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Postprint available at:
http://www.zora.uzh.ch

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http://www.zora.uzh.ch

Originally published at:
Gastroenterology 2009, 136(3):872-882.e3.
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Abstract

BACKGROUND & AIMS: Hartnup amino acid transporter B(0)AT1 (SLC6A19) is the major luminal sodium-dependent neutral amino acid transporter of small intestine and kidney proximal tubule. The expression of B(0)AT1 in kidney was recently shown to depend on its association with collectrin (Tmem27), a protein homologous to the membrane-anchoring domain of angiotensin-converting enzyme (ACE) 2. METHODS: Because collectrin is almost absent from small intestine, we tested the hypothesis that it is ACE2 that interacts with B(0)AT1 in enterocytes. Furthermore, because B(0)AT1 expression depends on an associated protein, we tested the hypothesis that Hartnup-causing B(0)AT1 mutations differentially impact on B(0)AT1 interaction with intestinal and kidney accessory proteins. RESULTS: Immunofluorescence, coimmunoprecipitation, and functional experiments using wild-type and ace2-null mice showed that expression of B(0)AT1 in small intestine critically depends on ACE2. Coexpressing new and previously identified Hartnup disorder-causing missense mutations of B(0)AT1 with either collectrin or ACE2 in Xenopus laevis oocytes showed that the high-frequency D173N and the newly identified P265L mutant B(0)AT1 transporters can still be activated by ACE2 but not collectrin coexpression. In contrast, the human A69T and R240Q B(0)AT1 mutants cannot be activated by either of the associated proteins, although they function as wild-type B(0)AT1 when expressed alone. CONCLUSIONS: We thus show that ACE2 is necessary for the expression of the Hartnup transporter in intestine and suggest that the differential functional association of mutant B(0)AT1 transporters with ACE2 and collectrin in intestine and kidney, respectively, participates in the phenotypic heterogeneity of human Hartnup disorder.
Tissue-specific amino acid transporter partners ACE2 and Collectrin differentially interact with Hartnup mutations

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Short title: Hartnup disorder and ACE2

No conflict of interest exists

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Abbreviation List

AB, antibody
ACE2, angiotensin-converting enzyme 2
ace2 /- , ace2 female null mouse
ace2 /y , ace2 male null mouse
ace2 +/- , ace2 female wild type mouse
ace2 +/y , ace2 male wild type mouse
AP substrate, alkaline phosphatase substrate
B0AT1, Broad range neutral amino acid transporter 1
BBMV, brush border membrane vesicles
coll /-y , collectrin male null mouse
coll +/y , collectrin male wild type mouse
C-term, Carboxyl-terminal
HRP substrate, horseradish peroxidase substrate
KO, knock-out mouse
MES, 2-(N-morpholino)-ethanesulfonic acid
MTSEA-biotin, 2-(aminoethyl)-methanethiosulfonate-biotin
NMDG, N-methyl-D-glucamine
N-term, Amino-Terminal
PCR, polymerase chain reaction
PVDF, Polyvinylidenfluorid
SARS, Severe acute respiratory syndrome
SARS-CoV, SARS coronavirus
SDS, sodium dodecyl sulfate
SLC6A19, solute carrier family 6, member 19
SNP, Single nucleotide polymorphism
TEVC, two electrode voltage clamp
V_h, holding voltage
WT, Wild type
Abstract

Background and aims: Hartnup amino acid transporter B⁰AT1 (SLC6A19) is the major luminal sodium-dependent neutral amino acid transporter of small intestine and kidney proximal tubule. The expression of B⁰AT1 in kidney was recently shown to depend on its association with Collectrin (Tmem27), a protein homologous to the membrane anchoring domain of angiotensin converting enzyme 2 (ACE2). Since Collectrin is almost absent from small intestine, we tested the hypothesis that it is ACE2 that interacts with B⁰AT1 in enterocytes. Furthermore, since B⁰AT1 expression depends on an associated protein, we tested the hypothesis that Hartnup-causing B⁰AT1 mutations differentially impact on B⁰AT1 interaction with intestinal and kidney accessory proteins. Results: Immunofluorescence, co-immunoprecipitation, and functional experiments using wild type and ace2 null mice demonstrate that expression of B⁰AT1 in small intestine critically depends on ACE2. Co-expressing new and previously identified Hartnup disorder-causing missense mutations of B⁰AT1 with either Collectrin or ACE2 in Xenopus laevis oocytes shows that the high frequency D173N and the newly identified P265L mutant B⁰AT1 transporters can still be activated by ACE2 but not Collectrin coexpression. In contrast, the human A69T and R240Q B⁰AT1 mutants cannot be activated by either of the associated proteins although they function as wild type B⁰AT1 when expressed alone. Conclusion: We thus demonstrate that ACE2 is necessary for the expression of the Hartnup transporter in intestine and suggest that the differential functional association of mutant B⁰AT1 transporters with ACE2 and Collectrin in intestine and kidney, respectively, participates to the phenotypic heterogeneity of human Hartnup disorder.

