an as yet unidentified light subunit (4, 37) (Fig. 1A, arBAT panel, lane 2).

### FIGURE 1. Blue Native PAGE analysis of \( b^{0+} \) -AT-rBAT.

A. 1% digitonin extracts from distinct sources were supplemented with Blue Native sample buffer and loaded on 5–15% Blue Native gels for Western blot with antibodies against rBAT (\( b^{0+} \) AT or \( b^{0+} \) AT (\( b^{0+} \) AT), mouse kidney brush border membranes; 2, kidney brush border membranes from the null \( b^{0+} \) AT KO mouse; 3, total membranes from HeLa cells co-transfected with rBAT and \( b^{0+} \) AT; 4, the His-b\(^{0+} \) AT/rBAT concatamer expressed in HeLa cells was purified on a Ni\(^{2+} \) NTA chromatography column and eluted in imidazole buffer containing 0.1% digitonin. Preimmune antisera did not detect any band in the Blue Native gels (not shown). B, HeLa cells were co-transfected with rBAT, and \( b^{0+} \) AT and total membranes were isolated. The membranes were either solubilized with 1% digitonin (D), or after solubilization with 1% digitonin, 2% SDS was added for 30 min at 37 °C (S) in the absence (−) or in the presence (+) of DTT. The membranes were then supplemented with Blue Native sample buffer and loaded on 5–15% Blue Native gels for Western blot with antibodies against rBAT (\( b^{0+} \) AT) or \( b^{0+} \) AT (\( b^{0+} \) AT). C, the concatamer His-b\(^{0+} \) AT/rBAT was transfected in HeLa cells. Total membranes were isolated, solubilized with 1% digitonin, and applied to a Ni\(^{2+} \) NTA chromatographic column. The purified concatamer was eluted with imidazole buffer containing 0.1% digitonin and either left untreated (D) or treated with 2% SDS for 30 min at 37 °C (S) in the absence (−) or presence (+) of DTT. Blue Native sample buffer was added for loading on 5–15% Blue Native gels, and the Western blot was decorated with antibodies against the Xpress tag (\( \alpha \)Xpress) contiguous to the histidine tag of the concatamer. Longer periods in SDS or higher incubation temperatures did not modify the results. No other bands were detected in any of the gels, even with longer exposure times. Representative experiments from at least \( n = 4 \) experiments for each type of sample are shown. Ab, antibody; I, band I; II, Band II.
LSHAT-4F2hc heterodimers do not stably assemble into higher order oligomers. We cannot rule out that an equilibrium between xCT-4F2hc and [xCT-4F2hc]₂ exists, which is likely shifted to the heterodimeric form.

Blue Native PAGE does not escape general criticisms to detergent-based methods. Therefore, we applied DMS a primary amine permeable cross-linker to total membranes from HeLa cells expressing rBAT and b₀⁺/H₁₁₀₀₁/AT or 4F2hc and His-xCT. DMS shifted the b₀⁺/H₁₁₀₀₁/AT-rBAT heterodimer from 130 to 250 kDa in a dose-dependent manner, indicating that two heterodimers are in close contact (Fig. 3A). Cross-linking efficiency was very high (10 mM DMS quantitatively shifted b₀⁺/H₁₁₀₀₁/AT-rBAT to the 250 kDa band), revealing that both the intracellular and the plasma membrane heterodimer, the sites of transport.

