

Themed Section: Transporters

REVIEW

The SLC36 family of proton-coupled amino acid transporters and their potential role in drug transport

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Members of the solute carrier (SLC) 36 family are involved in transmembrane movement of amino acids and derivatives. SLC36 consists of four members. SLC36A1 and SLC36A2 both function as H⁺-coupled amino acid symporters. SLC36A1 is expressed at the luminal surface of the small intestine but is also commonly found in lysosomes in many cell types (including neurones), suggesting that it is a multipurpose carrier with distinct roles in different cells including absorption in the small intestine and as an efflux pathway following intralysosomal protein breakdown. SLC36A1 has a relatively low affinity (K_m 1–10 mM) for its substrates, which include zwitterionic amino and imino acids, heterocyclic amino acids and amino acid-based drugs and derivatives used experimentally and/or clinically to treat epilepsy, schizophrenia, bacterial infections, hyperglycaemia and cancer. SLC36A2 is expressed at the apical surface of the human renal proximal tubule where it functions in the reabsorption of glycine, proline and hydroxyproline. SLC36A2 also transports amino acid derivatives but has a narrower substrate selectivity and higher affinity (K_m 0.1–0.7 mM) than SLC36A1. Mutations in SLC36A2 lead to hyperglycinuria and iminoglycinuria. SLC36A3 is expressed only in testes and is an orphan transporter with no known function. SLC36A4 is widely distributed at the mRNA level and is a high-affinity (K_m 2–3 μ M) transporter for proline and tryptophan. We have much to learn about this family of transporters, but from current knowledge, it seems likely that their function will influence the pharmacokinetic profiles of amino acid-based drugs by mediating transport in both the small intestine and kidney.

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Abbreviations

AIB, α -aminoisobutyric acid or 2-aminoisobutyric acid; BBMV, brush-border membrane vesicles; BCECF, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; BDGP, Berkeley *Drosophila* Genome Project; β -ABA, β -aminobutyric acid; EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; HGNC, HUGO Gene Nomenclature Committee; HUGO, Human Genome Organisation; MeAIB, α -(methylamino)isobutyric acid or 2-(methylamino)isobutyric acid; OMIM, Online Mendelian Inheritance in Man; PACAP, pituitary adenylate cyclase-activating polypeptide; S1611, 3-[2-(3-guanidino-2-methyl-3-oxo-propenyl)-phenyl]-N-isopropylidene-2-methyl-acrylamide dihydro-chloride; SLC, solute carrier; TEVC, two-electrode voltage-clamp; THPO, 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridin-3-ol; VIP, vasoactive intestinal peptide; 8-br-cAMP, 8-bromoadenosine-3',5'-cyclic monophosphate

Introduction

To date, the Human Genome Organisation (HUGO) has categorized 378 membrane transporters into 51 solute carrier

(SLC) families (Hediger *et al.*, 2004). A transporter is assigned to a specific SLC family if it has 20% or more amino acid sequence identity to the other members of that family (Hediger *et al.*, 2004). The ability to transport amino acids

across cellular membranes is a fundamental function of all animal and plant cells as amino acids play important roles in many essential biological functions such as protein synthesis, neurotransmission, cell growth and nitrogen metabolism. Historically, amino acid transport systems have been identified and characterized functionally and thus named due to some key functional characteristic (such as substrate specificity, ion dependency or exchange properties) (Christensen, 1990; Palacin *et al.*, 1998). More recently, amino acid transporters have been identified at the molecular level (Palacin *et al.*, 1998; Bröer, 2008a,b). Current analysis of the SLC families identifies, in mammals, 59 functional amino acid transport proteins that are grouped into 12 separate SLC families. Orphan transporters are present in many of these families, suggesting that the total number of mammalian amino acid transport proteins is actually much greater than the 59 identified to date.

The purpose of this review is to summarize current knowledge of the SLC36 family of amino acid transporters. This family consists of four members, two of which, SLC36A1 and SLC36A2, have been characterized in some detail. The SLC36A1 and SLC36A2 transporters are unusual both in their ion coupling and substrate selectivity. They function as H⁺/amino acid symporters, unlike the vast majority of mammalian amino acid transporters that function as exchangers or Na⁺/amino acid symporters. SLC36A1 is the most thoroughly characterized of the SLC36 transporters and is expressed at the luminal surface of the human small intestine. SLC36A1 transports a broad range of amino acids and many orally-active amino acid-based drugs and derivatives (including a large number of GABA- and proline-related compounds) (Table 1) that can be used experimentally and/or clinically to treat infections, diabetes, epilepsy, schizophrenia, homocystinuria, cancer cell growth (*in vitro*) and in photodynamic therapy, fluorescent diagnosis and fluorescent-guided resection of cancer. SLC36A2 is expressed at the apical membrane in the renal proximal tubule and is involved in reabsorption of

amino acids and derivatives from the renal filtrate. Defects in SLC36A2 cause iminoglycinuria (excess urinary proline, hydroxyproline and glycine) and hyperglycinuria (isolated excess urinary glycine). It seems likely that function of both transporters will influence the pharmacokinetic profiles of amino acid-based drugs by mediating absorption in the small intestine and reabsorption in the kidney.