Key words: Epithelial transport, enterocyte, amino acid transport, renin-angiotensin system, hereditary aminoaciduria, intestinal absorption
Introduction

Hartnup is an autosomal recessive disorder caused by mutations in the SLC6A19 gene that encodes the main epithelial neutral amino acid transporter B₀AT₁². This disorder is characterized by neutral aminoaciduria due to impaired amino acid transport in kidney proximal tubule epithelial cells whereas the extent of intestinal transport impairment appears to be less consistent. Various other clinical symptoms such as pellagra-like rash, cerebellar ataxia or other neurological dysfunctions may be present in affected individuals, while other subjects remain, besides aminoaciduria, asymptomatic. Phenotypic variability might be partially explained by the differential impact of various mutations and the frequent compound heterozygosity.

Differential phenotypic effects of mutations could arise from differential interactions of B₀AT₁ with tissue-specific modulatory and/or associated proteins. We and others have previously shown that B₀AT₁ requires association with Collectrin for luminal surface expression in kidney proximal tubule, whereas Collectrin is nearly absent in small intestine, the other major site of B₀AT₁ expression³,⁴. Intriguingly, the closest homolog of Collectrin is the angiotensin-converting enzyme 2 (ACE2), which functions as a key carboxypeptidase enzyme in the renin angiotensin system. ACE2 is involved in heart and kidney pathologies⁵ and has been identified as the SARS receptor in vitro⁶ and in vivo⁷. Importantly, previous studies have reported ACE2 expression and SARS-CoV replication in human small intestine⁸,⁹.

We first investigated whether ACE2 associates with B₀AT₁ in mouse small intestine. Immunofluorescence, co-immunoprecipitation and functional data presented here clearly demonstrate that ACE2 is the specific partner of B₀AT₁ in small intestine and thus that the accessory protein of B₀AT₁ is tissue specific.

We then tested the influence of Collectrin and ACE2 on the function of human B₀AT₁ Hartnup-causing mutations in the *Xenopus laevis* oocyte expression system. Our results
suggest that specific defects in interaction with tissue specific accessory proteins may lead to
differential defects in intestinal versus kidney (re)absorption of neutral amino acids and
thereby participate to the variability of phenotypes observed in Hartnup subjects.

Methods

Animals

The \textit{ace2} and \textit{collectrin} wild-type (WT) and knock-out (KO) mice were housed in standard
conditions and fed a standard diet. Generation of the KO mice was described elsewhere \cite{3, 5}.
Animals were either anaesthetised and perfused with a fixative solution for localization
studies or euthanized to remove small intestine and kidneys. Scraped small intestine mucosa
cells and total kidneys were frozen in liquid nitrogen for subsequent RNA extraction or brush
border membrane vesicles (BBMV) preparation. All procedures for mice handling were
according to the Swiss Animal Welfare laws and approved by the Kantonales Veterinäramt
Zürich.

Organ fixation

Male mice were anesthetized with ketamine and xylazine (90 mg/kg body weight, Narketan
10, Vétoquino, Lure, France) and Xylazine (10 mg/kg body weight, Xylazin, Streuli, Uznach,
Switzerland) intraperitoneally and perfused through the left cardiac ventricle with phosphate-
buffered saline (PBS, phosphate buffer, pH 7.4) followed by a buffered paraformaldehyde
solution (4\%, pH 7), as previously described \cite{10}. Small intestine was then harvested, incubated
overnight in paraformaldehyde solution, washed several times with PBS and stored in PBS-
0.02\% sodium azide at 4\°C. Tissues were then mounted with Kryostat OCT (Medite,
Nunningen, Switzerland), frozen in liquid propane and stored at – 80\°C.

Immunofluorescence

Immunofluorescence was performed as previously described \cite{11}. Primary antibodies were
diluted (1:200) for rabbit affinity purified anti-mouse B\textsuperscript{0}AT1 \cite{11}, and (1:100) for affinity
purified goat anti-mouse ACE2 (R&D Systems, Minneapolis, USA). Secondary antibodies were diluted (1:500) for Alexa Fluor 488 donkey anti-goat IgG and Alexa Fluor 594 donkey anti-rabbit IgG (Molecular Probes, Invitrogen, Carlsbad, USA). Digital images were viewed by using a Nikon Eclipse TE300 epifluorescence microscope (Nikon Instruments Inc., Melville, USA) equipped with a DS-5M Standard CCD camera (Nikon) and acquired with NIS-Elements (Nikon).