FIGURE 2. Blue Native PAGE analysis of 4F2hc-associated heterodimers. A, HeLa cells were transfected with 4F2hc and His-xCT (lanes 1–3), the concatamer His-xCT/4F2hc (lanes 4–6), or 4F2hc with the concatamer myc-xCT/xCT (lanes 7 and 8) (each transfection corresponds to a distinct gel). Total membranes were isolated and either solubilized with 1% digitonin (D), or after solubilization with 1% digitonin, 2% SDS was added for 30 min at 37 °C (S) in the absence (−) or in the presence (+) of DTT. The samples were supplemented with Blue Native sample buffer and loaded on 5–15% Blue Native gels for Western blot with antibodies against the Xpress or the Myc tag (αXpress or αmyc). The asterisk (*) indicates the high molecular weight band in lanes 4–8. A representative experiment from n = 4 is shown. B, HeLa cells were co-transfected with 4F2hc and myc-LAT2. Total membranes were isolated and re-suspended in reaction buffer containing the concentrations indicated of the cross-linking agent DMS (− indicates no DMS). The reaction was stopped, supplemented with SDS sample buffer without DTT, and loaded on SDS-PAGE for Western blotting with antibodies against rBAT (αrBAT). The results were the same with the antibody against b₀⁺/H₁₁₀₀₁/AT. C, the same samples in A were supplemented with SDS sample buffer with DTT and detected with the two antibodies. D, HeLa cells were co-transfected with 4F2hc and His-xCT and were treated as in A. Western blot was performed with antibodies against the Xpress tag (αXpress) contiguous to the His tag of the xCT subunit. Equal protein amounts were loaded on each lane of the same gel. We did not observe higher molecular weight cross-linking bands. Ab, antibody. Representative experiments from n = 4 are shown.

FIGURE 3. Cross-linking of b₀⁺/H₁₁₀₀₁/AT-rBAT and xCT-4F2hc. A, HeLa cells were co-transfected with rBAT and b₀⁺/H₁₁₀₀₁/AT. 36 h later total membranes were isolated and re-suspended in reaction buffer containing the concentrations indicated of the cross-linking agent DMS (− indicates no DMS). The reaction was stopped, supplemented with SDS sample buffer without DTT, and loaded on SDS-PAGE for Western blotting with antibodies against rBAT (αrBAT). The results were the same with the antibody against b₀⁺/H₁₁₀₀₁/AT. B, the same samples in A were supplemented with SDS sample buffer with DTT and detected with the two antibodies. C, HeLa cells were co-transfected with 4F2hc and His-xCT and were treated as in A. Western blot was performed with antibodies against the Xpress tag (αXpress) contiguous to the His tag of the xCT subunit. Equal protein amounts were loaded on each lane of the same gel. We did not observe higher molecular weight cross-linking bands. Ab, antibody. Representative experiments from n = 4 are shown.
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In Vivo Analysis of the Quaternary Structure of HAT—We applied the sensitized FRET emission method to study oligomerization in vivo (40) in HeLa cells transiently transfected with N-terminal (YFP or CFP)-tagged versions of the concatamers b0, + AT/rBAT (see Fig. 4A) and xCT/4F2hc (see below). CFP-b0, + AT/rBAT and YFP-b0, + AT/rBAT completely co-localized at the plasma membrane and intracellular sites (Fig. 4B), and induced cystine transport activity (data not shown). Positive CFP→YFP FRETc signals were detected at the plasma membrane when CFP- and YFP-b0, + AT/rBAT were co-expressed (Fig. 4B), suggesting close proximity of the N termini of the two fusion proteins (i.e. the N termini of the b0, + AT subunits) and that b0, + AT-rBAT forms dimers or higher order oligomers at the cell surface. As a control, we measured FRET in cells co-expressing YFP-b0, + AT/rBAT and an unrelated integral membrane protein, the receptor for the epidermal growth factor tagged with CFP (CFP-EGFR) (40). Despite the co-localization of the two proteins at the plasma membrane (Fig. 4B), no FRETc signals were detected, indicating that they did not form complexes.

Because the efficiency of energy transfer depends on the amount of donor and acceptor capable of interaction, FRETc values were divided by the product of YFP and CFP intensities on a pixel-by-pixel basis to obtain normalized FRET (FRETN) at the plasma membrane. FRETN values in cells co-expressing CFP- and YFP-b0, + AT/rBAT (23.3 (E = 0.5) ± 3.6 (E = 0.5)) were substantially higher than in control cells co-expressing YFP b0, + AT/rBAT and CFP-EGFR (3.5 (E = 0.5) ± 1.2 (E = 0.5)) (Fig. 4C).