Nomenclature

The first cDNA corresponding to a member of SLC36 (SLC36A1) was isolated from rat brain and named LYAAT1 (for lysosomal amino acid transporter 1) (Sagné *et al.*, 2001; Wreden *et al.*, 2003). SLC36A1 has since been cloned from mouse (Boll *et al.*, 2002), human (Chen *et al.*, 2003a) and rabbit (Miyauchi *et al.*, 2005), and the transport system is most commonly known as PAT1 for proton-coupled amino acid transporter 1 (see Table 1). The carrier was named PAT1 because it represents the molecular identification of a H⁺-coupled amino acid transporter, named system PAT, characterized previously at the brush-border membrane of monolayers of the human intestinal epithelial cell line Caco-2 (Thwaites *et al.*, 1993a; 1993b; 1994; 1995a; 2000; Ranaldi *et al.*, 1994; Thwaites and Stevens, 1999). System PAT has the same substrate specificity and relative selectivity as the imino acid carrier (Anderson *et al.*, 2004), a transport system characterized at the luminal surface of the rat small intestine (Munck, 1966; Munck *et al.*, 1994), suggesting that they represent the human and rat forms of the same carrier. The nomenclature in this field has been used inconsistently over the years, and the rat imino acid carrier has been known by many different names including the glycine–proline carrier, sarcosine carrier, system 4 and methionine-insensitive ‘sarcosine–glycine–proline’ system (Newey and Smyth, 1964; Wiseman, 1968; Daniels *et al.*, 1969a,b; Thompson *et al.*, 1970; De la Noüe *et al.*, 1971). A detailed review of the physi-

Table 1

The SLC36 family of mammalian amino acid transporters

Gene*	Protein	Aliases	Examples of natural substrates, drugs and related derivatives
SLC36A1	PAT1	imino acid carrier LYAAT-1 Tramdorin 3	Small, unbranched, zwitterionic α -, β - and γ -amino and imino acids; N-methylated amino acids; heterocyclic amino acids containing four- to six-membered rings. Substrates include glycine, proline, alanine, <i>trans</i> -4-hydroxy-proline, β -alanine, taurine, sarcosine, betaine, GABA, D-proline, D-alanine, D-cysteine, D-serine, MeAIB, AIB, β -ABA, nipecotic acid, guvacine, THPO, isonipecotic acid, muscimol, gaboxadol, vigabatrin, 1-aminocyclopropanecarboxylic acid, 3-amino-1-propanesulphonic acid, L-azetidine-2-carboxylic acid, 3,4-dehydro-D,L-proline, L- and D-cycloserine, 5-aminolevulinic acid, β -guanidinopropionic acid, and short-chain fatty acids (butyrate, acetate, propionate).
SLC36A2	PAT2	Tramdorin 1	Small, unbranched, dipolar amino and imino acids and heterocyclic amino acids containing four- to five-membered rings. Substrates include glycine, alanine, L- and D-proline, <i>trans</i> -4-hydroxy-proline, sarcosine, MeAIB, D- and L-cycloserine, L-azetidine-2-carboxylic acid, 3,4-dehydro-D,L-proline, and short-chain fatty acids (acetate, propionate, butyrate).
SLC36A3	PAT3	Tramdorin 2	
SLC36A4	PAT4	LYAAT-2	Proline, tryptophan

*The full list of SLC tables can be found at the HUGO Gene Nomenclature Committee (HGNC) Database at: <http://www.bioparadigms.org/slc/intro.htm>

ology, function and history of SLC36A1, system PAT and the imino acid carrier, can be found in Thwaites and Anderson (2007a).

SLC36A2 was cloned from mouse (Boll *et al.*, 2002), rat (Birmingham *et al.*, 2002; Chen *et al.*, 2003b) and human (Boll *et al.*, 2003b). The most commonly used abbreviation is PAT2 (for proton-coupled amino acid transporter 2), although it has also been named Tramdorin 1 for transmembrane domain rich protein 1 (Table 1). The clearest evidence for a physiological role of PAT2 is in the reabsorption of amino acids in the renal proximal tubule as defective PAT2 function leads to the amino acid uria iminoglycinuria [Online Mendelian Inheritance in Man (OMIM) 242600] (Bröer *et al.*, 2008). Prior to molecular identification, there was little physiological evidence for a PAT2-like transporter except that the functional characteristics of PAT2 are similar to a transport system detected in preparations (cerebral cortical slices, homogenates, synaptosomes) of rat brain (Johnston and Iversen, 1971; Logan and Snyder, 1971; Bennett *et al.*, 1974; Rubio-Aliaja *et al.*, 2004; Kennedy *et al.*, 2005).

There are two additional members of the SLC36 family, SLC36A3 and SLC36A4 (Boll *et al.*, 2003b; Chen *et al.*, 2003b; Wreden *et al.*, 2003; Birmingham and Pennington, 2004). SLC36A3 was cloned from mouse and named PAT3 (Boll *et al.*, 2003b) and remains an orphan transporter with no known function. SLC36A4 was cloned from rat and named LYAAT2 (Wreden *et al.*, 2003) but is more commonly known as PAT4 (see Table 1). PAT4 was actually one of the earliest SLC36 sequences to be identified from the mouse, rat and human genomes (Boll *et al.*, 2003b; Chen *et al.*, 2003b; Wreden *et al.*, 2003). Nevertheless, despite intense study by a number of investigators, the transport function of PAT4 has remained unknown until recently. The results of a recent study (Pillai and Meredith, 2011) suggest that human PAT4 might also function as an amino acid transporter.

The use of abbreviations in the literature can unintentionally cause confusion for the reader as the PAT1 abbreviation is used to describe several other genes and proteins. For example, an anion exchanger within the SLC26 family (SLC26A6) is known not only as CFEX for chloride-formate exchanger (Knauf *et al.*, 2001) but also PAT1 for putative anion transporter (Greeley *et al.*, 2001). To prevent further confusion in the literature, we advocate the use of SLC names alongside the more commonly used transporter names and abbreviations.

Pharmacology and function

Amino acid and drug transport via members of the SLC36 family are usually measured by uptake of [^3H] or [^{14}C] labelled ligands (e.g. Thwaites *et al.*, 1993a, 1995c; Sagné *et al.*, 2001; Boll *et al.*, 2002; Chen *et al.*, 2003a; 2003b; Wreden *et al.*, 2003; Anderson *et al.*, 2004). If a compound is unavailable in radiolabelled form, evidence for its transport via the carrier can be determined by the relative ability of the compound to *cis*-inhibit (when present in the ipsilateral compartment) and *trans*-stimulate (when present at the contralateral side of the membrane) the uptake of a standard substrate (e.g. Thwaites *et al.*, 1995c; 2000; Boll *et al.*, 2002; 2003a; Anderson *et al.*,

2004, 2010; Foltz *et al.*, 2004b; 2005; Kennedy *et al.*, 2005; Metzner *et al.*, 2006; Edwards *et al.*, 2011).