**Brush Border Membrane preparations**

Brush Border Membrane Vesicles (BBMV) were prepared from small intestine mucosa cells and kidneys using the Mg²⁺ precipitation technique as described elsewhere.

**Western blotting**

Western Blotting was performed as previously described. Primary antibodies were diluted to: 1:1000 goat affinity purified anti-mouse ACE2 (R&D), 1:1000 goat affinity purified anti-human ACE2 (R&D), 1:2000 or 1:1000 for rabbit affinity purified anti-mouse or anti-human B₀AT1, respectively (Pineda, Berlin, Germany) and 1:10000 for mouse anti-mouse β-actin (Sigma, St Louis, USA). Secondary antibodies were diluted (1:5000) for ECL™ anti-rabbit or anti-goat IgG Horseradish Peroxidase linked fragment from donkey or mouse, respectively (Amersham Biosciences, Piscataway, USA and Pierce, Rockford, USA.) or anti-mouse IgG Alkaline Phosphatase Conjugate from mouse (Promega, Madison, USA). Antibody binding was detected with Immobilon Western Chemiluminescent HRP or AP substrate (Millipore, Billerica, USA) and chemiluminescence visualized with a DIANA III camera (Raytest, Dietikon, Switzerland).

**Intestinal Ring Uptake**

Uptake of radiolabelled L-isoleucine was performed as previously described on ileum segments, with slight modifications. Briefly, everted ileum segments were incubated in bubbling (Oxycarbon) Krebs-Tris Buffer (pH 7.4) containing 1mM L-isoleucine (1 μCi ¹⁴C-L-Ile/ml) for 5 min at 37°C. Ileum segments were dried at 55°C O/N on cellulose (Sartorius
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AG, Goettingen, Germany) and weighed. Segments were then lysed in 0.75 N NaOH for 6 h, neutralised with 10N HCl and the radioactivity was determined by liquid scintillation. Na\textsuperscript{+} was replaced by N-methyl-D-glucamine (NMDG) in the condition without Na\textsuperscript{+}. Amino acid transport was expressed relative to dry tissue weight.

**Identification of new Hartnup mutations**

After informed consent genomic DNA was obtained from whole blood of Hartnup patients as previously published\textsuperscript{1}. Intronic primers to sequence exons and splice sites of each exon were chosen. Primer sequences are available on request. PCR products of each exon were separated by electrophoresis on agarose gels and specific bands removed for DNA isolation. This DNA was sequenced in both directions using a Beckman Coulter CEQ8000 (Beckman Coulter, Fullerton, USA) following the manufacturer's protocol. The absence of each recognized missense mutation was confirmed in 100 ethnically matched control alleles.

**Isolation and subcloning of cDNAs and site-directed mutagenesis**

Human (h) B\textsuperscript{0}AT1, ACE2 and Collectrin cDNAs were amplified from human kidney Marathon-Ready cDNA (Clontech, Mountain View, CA, USA) by PCR using a proofreading polymerase (Pfu, Promega, Madison, USA). The primers used are available upon request. The amplified ACE2 and Collectrin cDNAs were subcloned in pcDNA3; mouse (m) ACE2 and Collectrin in pcDNA 3.1 hygromycin (Invitrogen, Carlsbad, USA). The B\textsuperscript{0}AT1 was subcloned into a pBlueScript modified *Xenopus laevis* expression vector containing both the 5’ and 3’ ends of the β-globin gene (KSM) kindly provided by Dr. Leila Virkki. For expression in *Xenopus laevis* oocytes the plasmids were linearized and used as template for RNA synthesis (mMESSAGEmMACHINE\textsuperscript{TM}, Ambion, Austin, USA). The mutation in human B\textsuperscript{0}AT1 and ACE2 was performed using quick changes site-directed mutagenesis according to the manufacturer (Stratagene, La Jolla, USA). The mutation ACE2-R273Q was shown previously to be catalytically inactive but normally expressed\textsuperscript{14}. The B\textsuperscript{0}AT1 mutations were done on the SNP V252I, which has been shown to behave like the wild type.
**Homology model**

The homology model of human B⁰AT1 transport was based on the crystal structure of LeuTα (NP_214423) as a template using the I-TASSER server. The pdb file was visualized using Pymol (DeLano Scientific LLC, Palo Alto, USA).

**Transport studies in Xenopus laevis oocytes.**

Expression studies and influx assays using radiolabeled amino acid tracer were performed in *X. laevis* oocytes after 5 to 9 days expression, as described previously. Data is expressed in pmol/h/oocyte and values obtained for non-injected oocytes are subtracted.