We also measured FRET in HeLa cells co-expressing the YFP-xCT/4F2hc and CFP-xCT/4F2hc concatamers. Localization of the fusion proteins was similar to that observed for YFP- and CFP-b0, + AT/rBAT, and they also induced cystine transport activity (not shown). The FRETN value (7.8 (E = 0.5) ± 2.6 (E = 0.5)) was significantly different from the values obtained when YFP-xCT/4F2hc was co-expressed with CFP-EGFR (which were zero) but lower than for b0, + AT/rBAT (see above).

where b0, + AT-rBAT is found in HeLa cells,9 were in the same oligomeric state. Under reducing conditions the size of rBAT was not modified by the cross-linker, whereas a dose-dependent increase in the dimeric form of b0, + AT was observed (Fig. 3B). Therefore, two or more lysines on two b0, + AT subunits must be in close contact (no more than 11 Å). In addition, under reducing conditions b0, + AT ran as a monomer and as an SDS-resistant dimer even in the absence of cross-linker, consistent with the two bands observed in non-reducing conditions (compare Figs. 3, A and B) (4). DMS had no effect on the xCT-4F2hc heterodimer (Fig. 3C). Under non-reducing conditions, the 125-kDa band represented most (>90%) of the total xCT-

9 E. Fernández, M. Palacín, and J. Chillarón, unpublished data.
Finally, we also determined FRET efficiency (E) with an independent approach; that is, the acceptor photobleaching FRET method (34, 35). In YFP- and CFP-b0.5-AT/rBAT co-expressing cells, E was 12.9 ± 2.2%; in contrast, in YFP- and CFP-xCT/4F2hc co-expressing cells, E was not different from zero (2.9 ± 2.9%). These results are consistent with the presence of b0.5+AT-rBAT oligomers at the cell surface. Some xCT-4F2hc might be in an oligomeric state, which forms either less efficiently or is less stable than b0.5+AT-rBAT oligomers.

The Transport Unit of Systems b0.5+ and xC− Is the Single Heterodimer—To study the b0.5+AT-rBAT transport unit we first generated functional b0.5+AT transporters either sensitive or insensitive to a sulphydryl reagent (41–43). Among the reagents tested, only MTSEA completely abolished b0.5+AT-rBAT-mediated uptake (Fig. 5A). Because b0.5+AT alone is functional in a reconstituted system (21), we reasoned that the residues responsible for MTSEA inactivation may reside in b0.5+AT. Therefore several b0.5+AT Cys to Ser mutants were analyzed. The C321S b0.5+AT mutant was insensitive to MTSEA (Fig. 5A), and in reconstitution experiments its activity was not significantly different to that shown by the wild type (Fig. 5B). Because the structural data indicated a heterotetrameric structure for b0.5+AT-rBAT, we constructed dimeric concatamers between the wild-type and the C321S b0.5+AT (Wt-CS and CS-Wt), so that when expressed the wild type and the C321S b0.5+AT would be represented in equal proportions. Concatamers of two wild-type subunits (Wt-Wt) and two C321S subunits (CS-CS) were also constructed. The four concatamers were expressed together with rBAT in HeLa cells. They displayed the expected mobility in Western blots, and no single b0.5+AT subunits were observed (data not shown). The transport function of the concatamers was 15–30% of the activity of b0.5+AT-rBAT (supplemental Fig. A1), caused at least in part to their lower expression (less than 50%, data not shown) compared with b0.5+AT-rBAT. The transport activity of the concatamers was measured after preincubation with MTSEA or vehicle (Fig. 5C). In a monomorphic functional unit, MTSEA addition would result in a 50% loss of transport activity, whereas a total loss of function would be expected for a dimeric functional unit. After incubation with MTSEA, the wild-type and the C321S concatamers were completely inhibited or unaffected, respectively. In contrast, the activity of the combined concatamers after this treatment was close to 50% (Fig. 5C), suggesting that the transport unit of system b0.5+ is the heterodimer b0.5+AT-rBAT.