Both PAT1 and PAT2 transport zwitterionic amino acids in a 1:1 stoichiometry with a H^+ (Thwaites *et al.*, 1994; Boll *et al.*, 2002; Wreden *et al.*, 2003), meaning that both carriers are rheogenic (i.e. able to generate current) and membrane potential sensitive. The movement of the coupling ion (in this case a H^+) has been used to measure PAT1 function at the apical membrane of human intestinal epithelial Caco-2 cell monolayers where PAT1-mediated H^+ -influx has been measured as a decrease in pH, in cells loaded with the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (e.g. Thwaites *et al.*, 1993a; 1995c; 2000; Anderson *et al.*, 2004; Abbot *et al.*, 2006); an increase in inward short-circuit current in voltage-clamped Caco-2 cell monolayers in Ussing chambers (e.g. Thwaites *et al.*, 1993a,b; 1994; 1995b,c; 2000); a change in membrane potential using a red membrane potential-sensitive dye (Metzner *et al.*, 2005, 2006). In cultured isolated rat hippocampal neurones, PAT1 function has been detected using pH-sensitive green fluorescent protein (Wreden *et al.*, 2003).

To allow detailed functional characterization of either PAT1 or PAT2 in isolation, the transporters have been expressed heterologously either in mammalian cell lines (e.g. COS-7, HRPE or HeLa cells) (Sagné *et al.*, 2001; Chen *et al.*, 2003a; 2003b; Wreden *et al.*, 2003; Miyauchi *et al.*, 2005) or in *Xenopus laevis* oocytes (e.g. see Boll *et al.*, 2002; 2003a; Chen *et al.*, 2003b; Wreden *et al.*, 2003; Anderson *et al.*, 2004, 2009, 2010; Foltz *et al.*, 2004a, 2005; Kennedy *et al.*, 2005; Metzner *et al.*, 2005; 2009; Abbot *et al.*, 2006; Edwards *et al.*, 2011). Expression in *X. laevis* oocytes allows detailed kinetic analysis of transporter function by measurement of substrate-induced inward current (H^+ movement) using electrophysiological techniques such as two-electrode voltage-clamp (TEVC) or giant patch clamp (Boll *et al.*, 2002; 2003a; Wreden *et al.*, 2003; Anderson *et al.*, 2004; 2010; Foltz *et al.*, 2004a; 2005; Kennedy *et al.*, 2005). H^+ movement has also been measured using H^+ -sensitive intracellular electrodes in oocytes (Boll *et al.*, 2002; Foltz *et al.*, 2004a) or BCECF in PAT1-transfected HeLa cells (Wreden *et al.*, 2003).

SLC36A1

PAT1 transport has been characterized using cDNA clones from human, rat, mouse and rabbit, and no marked differences in transport (substrate specificity or substrate affinity) have been observed between these orthologues. PAT1 is a H^+ -coupled, pH-dependent, low-affinity transporter (K_m approximately 1–10 mM) of small, unbranched, zwitterionic α -, β - and γ -amino and imino acids; N-methylated amino acids; and heterocyclic amino acids containing four- to six-membered rings (Thwaites and Anderson, 2007a,b). Substrates include glycine, proline, alanine, *trans*-4-hydroxyproline, β -alanine, taurine, sarcosine, betaine and GABA; the D-amino acids D-proline, D-alanine, D-cysteine and D-serine; and the amino acid derivatives α -(methylamino)isobutyric acid (MeAIB), α -aminoisobutyric acid (AIB) and β -aminobutyric acid (β -ABA) (see Table 1) (Sagné *et al.*, 2001; Boll *et al.*, 2002; 2003a; Chen *et al.*, 2003a; Wreden *et al.*, 2003; Anderson *et al.*, 2004; 2009; Miyauchi *et al.*, 2005; Abbot *et al.*, 2006). This substrate specificity is identical to that described for system PAT at the apical membrane of

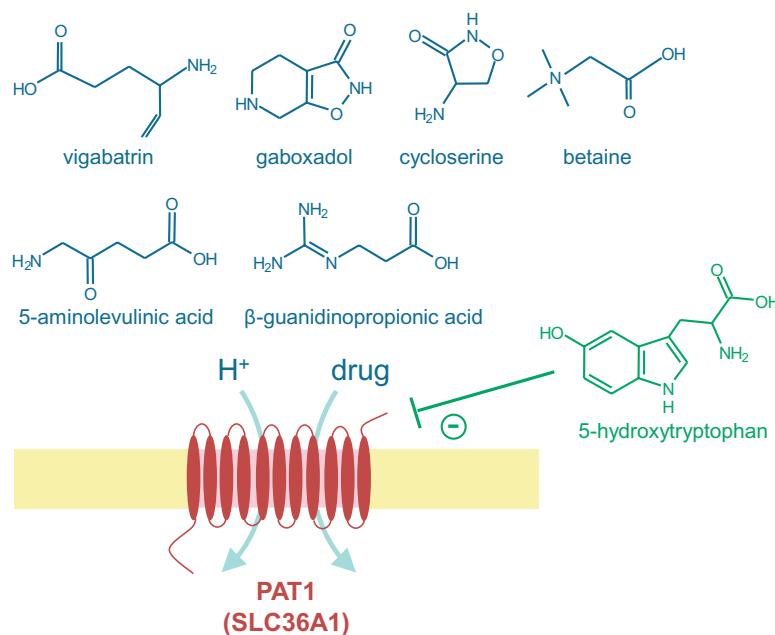


Figure 1

The H^+ -coupled amino acid transporter PAT1 (SLC36A1) can transport a number of compounds with potential therapeutic value (shown in blue). Several analogues of tryptophan such as 5-hydroxy-L-tryptophan (shown in green) are non-transported inhibitors of PAT1.

human Caco-2 cell monolayers (Thwaites *et al.*, 1993a; 1994; 1995b,c; Thwaites and Stevens, 1999; Anderson *et al.*, 2004, 2009; Metzner *et al.*, 2004, 2006) and the imino acid carrier at the luminal surface of the rat small intestine (Newey and Smyth, 1964; Munck, 1966; Wiseman, 1968; Daniels *et al.*, 1969a,b; Thompson *et al.*, 1970; De la Noüe *et al.*, 1971; Munck *et al.*, 1994; Anderson *et al.*, 2004; Iñigo *et al.*, 2006). A comprehensive review of the relationship between the imino acid carrier, system PAT and PAT1 can be found in Thwaites and Anderson (2007a). The most detailed investigations of the substrate specificity of this carrier can be found in Thwaites *et al.* (1995c), Boll *et al.* (2003a) and Metzner *et al.* (2006). In addition to rheogenic H^+ /zwitterionic amino acid transport, PAT1 can also function as an electroneutral transport system for H^+ and fatty acids including acetate, propionate and butyrate (Foltz *et al.*, 2004a).