**Electrophysiology using two-microelectrode voltage clamp**

The two-microelectrode voltage clamp (TEVC) technique was used for the recording of whole-cell currents from *X. laevis* oocytes. Recordings were performed at room temperature 5-9 days after injection with cRNA. Recordings were carried out as previously described at a membrane holding potential (V<sub>h</sub>) of ~50 mV. To control for batch variation in transporter expression and measured current, the data from the selectivity experiment were normalized to I<sub>L-Ile 10mM</sub>. Pooled data are shown as mean ± SEM where n represents the number of pooled cells. Experimental protocols were repeated at least twice. Nonlinear regression calculations were performed using GraphPad Prism™ Version 4.0 (GraphPad Inc., San Diego, USA).

**Labelling of surface proteins in Xenopus oocytes**

Surface labelling of oocytes expressing human B⁰AT1 alone or co-expressed with mouse ACE2 or human Collectrin was performed using MTSEA-Biotin (Sigma-Aldrich, Switzerland) as previously described. Samples were separated on a 10% SDS gel and immunoblotted with affinity purified rabbit anti-human B⁰AT1 antibody (Pineda). Signal intensity was quantified with the AIDA Image Analyzer (Raytest).

**Immunoprecipitations**
Mouse brush border membrane proteins: BBMVs were incubated with serum anti-B\textsuperscript{0}AT1 polyclonal antibody\textsuperscript{11} in EBC solution (20 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP40) at 4°C on a rotator. The immunocomplexes were coupled to Immobilized Protein A/G beads (Pierce) O/N at 4°C on a rotator. The beads were washed with NET-N solution (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% NP40, 1 mM EDTA), and the immunoprecipitate loaded on a polyacrylamide gel. Western Blot was performed as described above with anti-mouse B\textsuperscript{0}AT1 antibody to check for B\textsuperscript{0}AT1 immunoprecipitation or anti-mouse ACE2 antibody to test ACE2 co-immunoprecipitation. To avoid background staining, Protein A/G coupled to Alkaline Phosphatase (AP) was used as a secondary antibody (Pierce).

*Xenopus laevis* oocytes: Immunoprecipitation of human B\textsuperscript{0}AT1, B\textsuperscript{0}AT1 mutants and human ACE2 was performed as previously described\textsuperscript{18}. Briefly oocytes were lysed in EBC buffer (as described for BBMV) and the supernatant was first incubated with serum anti-human B\textsuperscript{0}AT1 and subsequently with Immobilized Protein A/G beads (Pierce) O/N at 4°C on a rotator. Western Blot was performed as described above to test for human ACE2 co-immunoprecipitation.

**Statistics**

Data are presented as means ± standard error of the mean (SEM). Analyses were done by running the GraphPad Prism 4.0 software (GraphPad). Between-group comparisons were performed by Student's unpaired \textit{t} test. Multiple comparisons within groups were performed by repeated-measures oneway ANOVA, followed by Tukey post test. Statistical significance was accepted at \(P < 0.05\).

**Results**

ACE2 is the intestinal partner of the amino acid transporter B\textsuperscript{0}AT1
We first assessed the potential *in vivo* role of ACE2 as intestinal B\(^0\)AT1 associated protein by investigating the B\(^0\)AT1 expression in *ace* 2 null mice \(^5\). Remarkably, B\(^0\)AT1 protein was completely absent in small intestine brush border membranes of mice lacking ACE2 (Figure 1A, B) whereas it was normally expressed in kidney (Figure 1B). This organ distribution of B\(^0\)AT1 in *ace* 2 null mice mirrors the situation observed in *collectrin* null mice, where B\(^0\)AT1 is absent in kidney and, as shown in this study, normally expressed in small intestine (Figure 1B). Furthermore, ACE2 was co-immunoprecipitated with B\(^0\)AT1 from intestinal brush border membranes of wild type mice, demonstrating *in vivo* interaction (Figure 1C).

The function of B\(^0\)AT1 was also shown to depend on ACE2 in mouse intestine. We observed that the lack of B\(^0\)AT1 protein expression in the intestine of *ace* 2\(^{−/−}\) mice abolished the Na\(^+\)-dependant portion of L-isoleucine transport measured in intestinal rings (Figure 1D). The total (in the presence of Na\(^+\)) but not the sodium-independent transport was dramatically reduced when compared to the wild type littermates. The equivalent effect was previously shown in kidney brush border membrane vesicles of *collectrin* \(^{−/−}\) mice, where the transport was also reduced \(^3\). These data show that ACE2 is essential for expression and function of B\(^0\)AT1 in intestine whereas Collectrin controls B\(^0\)AT1 expression in the kidney.