xCT is completely inactivated by the sulphydryl reagent pCMB, whereas the fully functional C327S xCT mutant is unaffected (22). We constructed dimeric concatamers between the wild-type and the C327S xCT in both orientations. Blue Native gels showed that these concatamers had the expected size, and no single xCT subunits were observed (see Fig. 2A). Western blots confirmed this finding (data not shown). The concatamers cRNAs were injected together with 4F2hc in Xenopus oocytes. The activity of the concatamers was 20–30% compared with the wild-type or C327S xCT (supplemental Fig. A2). After exposure to pCMB, the wild-type and the C327S concatamers were completely inhibited or unaffected, respectively. In contrast, the combined concatamers showed an inhibition very close to the theoretical 50% (Fig. 6). In addition, when a 1:1 ratio of wild-type and C327S xCT cRNAs was injected together with 4F2hc, transport was inhibited by 47.9 ± 2% after treatment with pCMB (supplemental Fig. B). Therefore, systems b0.5+ and xC− have a common functional unit, the single heterodimer.

rBAT Promotes Oligomerization—To uncover determinants of b0.5+AT-rBAT oligomerization, we compared the mobility of b0.5+AT/4F2hc, His-xCT/rBAT, b0.5+AT/rBAT, and His-xCT/4F2hc concatamers on Blue Native gels (Fig. 7). The chimaeras showed 28 ± 5% (b0.5+AT/4F2hc) and 36 ± 3% (His-xCT/4F2hc)
rBAT) activity compared with their controls (supplemental Fig. C). b0, +AT/rBAT forms an oligomer at the plasma membrane, with a minimal heterotetrameric structure. We used initially Blue Native PAGE because it seems to maintain the oligomeric structures of several membrane protein complexes solubilized in non-ionic detergents such as dodecylmaltoside and digitonin (28–30, 38, 44). On Blue Native gels the b0, +AT/rBAT complex was detected as a single 535-kDa band both in kidney membranes and transfected HeLa cells. More important, the complex was partially dissociated by SDS only to a smaller band of about half the size, strongly suggesting a heterotetrameric structure. Strikingly, urea did not dissociate the complex, indicating a very stable interaction between the two heterodimers. A similar property was reported for His-xCT/rBAT (44). Moreover, DTT in the absence of SDS did not dissociate the complex, indicating a very stable interaction between the two heterodimers. A similar property was reported for the mitochondrial 400-kDa general import pore (GIP) complex (44). Moreover, DTT in the absence of SDS did not dissociate the complex despite complete reduction of the intersubunit disulfide bond (data not shown), suggesting that the stability of the heterotetramer is independent from the integrity of that link. The interaction between the heterodimers may be of high affinity, as only the 535-kDa species was found on Blue Native gels and because the cross-linker DMS quantitatively linked two (and only two) b0, +AT/rBAT heterodimers on non-solubilized total membranes. DMS cross-linking indicated that the heterotetramer was the main structure in vivo. Additional evidence for oligomerization of the b0, +AT/rBAT on the cell surface of live cells came from FRET analysis, which showed significant association of CFP- and YFP-b0, +AT/rBAT. FRET did not occur by random association because it was negligible after co-expression with an unrelated, but co-localized, plasma membrane protein, the EGFR. Taken together, the evidence is consistent with a high affinity and stable heterotetrameric b0, +AT/rBAT on the cell surface in vivo. It is worth mentioning that the size of rBAT associated with an unidentified subunit in brush border membranes (4, 37) was also consistent with a heterotetramer (Fig. 1A, rBAT panel, lane 2).

Because the functional unit of HAT is the single heterodimer (see below for the discussion), it is hard to propose a functional role for the heterotetramer in system b0, + activity. An alternative possibility is that oligomerization provides a late quality control step in the biogenesis pathway of the transporter. Preliminary data obtained in our laboratory10 is consistent with this hypothesis, because the oligomerization of b0, +AT/rBAT

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seems to occur just before or concomitant with the exit from the endoplasmic reticulum.