The low-affinity, high-capacity, transport characteristics and relatively broad substrate specificity identify PAT1 as a potential pathway for transport of orally delivered hydrophilic amino acid-based drugs across the luminal surface of the human small intestinal wall, the initial barrier to effective drug delivery (Figure 1). In particular a number of GABA- and proline-related compounds have been identified as PAT1 substrates (Ranaldi *et al.*, 1994; Thwaites *et al.*, 1995a; 2000; Anderson *et al.*, 2004; Metzner *et al.*, 2004; 2006; 2009; Abbot *et al.*, 2006; Larsen *et al.*, 2008; 2009; 2010). PAT1 substrates with therapeutic, or potential therapeutic, value include nipecotic acid, guvacine and THPO (GAT transporter inhibitors) (Thwaites *et al.*, 2000; Anderson *et al.*, 2004; Abbot *et al.*, 2006; Larsen *et al.*, 2008); isonipecotic acid, muscimol and gaboxadol (GABA_A receptor agonists) (Thwaites *et al.*, 2000; Anderson *et al.*, 2004; Larsen *et al.*, 2008; 2009; 2010); vigabatrin (a GABA-transaminase inhibitor) (Abbot *et al.*,

2006); 1-aminocyclopropanecarboxylic acid (a partial agonist for the NMDA receptor) (Thwaites *et al.*, 2000); 3-amino-1-propanesulphonic acid (an anticraving agent) (Thwaites *et al.*, 2000; Metzner *et al.*, 2004); L-azetidine-2-carboxylic acid and 3,4-dehydro-D,L-proline (inhibitors of collagen synthesis) (Metzner *et al.*, 2004, 2006); L-cycloserine (an inhibitor of cancer cell growth *in vitro*) (Anderson *et al.*, 2004; Metzner *et al.*, 2006); 5-aminolevulinic acid (used in photodynamic therapy, fluorescent diagnosis and fluorescent-guided resection of cancer) (Anderson *et al.*, 2010); D-serine (used to treat schizophrenia) (Thwaites *et al.*, 1995a,c; Boll *et al.*, 2002; Chen *et al.*, 2003a); D-cycloserine (used as an antibiotic and in the treatment of schizophrenia) (Ranaldi *et al.*, 1994; Thwaites *et al.*, 1995a; Anderson *et al.*, 2004); betaine (used in the treatment of homocystinuria) (Thwaites *et al.*, 1995c; Boll *et al.*, 2003a); and β -guanidinopropionic acid (an anti-hyperglycaemic agent) (Metzner *et al.*, 2009) (Figure 1; Table 1).

The compounds listed above are all transported substrates of PAT1 and will thus compete for transport. In contrast, Brandsch and colleagues identified that L-tryptophan and the derivatives tryptamine, 5-hydroxy-L-tryptophan, serotonin and indole-3-propionic acid are non-transported, competitive inhibitors of PAT1 (K_i 0.9–6.1 mM) (Figure 1) (Metzner *et al.*, 2005). 5-Hydroxy-L-tryptophan has been used to identify the relative contribution of PAT1 and the di/tripeptide transporter PepT1 (SLC15A1) to uptake of 5-aminolevulinic acid at the apical membrane of human intestinal Caco-2 cell monolayers (Anderson *et al.*, 2010). In addition, L-tryptophan and 5-hydroxy-L-tryptophan have been used *in vivo* in studies in dogs and rats, respectively, to reduce gaboxadol absorption (Larsen *et al.*, 2009, 2010). These non-transported inhibitors are likely to prove highly useful tools to aid in the identifi-

cation of the relative contribution of PAT1 to substrate transport in membranes and tissues expressing multiple transporters with overlapping substrate specificity. However, the absolute value of the compounds may be restricted to some degree as the affinity for PAT1 is relatively low and, as yet, it is not known how many other carriers may also be inhibited by these compounds. For example, another tryptophan derivative, α -methyl-D,L-tryptophan, acts as a relatively high affinity (IC_{50} 250 μ M) non-transported inhibitor of the amino acid transporter ATB⁰⁺ (SLC6A14) (Karunakaran *et al.*, 2008).

PAT1 can be considered as a low affinity multipurpose transporter with substrate specificity that overlaps with many of the SLC6 family of Na⁺- and Cl⁻-dependent neurotransmitter transporters including GAT1 (SLC6A1), GlyT2 (SLC6A5), TauT (SLC6A6), PROT (SLC6A7), CT1 (SLC6A8), GlyT1 (SLC6A9), GAT3 (SLC6A11), BGT1 (SLC6A12), GAT2 (SLC6A13), ATB⁰⁺ (SLC6A14), SBAT1 (SLC6A15), NTT4 (SLC6A17), XT2 (SLC6A18), B⁰AT1 (SLC6A19) and SIT1 (SLC6A20). In general, the SLC6 transporters are highly selective and have a much higher affinity for their substrates compared to PAT1. Therefore, we can speculate that the role of PAT1 is maybe to function as a high-capacity route for mass transfer of substrates in tissues where the high-affinity SLC6 carriers might be saturated. Evidence to date suggests that PAT1 is not generally co-expressed at the same membrane surfaces as SLC6 members except at the luminal surface of the small intestine where TauT, CT1, ATB⁰⁺, B⁰AT1 and SIT1 are expressed to varying degrees. The relative roles of PAT1 and other carriers in neutral amino acid transport in the intestine and kidney are reviewed elsewhere (Thwaites and Anderson, 2007a; Br  er, 2008a,b).