**ACE2 and Collectrin increase the function of human Hartnup transporter B\(^0\)AT1 *in vitro***

The tissue-specific associated proteins Collectrin and ACE2 expressed in *Xenopus laevis* oocytes mimicked their effect *in vivo* on B\(^0\)AT1 by stimulating its transport function. Expressed alone in oocytes human B\(^0\)AT1 induced a low amino acid transport rate as shown by us and others previously \(^1,2\). We show here that its co-expression with mouse or human ACE2, or human Collectrin increases the transport function ~10-fold (Supplementary Figure 1A; see supplementary material online at www.gastrojournal.org ). The mouse ortholog of Collectrin did not activate human B\(^0\)AT1, although it has been shown to be effective on
mouse B₀AT₁. We have as yet no explanation for this differential impact of the collectrin orthologs in the X. laevis expression system. Different from Collectrin, ACE2 has one catalytic site. It is a carboxypeptidase, and converts Angiotensin (Ang) I and II to Ang (1-9) and (1-7), respectively, functionally antagonizing its homolog ACE. We analyze the role of the catalytic activity of ACE2 in the functional interaction with B₀AT₁ (Supplementary Figure 1B; see supplementary material online at www.gastrojournal.org). The catalytically dead mutant (ACE2 R273Q)¹⁴ was as efficient as the wild type in enhancing the B₀AT₁ function suggesting that it is not necessary for the interaction. This was expected since human Collectrin shares 48% identity with ACE2 at the level of its carboxyl-terminal membrane anchor region but entirely lacks the extracellular peptidase domain.²¹

The amino acid selectivity, ion dependence and kinetic characteristics of human B₀AT₁ co-expressed with ACE2 are, besides a much higher maximal transport rate, very similar to those previously published for human B₀AT₁ expressed alone.¹ (Supplementary Figure 2A-E; see supplementary material online at www.gastrojournal.org).

**Differential interaction of accessory proteins with B₀AT₁ Hartnup mutations**

To address the question whether Hartnup mutations impact on the interaction of B₀AT₁ with its accessory proteins ACE2 and/or Collectrin, we expressed missense mutations alone or together with either accessory protein in X. laevis oocytes and analysed their transport function and surface expression. Beside five previously described missense mutations and one non-synonymous single nucleotide polymorphism (SNP), we characterized four new missense mutations identified in patients with neutral aminoaciduria characteristic of Hartnup disorder belonging to 4 different families (Table 1), (Supplementary Figure 3A-D; see supplementary material online at www.gastrojournal.org).¹ ². The non-synonymous SNP V252I essentially behaved as wild-type B₀AT₁ when co-expressed with Collectrin and ACE2 (Figure 2A upper and middle panels). Interestingly, the observed two to three-fold increase in B₀AT₁ surface expression suggested that it is not necessary for the interaction.
expression measured by surface biotinylation only partially explains the almost ten-fold increase in surface transport function induced by co-expression with Collectrin or ACE2 (Figure 2A lower panel and representative Western blot).

Surprisingly, Collectrin and ACE2 co-expression differentially impacted on the surface expression and function of the two B0AT1 mutants D173N and P265L (Figure 2B). In both cases, co-expression with ACE2 increased the transport rate whereas co-expression with Collectrin either had the opposite effect or no effect (Figure 2B upper panel). The transport function of these mutants was shown to be Na+-dependent as for wild-type B0AT1 (Figure 3 upper and middle panels). Surface biotinylation experiments suggested that the differential function of these B0AT1 mutants with the two associated proteins is due, in the case of D173N, to a difference in surface expression, whereas the surface expression appeared in the case of P265L to be similar with both associated protein (Figure 2B lower panel and representative Western blot). Finally, the mutants D173N and P265L co-precipitated with human ACE2, confirming that they interact with ACE2 (Figure 2D).

We next analyzed the two B0AT1 mutations A69T and R240Q that when expressed alone display the same L-isoleucine transport function and ion dependence as wild type B0AT1, whereas their transport function was not activated in the presence of either associated protein (Figure 2C upper and middle panels and Figure 3 lower panel). In the case of the newly described A69T, this was particularly surprising, since its surface expression was increased two to three-fold by co-expression with both accessory proteins (Figure 2C lower panel and representative Western blot) and its interaction with ACE2 confirmed by co-immunoprecipitation (Figure 2D). In contrast, the surface expression of R240Q did not appear to be increased by the presence of the accessory proteins (Figure 2C lower panel and representative Western blot), although the mutant transporter was co-immunoprecipitated with human ACE2 to some extent (Figure 2D).
The mutations R57C, L242P, and E501K have previously been tested by expression in *X. laevis* oocytes, and showed no transport capacity\(^1,2\). Co-expression of these mutants with Collectrin and ACE2 did not change their impaired function and did not affect their cell surface expression (Figure 4). Similarly, the newly described mutations G93R and P579L lack function also in the presence of associated proteins. However, the surface expression of G93R was selectively increased by ACE2 (Figure 4 lower panel and representative Western blot).