In contrast to b0⁺-AT-rBAT, the results suggest that xCT-4F2hc and LAT2–4F2hc heterodimers do not efficiently oligomerize and/or that the formed oligomers are not as stable as the b0⁺-AT-rBAT heterotetramers. Actually, the faint bands of heterotetrameric xCT/4F2hc detected in Blue Native gels and the positive FRET value for xCT/4F2hc (not confirmed by the acceptor photobleaching FRET method) are the only data supporting oligomerization of xCT-4F2hc. Moreover, despite the similar distribution of lysine residues in b0⁺-AT and xCT, xCT-4F2hc was not cross-linked by DMS, and the positive FRET value was lower than for b0⁺-AT-rBAT. Anyway, caution must be taken concerning the oligomerization of LSHAT-4F2hc heterodimers. For instance, digitonin may dissociate oligomers of LSHAT-4F2hc. In addition, most tissues and cell lines express 4F2hc and one or more 4F2hc-associated LSHAT (1, 2), which may compete with the xCT-4F2hc FRET constructs and reduce the FRET signal. Other factors may decrease FRET signal, such as interactions of the xCT N termini with other proteins. As a working hypothesis we propose that 4F2hc-associated heterodimers do not stably oligomerize and/or that the heterodimer ↔ oligomer equilibrium is strongly shifted to the heterodimeric form.

The two b0⁺-AT light subunits were cross-linked by DMS, suggesting that they are in close contact within the b0⁺-AT-rBAT oligomer (Fig. 3). However, the heavy subunit rBAT appeared to be the major determinant of the oligomerization, as its presence was sufficient to confer a heterotetrameric structure to the xCT/4F2hc concatamer (Fig. 7). rBAT may facilitate or stabilize the interaction between the two light subunits either by exposing a dimerization surface on b0⁺-AT and xCT and/or by prior dimerization of rBAT itself. The appearance of the heterotetramer band on the b0⁺-AT/4F2hc concatamer (Fig. 7) suggests that b0⁺-AT may tend to oligomerize, and 4F2hc could prevent a high affinity interaction. Alternatively, 4F2hc-associated heterodimers may oligomerize depending on the context. In this regard it has been shown that 4F2hc interacts with integrins (19), and this interaction requires the clustering of 4F2hc (45).

Here we provide the first strong evidence indicating that the single heterodimer is the functional unit of HAT (where the light subunit is the transporter itself (21)). This is based on the distinct sensitivity to sulfhydryl reagents of the wild-type transporters and the Cys-to-Ser mutants, which otherwise are functionally very similar (Ref. 22, and see “Results”). We assume that if the activity of one subunit of an oligomeric functional unit is inhibited through chemical modification, the transport of the entire functional complex is blocked. Given this assumption, the experiments are compatible with a functional unit composed by a single heterodimer both for b0⁺-AT-rBAT and xCT-4F2hc. The ~50% loss of function observed (Figs. 5C and 6) is best explained by the presence of two functionally independent transport pathways per concatamer. The low expression level of the concatamers used for the functional studies precluded a more detailed kinetic transport analysis. Therefore, we cannot exclude, especially for b0⁺-AT-rBAT (which is a heterotetramer; see above), some degree of cooperativity between the two b0⁺-AT subunits. An alternative proposal is that the dimeric concatamers associate themselves into tetrameric structures (four light subunits) where the only functional dimers are constituted by the unlinked light subunits. If the association occurs at random, the reagents would inactivate 75% of the transport activity, which is excluded by the data. Moreover, DMS cross-linking of the b0⁺-AT/b0⁺-AT concatamers failed to show any cross-linked band (data not shown).

It has been shown that system b0⁺-AT from the chicken small intestine shows a sequential exchange mechanism compatible with the formation of a ternary complex (46). If this applies to the expressed b0⁺-AT-rBAT complex, then export and import pathways should co-exist simultaneously in the proposed functional unit (i.e. the heterodimer) and, therefore, in a single b0⁺-AT catalytic subunit. Interestingly, mitochondrial carriers have a similar transport mechanism, although each transport pathway resides in a single six-transmembrane domain subunit of the functional dimeric carrier (47, 48). Clearly, studies with purified and reconstituted HAT will be necessary to integrate the transport mechanism, the functional unit, and the structure of these transporters in a unique model.

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