SLC36A2

PAT2, like PAT1, is a rheogenic H⁺-coupled, pH-dependent, transporter of small, unbranched, dipolar amino and imino acids but has a higher affinity (K_m 0.1–0.7 mM) than PAT1 and has a relatively restricted substrate profile (Table 1). PAT2 substrates include glycine, alanine, L- and D-proline, *trans*-4-hydroxy-proline, sarcosine and the amino acid derivative MeAIB (Boll *et al.*, 2002; Chen *et al.*, 2003b; Rubio-Aliaga *et al.*, 2004; Foltz *et al.*, 2004b; Kennedy *et al.*, 2005; Br  er *et al.*, 2008; Edwards *et al.*, 2011). Like PAT1, PAT2 also transports amino acids containing a free carboxyl group and small side chain but has a preference for the amino group in the α position, is more selective for L- over D-enantiomers and is less tolerant of N-methylation (Foltz *et al.*, 2004b; Kennedy *et al.*, 2005). The K_m values estimated from transport measurements using the mouse, rat or human PAT2 transporters are almost identical. PAT2 transports a number of heterocyclic compounds with four- or five-membered ring structures including some with potential in therapy such as D-cycloserine, L-cycloserine, L-azetidine-2-carboxylic acid and 3,4-dehydro-D,L-proline, all mentioned above (Table 1) (Kennedy *et al.*, 2005; Edwards *et al.*, 2011). Like PAT1, PAT2 can also function in an electroneutral mode whereby it cotransports a H⁺ and a short-chain fatty acid (such as acetate, propionate or butyrate) but with relatively low affinity (Foltz *et al.*, 2004a). As observed with PAT1, a number of tryptophan derivatives can also inhibit PAT2 in a non-transported manner, with 5-hydroxy-L-tryptophan and α -methyl-D,L-

tryptophan having the greatest affinities (IC_{50} 1.6 and 3.5 mM respectively) (Edwards *et al.*, 2011). Although relatively low affinity inhibitors, these two tryptophan derivatives should help discriminate between PAT1 and PAT2 transport, as 5-hydroxy-L-tryptophan has a preference for PAT1, whereas the two compounds inhibit PAT2 with similar potency (Edwards *et al.*, 2011). The most detailed investigations of the substrate specificity of PAT2 can be found in Boll *et al.* (2002), Foltz *et al.* (2004b), Kennedy *et al.* (2005) and Edwards *et al.* (2011).

In a similar manner to PAT1 in the small intestine, the function of PAT2 in the renal proximal tubule is likely to overlap to some extent with members of the SLC6 family including XT2 (SLC6A18), B⁰AT1 (SLC6A19) and SIT1 (SLC6A20), all of which are likely to function in the reabsorption of glycine and imino acids from the renal filtrate.

SLC36A4

PAT4 function has not been characterized in as much detail as PAT1 and PAT2. However, PAT4 appears to function by facilitated diffusion in an electroneutral, Na⁺-independent, manner with a relatively high affinity for its substrates proline (K_m 3 μ M) and tryptophan (K_m 2 μ M). The pH dependence is distinct from PAT1 and PAT2, with transport being maximal at pH 7.4 and reduced at pH 5.5 and pH 8.4 (Pillai and Meredith, 2011).

Distribution: localization and function

SLC36A1

As determined by Northern blot and RT-PCR, PAT1 mRNA expression appears to be ubiquitous in human (Chen *et al.*, 2003a; Anderson *et al.*, 2004, 2009; Bermingham and Pennington, 2004), rat (Sagn   *et al.*, 2001), mouse (Boll *et al.*, 2002; 2003b) and rabbit (Miyauchi *et al.*, 2005) tissues. High levels of expression are found in both small intestine and brain in human and mouse (Boll *et al.*, 2002; Chen *et al.*, 2003a; Bermingham and Pennington, 2004). PAT1 mRNA was expressed in all paired normal and tumour samples from colonic tissue from 18 patients (Anderson *et al.*, 2010). PAT1 mRNA is expressed in neurones in most areas of the rat CNS (Agulhon *et al.*, 2003). *In situ* hybridization in rat brain identified the highest PAT1 mRNA expression in the cortex, thalamus, pyramidal cells of the hippocampus and Purkinje cells of the cerebellum, suggesting that PAT1 is expressed in both glutamatergic and GABAergic neurones (Sagn   *et al.*, 2001; Wreden *et al.*, 2003). For a detailed description of PAT1 mRNA distribution in the brain, see Agulhon *et al.* (2003).

PAT1 protein is expressed at the brush-border membrane in human small intestine, rat small intestine and human intestinal epithelial Caco-2 cell monolayers (Chen *et al.*, 2003a; Anderson *et al.*, 2004). In contrast, in the mouse kidney, PAT1 protein is found in sub-apical compartments within the S1 segment of the proximal tubule (Vanslambr  uck *et al.*, 2010). This intracellular localization is consistent with the earlier demonstration of predominant lysosomal localization in neuronal tissues in rat brain, although PAT1 immunoreactivity was also found in other intracellular organelles (e.g. Golgi, endoplasmic reticulum)

and occasionally the plasma membrane (Sagné *et al.*, 2001; Agulhon *et al.*, 2003). In rat hippocampal neurones in culture, PAT1 immunoreactivity is also colocalized at the plasma membrane with markers for the exocyst (a complex involved in targeting and tethering secretory vesicles to the plasma membrane) (Wreden *et al.*, 2003). Lysosomal localization of PAT1 is also observed in the mouse hypothalamic GT1-7 and neuroblastoma N2a cell lines (Pimpinelli *et al.*, 2005). In human macrophages and NIH3T3 cells, PAT1 colocalized with the haematopoietic cell kinase p61Hck in lysosomes and also, partially, in podosomes (Cougoule *et al.*, 2005). In PAT1-transfected cells (HeLa, PC12 and COS7), PAT1 colocalizes with the lysosomal protein LAMP1 (Wreden *et al.*, 2003).