**Discussion**

Recently we have reported that Collectrin (Tmem27) associates with and controls the expression of B\(^0\)AT1 in kidney proximal tubule\(^3,4\). In this study, we demonstrated a novel and unexpected function of the important renin-angiotensin system enzyme ACE2 that associates with the luminal amino acid transporter B\(^0\)AT1 in small intestine and thereby controls its surface expression and function. B\(^0\)AT1 associates in a tissue specific manner with ACE2 in small intestine and with Collectrin in the kidney. Thus, *ace2* and *collectrin* null mice can be used to study the role of the neutral amino acid transporter B\(^0\)AT1 in kidney and intestine independently.

The same effect was observed on human B\(^0\)AT1 ortholog expressed in the heterologous expression systems, *Xenopus laevis* oocytes. The co-expression of the two accessory proteins, ACE2 or Collectrin, increased the transport rate and the cell surface expression of the transporter. The analysis of the function and surface expression of B\(^0\)AT1-Hartnup causing mutations expressed alone or together with the two partner proteins allowed us to discriminate classes of mutants that might differentially impact on the phenotype of the patients.

The Hartnup-causing mutations in B\(^0\)AT1 are localized throughout the protein, as shown on the homology model depicted in Figure 5. This is also the case for the largest class of mutations mentioned here, the “dead” mutants, three of which were published earlier (R57C, L242P, E501K\(^1,2\)) and two of which are newly described here (G93R and P579L).
(Supplementary Figure 3 C and D; see supplementary material online at www.gastrojournal.org). These mutants do not display any measurable function which is consistent with the fact that, with one exception, they do not reach the plasma membrane, neither when expressed alone, nor upon co-expression with associated proteins.

The second class of mutants corresponds to those which expressed alone function as wild type B^0^AT1 but are not stimulated by either associated protein. In the case R240Q, its surface expression is not increased upon co-expression with ACE2 or Collectrin, but co-immunoprecipitation demonstrates that its interaction with ACE2 is not abolished. Assuming a qualitative effect of the R240Q mutation on this interaction, our observation is nonetheless potentially compatible with the hypothesis recently put forward by the group of Broer, namely that the localization of this mutation at the surface of B^0^AT1 might impact on the interaction with accessory proteins. Further studies are necessary to clarify the mechanism by which this mutation, initially classified as a SNP, prevents the normal activation of B^0^AT1 by the accessory proteins. In contrast, in the case of the other functional mutant of this class A69T, an increase in cell surface expression of this mutant B^0^AT1 was demonstrated upon interaction with the associated proteins (Figure 2C and D). One possible explanation of why the increase in surface-expression did not lead to an increase in transport rate is that ACE2/A69T-B^0^AT1 heterodimers reaching the cell surface remain inactive, meaning that the associated protein inhibits the function of A69T-B^0^AT1. The fact that the residue mutated in A69T is part of a highly conserved motif (NGGGAF) shown to undergo conformational changes during the transport cycle is compatible with this hypothesis. Additionally, that the co-expression of ACE2 or Collectrin apparently activates the transport rate of wild type B^0^AT1 several-fold more than it increases its surface expression (~10-fold versus ~2.5-fold) also suggests that associated proteins may impact on transporter function, though normally by increasing the cycling rate.
The most intriguing class of mutants in regards to its potential phenotypic impact is represented by D173N and the newly described P265L. These mutants differentially interact with the two tissue-specific accessory proteins such that their function is stimulated only by the intestine-specific associated protein ACE2. Interestingly, the D173N allele is relatively frequent in unrelated Hartnup pedigrees and can also be observed in healthy Caucasians with a high heterozygote frequency (1:122 healthy individuals) \(^2\), \(^{24}\). We know of no potential selective advantage for heterozygous carriers that explains the persistence and geographic spread of this mutant allele \(^{25},^{26}\). The selective functional interaction of D173N with the intestinal accessory protein ACE2 might prevent deleterious effects due to a lack of amino acid absorption and thereby allowing the “survival” of this frequent allele. Although as yet no evidence of genotype-phenotype relationship performed in patients were reported, studies performed long before the identification of the Hartnup transporter have shown that intestinal amino acid absorption differs between Hartnup subjects \(^{27},^{28}\).