A role for PAT1 in amino acid and drug absorption across the small intestinal wall is supported by evidence for PAT1 function at the brush-border membrane from studies in human Caco-2 cell monolayers (Nicklin *et al.*, 1992; Thwaites *et al.*, 1993a,b; 1994; 1995a; 2000; Ranaldi *et al.*, 1994; Thwaites and Stevens, 1999; Chen *et al.*, 2003a; Boll *et al.*, 2003a; Anderson *et al.*, 2004; 2009; 2010; Metzner *et al.*, 2004; 2005; 2006; 2009; Anderson and Thwaites, 2005; 2007; Abbot *et al.*, 2006; Larsen *et al.*, 2008; 2009), rat small intestine (Newey and Smyth, 1964; Munck, 1966; Wiseman, 1968; Daniels *et al.*, 1969a,b; Thompson *et al.*, 1970; De la Noüe *et al.*, 1971; Munck *et al.*, 1994; Anderson *et al.*, 2004; Iñigo *et al.*, 2006), lizard duodenum (Díaz *et al.*, 2000) and eel intestine (Ingrosso *et al.*, 2000); for a review, see Thwaites and Anderson (2007a). However, PAT1 may not be functional at the apical membrane of rabbit and guinea pig small intestine (Anderson *et al.*, 2004; for review of species differences in intestinal amino acid absorption, see Munck and Munck, 1994; Thwaites and Anderson, 2007a).

There is evidence for PAT1-like transport in brush-border membrane vesicles (BBMV) prepared from rabbit renal pars convoluta (Røigaard-Petersen and Sheikh, 1984; Rajendran *et al.*, 1987; Røigaard-Petersen *et al.*, 1987; 1989; 1990; Jessen *et al.*, 1988a,b; 1989; 1991; Jessen and Sheikh, 1991; Wunz and Wright, 1993; Miyauchi *et al.*, 2005), although these observations are discussed below in more detail in relation to the recent identification of PAT2 function in the mouse and human kidney (Brøer *et al.*, 2008; Vanslambrouck *et al.*, 2010).

PAT1 function has been measured at the plasma membrane of dissociated rat hippocampal neurones in culture, suggesting that it could play a role in neurotransmitter transport in the brain (Wreden *et al.*, 2003). However, the dominant lysosomal localization in most neurones, and other cells, points to a more general role in efflux of lysosomal proteolysis products into the cytosol (Sagné *et al.*, 2001).

PAT1 is thought to be involved in the control of growth and proliferation. Over expression of human PAT1 in the differentiating eye of *Drosophila melanogaster* leads to an increase in ommatidial size (Heublein *et al.*, 2010), as observed previously with PAT-like *Drosophila* transporters (Goberdhan *et al.*, 2005). In the human MCF-7 and HEK-293 cell lines, PAT1 protein is intracellular and is partially associated with an endosomal compartment (Heublein *et al.*, 2010). siRNA knockdown of PAT1 expression decreased cell proliferation possibly through a decrease in mTORC1 signalling, whereas overexpression of PAT1 led to an increased rate of

proliferation. Thus, an additional proposed function of PAT1 is as an intracellular amino acid sensor, similar observations being made with human PAT4 (Heublein *et al.*, 2010).

SLC36A2

PAT2 mRNA expression, as detected by Northern blot, RT-PCR and qPCR, is less widespread than PAT1 but is detected in heart, lung, kidney, skeletal muscle, spleen and brain in mouse (Boll *et al.*, 2002; 2003b; Rubio-Aliaga *et al.*, 2004; Vanslambrouck *et al.*, 2010); adipose tissue, spleen, lung, skeletal muscle, kidney, heart and brain in rat (Chen *et al.*, 2003b; Sundberg *et al.*, 2008); and kidney and skeletal muscle in human (Birmingham and Pennington, 2004; Nishimura and Naito, 2005). *In situ* hybridization localized PAT2 mRNA to bone marrow and fat pad in mouse (Birmingham and Pennington, 2004).

PAT2 immunoreactivity has been detected at the brush-border membrane of the S1 segment of the proximal tubule in human kidney (Brøer *et al.*, 2008) and along the length of the proximal tubule (S1–S3 segments) in mouse (Vanslambrouck *et al.*, 2010). PAT2 protein has been localized to paranodes and incisures in myelinated fibres of rat sciatic nerve (Birmingham *et al.*, 2002). In mouse spinal cord and brain, PAT2 protein was absent from lysosomes but was detected in endoplasmic reticulum, recycling endosomes and other cellular compartments including the plasma membrane (Rubio-Aliaga *et al.*, 2004). Transfection of a C-terminally tagged version of mouse PAT2 in HeLa cells led to PAT2 expression at the plasma membrane and a perinuclear, possibly endosomal, compartment whereas PAT2 was absent from lysosomes demonstrating a distinct sub-cellular distribution compared with PAT1 (Boll *et al.*, 2002). Birmingham and colleagues have suggested that this distinct distribution is due to the presence of a lysosomal targeting motif in PAT1, which is absent in PAT2 (Birmingham *et al.*, 2002).

There is less evidence for endogenous PAT2 function, compared with PAT1, although, as stated above, the transport characteristics of PAT2 (ion dependency, substrate specificity, substrate affinity) are very similar to those described in a series of studies using preparations of rat cerebral cortex and spinal cord (Johnston and Iversen, 1971; Logan and Snyder, 1971; Bennett *et al.*, 1974; see discussion sections in Rubio-Aliaga *et al.*, 2004; Kennedy *et al.*, 2005).

The loss or reduction of function observed in human PAT2 patients with iminoglycinuria is, at least in part, responsible for the increased loss of glycine, proline and hydroxyproline in urine, suggesting that an important role of PAT2 in humans is in the recovery of amino acids from the renal filtrate (Brøer *et al.*, 2008). This is supported by the immunocytochemical localization of PAT2 protein to the apical membrane of renal proximal tubule in both human and mouse (Brøer *et al.*, 2008; Vanslambrouck *et al.*, 2010).

A series of studies using BBMV prepared from the pars convoluta of the rabbit kidney have identified function of a PAT-like transport system indicative of a role in the renal reabsorption of amino acids (Røigaard-Petersen and Sheikh, 1984; Rajendran *et al.*, 1987; Røigaard-Petersen *et al.*, 1987; 1989; 1990; Jessen *et al.*, 1988a,b; 1989; 1991; Jessen and Sheikh, 1991; Wunz and Wright, 1993; Miyauchi *et al.*, 2005). The ion dependency is similar to that observed for both PAT1 and PAT2, but the substrate specificity and substrate affinity

(K_m 2.8–9.7 mM) are more like PAT1 than PAT2. Indeed, for a number of years, it has been considered that these studies were reporting transport via the same carrier (PAT1) characterized in human intestinal Caco-2 cell monolayers (for review, see Thwaites and Anderson, 2007a). However, the recent studies of PAT2 by Bröer and colleagues (Bröer *et al.*, 2008; Vanslambrouck *et al.*, 2010) emphasize the need to reevaluate the rabbit renal BBMVs studies. It is clear that in human and mouse, PAT2 is localized at the proximal tubule brush-border, whereas in mouse, PAT1 has a sub-cellular localization. The reason for this apparent discrepancy between the different studies is not clear but could be due to a number of reasons. It is possible that the expression of PAT1 and PAT2 in the proximal tubule show variation in different species such that PAT2 may be the carrier in human and mouse, whereas PAT1 fulfils the same function in rabbit. It is also possible that the BBMVs preparations used in the rabbit studies were contaminated with a PAT1-containing sub-cellular membrane. However, if that is the case, it might be expected that similar PAT1 contamination would be observed in the studies using BBMVs prepared from the pars recta, but no PAT-like function was observed in those BBMVs (e.g. Jessen *et al.*, 1988a; 1988b; 1989; 1991; Jessen and Sheikh, 1991). It may be that PAT1 is localized exclusively within a sub-cellular compartment only within the S1 segment, and this possibility is not inconsistent with the observations in mouse kidney by Vanslambrouck *et al.* (2010). The lack of apparent PAT2 function in the rabbit BBMVs studies could be because PAT2 is not expressed at the brush-border in rabbit proximal tubule, or that the transport system could not be detected under the experimental conditions used. These possibilities require further study, but it seems clear that in both human and mouse, it is PAT2, and not PAT1, that is involved in the renal reabsorption of glycine, proline and hydroxyproline (Bröer *et al.*, 2008; Vanslambrouck *et al.*, 2010).

SLC36A3

PAT3 mRNA (as detected by Northern blot and RT-PCR) is exclusively expressed in the testes in human (Birmingham and Pennington, 2004), mouse (Boll *et al.*, 2003b) and rat (Sundberg *et al.*, 2008).

SLC36A4

PAT4 mRNA expression appears to be ubiquitous in rat and human tissues (Nishimura and Naito, 2005; Sundberg *et al.*, 2008) and human cancer cell lines (Heublein *et al.*, 2010).

SLC36-related transporters in non-mammalian organisms

Although there are four SLC36 members in all mammalian genomes investigated thus far, there are greater numbers of SLC36-like genes in the genomes of some other organisms. Both Sagné *et al.* (2001) and Boll *et al.* (2003b) noted that there are a number of predicted PAT-like proteins in the fruit fly *D. melanogaster*. There are 11 members of the *Drosophila* PAT family, and the predicted proteins show 23–40% identity with human PAT1–4. Two of the *Drosophila* PAT-like transporters have been characterized following expression in *Xenopus* oocytes (Goberdhan *et al.*, 2005). Like mammalian forms of PAT1 and PAT2, one of the *Drosophila* transporters,

CG1139 or PAT, transported alanine, glycine and proline, transport being increased by a decrease in extracellular pH. The affinity of CG1139 (K_m 1.2–8.0 mM) was similar to mammalian forms of PAT1 (Goberdhan *et al.*, 2005). CG1139 mRNA (as detected by Northern blot) is enriched within the *Drosophila* body, suggesting that, like human PAT1, this PAT transporter could be expressed in the gut (Romero-Calderón *et al.*, 2007). In contrast, mRNA for the second transporter investigated, CG3424 (or PATH, for pathetic as a mutation led to reduced growth in flies), showed a ubiquitous expression, and *in situ* data, available through the Berkeley *Drosophila* Genome Project (BDGP), demonstrate expression in brain, muscle, the endocrine system and trachea (Romero-Calderón *et al.*, 2007). PATH showed different transport characteristics (alanine transport decreased as extracellular pH was lowered) and a higher affinity (K_m 3 μ M) compared with PAT1 and PAT2 but does have some similarity with PAT4. Modulation of both CG1139 and PATH expression in *Drosophila* is characterized by changes in eye and wing size, suggesting a role for the carriers in control of cell growth (Goberdhan *et al.*, 2005).

Following a blood meal, there is a change in mRNA levels in the midgut of the mosquito *Aedes aegypti* (Sanders *et al.*, 2003). Microarray analysis indicated an increase in expression of mRNA for AAEL007191, and this was confirmed by qPCR, which demonstrates a 16-fold increase in mRNA at 3 and 24 h post feeding (Evans *et al.*, 2009). Injection of this mRNA into *Xenopus* oocytes led to expression of a PAT-like amino acid transporter named AePAT1. Like mammalian forms of PAT1, AePAT1 is a low-affinity, Na⁺-independent, pH-dependent, rheogenic H⁺/amino acid symporter, but the substrate selectivity is distinct from mammalian PAT1 as AePAT1 transports glutamine (K_m 7.2 mM), tryptophan, serine and cysteine as well as the mammalian PAT1 substrate proline (Evans *et al.*, 2009). However, despite some functional differences, AePAT1 may well perform a similar function to human PAT1 in absorption of amino acids from diet, as AePAT1 protein is expressed at the apical membrane in both *A. aegypti* adult midgut (both male and female) and larval caecae. Western blot analysis of protein from adult midgut identifies a single band of approximately 51 kDa, which is similar to the predicted size of 53 kDa (Evans *et al.*, 2009). In females, a blood meal is required to initiate vitellogenesis, and it is likely that AePAT1 is important in providing nutrients for egg production and mosquito survival (Evans *et al.*, 2009).