Taken together, our data demonstrate that the expression and function of the main epithelial amino acid transporter is modulated by tissue-specific accessory proteins. Moreover, the intriguing fact that these accessory proteins can interact differentially with Hartnup mutations and thereby differentially affect kidney and intestinal amino acid transport function suggests that Collectrin and ACE2 may also contribute significantly to the phenotypic heterogeneity among individuals with Hartnup disorder.
Table 1: Missense mutations in the SLC6A19/human B<sup>0</sup>AT1 gene described in subjects with Hartnup disorder.

| Protein | wt mutation | Position from the atg | Position in the protein |
|---------|-------------|-----------------------|-------------------------|
| R57C<sup>1</sup> | Cgc | Tgc | 169 | 1st TMS |
| *A69T | Gcc | Acc | 205 | 2nd TMS |
| *G93R | Ggg | Agg | 277 | 2nd TMS |
| D173N<sup>2</sup> | Gac | Aac | 517 | 2nd EL |
| R240Q<sup>2</sup> | cGa | cAa | 719 | 3th EL |
| L242P<sup>2</sup> | cTg | cCg | 725 | 3th EL |
| *P265L | cCg | cTg | 794 | 6th TMS |
| E501K<sup>2</sup> | Gag | Aag | 1501 | 10th TMS |
| *P579L | Ccg | Tcg | 1735 | 6th EL |

New mutations (*) identified in this study. The previously published mutations by Kleta et al. <sup>1</sup> and Seow et al. <sup>2</sup> are also listed. R240Q was initially described as SNP by Seow et al.<sup>2</sup>. TMS = transmembrane segment, EL = extracellular loop.
Figure 1. ACE2 and B0AT1 co-localization and functional interaction.

(A) Immunofluorescence analysis shows that B0AT1 protein expression is lost in small intestine of ace2 null mice. Small intestine sections from ace2+/y and ace2-y/- were co-labelled with antibodies against ACE2 (green) and B0AT1 (red), and additionally viewed by phase contrast (PC). (B) Western blot analysis shows that B0AT1 is absent in small intestine but present in kidney brush border membranes of ace2 null mice, whereas B0AT1 is expressed in small intestine and absent from kidney brush border membranes in coll null mice. The loading of brush border membranes (2.5-20 μg) was tested using a β-actin antibody (βA). Note that male ace2+/y or ace2-y/- and female ace2+/+ or ace2-y/- mice were used with similar results. (C) Co-immunoprecipitation shows interaction of ACE2 with B0AT1 in mouse small intestine. Complexes were immunoprecipitated (IP) from small intestine brush border membrane vesicles (BBMVs) using anti-B0AT1 antibody (AB) and analysed by Western Blot (WB) using anti-mouse ACE2 antibody. (D) Na+-dependant uptake of L-isoleucine (L-Ile) is abolished in ileum segments from ace2-y/- mice. The transport was measured in the presence (empty bar) and in the absence (solid bar) of sodium. Data points represent means values of 4 intestinal ring uptakes from independent experiments ± SEM, ***p<0.001, ns = not significant.

Figure 2. Differential function of Hartnup mutations co-expressed with Collectrin or ACE2 in X. laevis oocytes. Wild type and mutant human B0AT1 are shown in this figure as three groups with different functional characteristics and labelled A-C. (A) The transport function and the surface expression of both wild type and non synonymous SNP V252I of B0AT1 are increased by co-expression with human Collectrin or mouse ACE2. (B) The relatively low transport activity of B0AT1 D173N and P265L mutants expressed alone is increased by co-expression with ACE2 but not with Collectrin. The surface expression of the D173N mutant is also significantly increased by co-expression with ACE2. (C) The wild type-like function of the A69T or R240Q mutants expressed alone is not increased in the
presence of Collectrin or ACE2 although; in the case of A69T its surface expression is increased. The upper panels of A-C show the transport function measured as radiolabelled L-isoleucine (L-Ile) uptake (means ± SEM of 13-31 oocytes from 3 independent experiments) and the middle panel the transport function as current measured by TEVC (9-11 oocytes from 3 batches). *P<0.05, ***P<0.001. The lower panels show the surface expression assayed by Western blot using surface-biotinylated proteins and a representative Western blot. The data correspond to the means ± SEM (n = 3-5 independent experiments) of the results normalized to the value obtained for B⁰AT1 expressed alone. For the transport experiments the significance was evaluated using ANOVA with Tuckey post-test and for the surface biotinylation the comparison of means was done by unpaired t test. (D) B⁰AT1 and the mutants D173N, P265L, R240Q and A69T interact with ACE2 in X. laevis oocytes. The transporters expressed alone or together with human ACE2 were immunoprecipitated (IP) from oocyte lysates using anti-human B⁰AT1 antibody and complexes were analysed by Western Blot (WB) using anti-human ACE2 antibody. NI = non-injected.

Figure 3. The ion dependency of D173N, P265L, A69T and R240Q co-expressed with ACE2 or Collectrin is not altered: Transport is Na⁺- but not Cl⁻-dependent. Wild type human B⁰AT1 (WT), and the mutants D173N, P265L, A69T and R240Q were expressed alone or with human Collectrin or mouse ACE2. The ion dependence was tested by substituting Na⁺ with NMDG (n-methyl-D-glucamine) and substituting Cl⁻ with gluconate. Function was assayed by influx of radiolabeled L-isoleucine (L-Ile). For each mutation, three independent experiments (15-24 oocytes) were pooled. Comparison of means was done by analysis of the variances (ANOVA) with Tuckey as post test. Data represent means ± SEM. * p<0.05, **p<0.01, ***p<0.001. ns, not significant.
**Figure 4.** The B⁰AT1 mutants R57C, G93R, L242P, E501K and P579L exhibit no transport activity when expressed alone or co-expressed with Collectrin or ACE2. Function was assayed by influx of radiolabeled L-isoleucine (L-Ile) (upper panels) or TEVC (middle panels). For each mutant three independent experiments (13-31 oocytes for influx and 9-11 for TEVC) were pooled. Comparison of means was done by analysis of the variances (ANOVA) with Tuckey as post test. Data represent means ± SEM. ns, not significant. Cell surface expression of labelled proteins (lower panels) was analysed by Western blot. The bands were quantified and data normalized to the values obtained from oocytes expressing B⁰AT1 proteins alone. For each mutation three to four independent experiments were pooled. Comparison of means was done by unpaired t test. Data represent means ± SEM. * p<0.05. ns, not significant.

**Figure 5.** Missense mutations in the SLC6A19 gene causing Hartnup disorder. The model of B⁰AT1 is based on the Slc6 bacterial homologue from *Aquifex aeolicus* LeuTₐₐ and the transmembrane segments are numbered. The localization of mutations causing a loss of surface expression (R57, G93, L242, E501, P579) are depicted in the left panel. The sites of mutations leading to a differential interaction with the two accessory proteins (D173 and P265) are depicted as black spheres on the middle panel, and the sites of the mutations preventing both accessory proteins of stimulating the transport function are depicted on the right panel (A69 and R240).

**Appendix**

**Supplementary data**

Supplementary data associated with this article can be found in the online version.
Acknowledgements

This project was supported by Swiss NF grant 31-108021/1 and the EUGINDAT (The European FP6 Project) to FV, and by a fellowship from the University Research Priority Program „Integrative Human Physiology“ at the University of Zurich. JMP is supported by grants from the Institute of Molecular Biotechnology of the Austrian Academy of Sciences, the Austrian Ministry of Sciences, and the EU network grant EuGeneHeart.

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Figure 1

A

ACE2  B°AT1  Merge  PC

ace2^{+/y}

ace2^{-/-}

B

ace2

Small Intestine

Kidney

WB: ACE2

WB: B°AT1

C

IP: B°AT1

AB beads

BBMV

WB: ACE2

D

L-lle transport (nmol/5min/mg tissue)

ace2^{+/y}

ace2^{-/-}
Figure 2

A

WT  V252I

L-ile transport (pmol/hour/ooocyte)

B

D173N  P265L

/ I-ile (nA)

C

A69T  R240Q

Surface expression (normalized)

D

IP: B^3AT1

ACE2

WB: ACE2
Figure 3

WT

|      | alone | Coll | ACE2 |
|------|-------|------|------|
| NaCl |       |      |      |
| NMDG-Cl |     |      |      |
| Na-Gluconate |   |      |      |

D173N

|      | alone | Coll | ACE2 |
|------|-------|------|------|
| NaCl |       |      |      |
| NMDG-Cl |     |      |      |
| Na-Gluconate |   |      |      |

P265L

|      | alone | Coll | ACE2 |
|------|-------|------|------|
| NaCl |       |      |      |
| NMDG-Cl |     |      |      |
| Na-Gluconate |   |      |      |

A69T

|      | alone | Coll | ACE2 |
|------|-------|------|------|
| NaCl |       |      |      |
| NMDG-Cl |     |      |      |
| Na-Gluconate |   |      |      |

R240Q

|      | alone | Coll | ACE2 |
|------|-------|------|------|
| NaCl |       |      |      |
| NMDG-Cl |     |      |      |
| Na-Gluconate |   |      |      |

Legend:
- **NaCl**
- **NMDG-Cl**
- **Na-Gluconate**
|                 | R57C | G93R | L242P | E501K | P579L |
|----------------|------|------|-------|-------|-------|
| **Surface expression (normalized)** | [ns] | [ns] | [ns]  | [ns]  | [ns]  |
|                 | [ns] | [ns] | [ns]  | [ns]  | [ns]  |
|                 | [ns] | [ns] | [ns]  | [ns]  | [ns]  |
| **I = I_m (nA)** | 0    | 0    | 0     | 0     | 0     |
|                 | 0    | 0    | 0     | 0     | 0     |
|                 | 0    | 0    | 0     | 0     | 0     |
| **I_e (pmol/hour/ocycle)** | 0    | 0    | 0     | 0     | 0     |
|                 | 0    | 0    | 0     | 0     | 0     |
|                 | 0    | 0    | 0     | 0     | 0     |

Legend:
- alone
- +Coll
- +ACE2

Figure 4