It is worth emphasizing that there are many more PAT-like transporters, or genes, in invertebrates such as *D. melanogaster* and *A. aegypti* than the four found in mammals (Sagné *et al.*, 2001; Boll *et al.*, 2003b; Goberdhan *et al.*, 2005; Evans *et al.*, 2009). Currently, we do not know whether any of the invertebrate transporters share the exact same functional characteristics as the mammalian transporters as, at present, only six different PAT-like transporters have been identified functionally (Table 2). Therefore, we do need to take care when assigning names and numbers to these transporters to avoid creating confusion in the literature. For example, AePAT1 functions in a similar way to mammalian forms of PAT1 and is expressed in a similar location in the gut. However, there are also marked differences in the substrate specificity, and we do not yet know whether or not any of the other mosquito transporters behave more like mammalian PAT1 than AePAT1.

Table 2

Functional PAT-related solute transporters in mammals and other animals

Protein	Species	Gene	Transport classification database ID*
PAT1	Rat, mouse, human, rabbit	<i>SLC36A1</i>	2.A.18.8.1
PAT2	Mouse, rat, human	<i>SLC36A2</i>	2.A.18.8.2
PAT4	Human	<i>SLC36A4</i>	
PAT	<i>Drosophila melanogaster</i>	<i>CG1139</i>	
PATH	<i>Drosophila melanogaster</i>	<i>CG3424</i>	2.A.18.8.3
AePAT1	<i>Aedes aegypti</i>	<i>AAEL007191</i>	

*Transport Classification Database (Saier *et al.*, 2006) at: <http://www.tcdb.org/>

Regulation

Acute influences on transporter activity

The function of any ion-coupled cotransporter that is driven by transmembrane ionic gradients depends exquisitely upon the ionic composition within the local microdomains bathing either side of the cell membrane in which the protein is resident. Thus, the function of a transport system, such as PAT1, which is driven by the H⁺-electrochemical gradient, will depend upon the local pH (chemical H⁺ gradient) at both intracellular and extracellular faces of the membrane and the membrane potential (electrical gradient) across that membrane. Activity of the transporter will thus be modulated rapidly by changes in either pH or membrane potential.

SLC36A1

The activity of PAT1 at the apical membrane of Caco-2 cell monolayers increases as the apical (extracellular) pH is reduced (e.g. Thwaites *et al.*, 1993a, 1995c; Thwaites and Stevens, 1999). When PAT1 (rat, mouse, human and rabbit forms) is expressed heterologously it functions as a pH-dependent, H⁺-coupled, Na⁺-independent amino acid transporter (Sagné *et al.*, 2001; Boll *et al.*, 2002; Chen *et al.*, 2003a; Miyauchi *et al.*, 2005). Clearly, the maintenance of the driving force (H⁺-electrochemical gradient) for PAT1 function is important as a reduction, through use of either the NH₄Cl prepulse-and-release manoeuvre (which will acidify the cell) (Thwaites and Stevens, 1999) or the protonophore nigericin (which will dissipate the gradient) (Sagné *et al.*, 2001; Wreden *et al.*, 2003), will reduce transport. The relationship between PAT1 function, pH and the driving force is, however, complex. A detailed investigation by Daniel and colleagues (Foltz *et al.*, 2005) using mouse PAT1 expressed in *Xenopus* oocytes indicates that the H⁺ binds first to the transport protein, which increases the affinity for the amino acid substrate. Hyperpolarization of the membrane potential increases substrate affinity and maximal transport. In addition, a decrease in extracellular pH decreases the K_m value for amino acid substrate binding (Foltz *et al.*, 2005). Therefore, both proton concentration and membrane potential control the affinity of the PAT1 transport protein for its substrates. PAT1 can also function bidirectionally, and the direction of

movement will depend upon the local H⁺ and substrate gradients, with the transporter having a greater affinity for substrates in the influx mode (Foltz *et al.*, 2005).

In human Caco-2 cell monolayers, PAT1-mediated amino acid-coupled H⁺-influx across the apical membrane leads to an intracellular acidification. This apical acidification activates the apical Na⁺/H⁺ exchanger NHE3 (SLC9A3), which recycles protons back across the apical membrane to maintain pH_i and the H⁺-electrochemical gradient (Thwaites *et al.*, 1994; 1995a; 1999; 2000; 2002). This functional coupling with NHE3 (known as 'functional cooperativity') means that in intact epithelia, such as human Caco-2 cells or rat small intestine, the Na⁺-independent PAT1 becomes partially dependent upon extracellular Na⁺ (Munck *et al.*, 1994; Anderson *et al.*, 2004) as NHE3 cannot function in the absence of Na⁺. Thus, any factor that decreases NHE3 activity will indirectly inhibit PAT1 function by decreasing maximal transport that, in turn, will ultimately reduce the intestinal epithelial absorptive capacity for amino acids and amino acid-based therapeutics (Anderson *et al.*, 2004; Anderson and Thwaites, 2005, 2007; for review, see Thwaites and Anderson, 2007a,b; Anderson and Thwaites, 2010). Such a reduction in PAT1 capacity is observed at the apical surface of Caco-2 cell monolayers in the presence of a variety of factors that inhibit NHE3 including the selective NHE3 inhibitor 3-[2-(3-guanidino-2-methyl-3-oxo-propenyl)-phenyl]-N-isopropylidene-2-methyl-acrylamide dihydro-chloride (S1611; Schwark *et al.*, 1998) and the amiloride analogue 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), and any factor that will increase [cAMP]_i, including forskolin, 8-bromoadenosine-3',5'-cyclic monophosphate (8-br-cAMP), the neurotransmitters vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP), and phosphodiesterase inhibitors such as caffeine (Anderson *et al.*, 2004; Anderson and Thwaites, 2005, 2007).

SLC36A2

The pH dependence of PAT2 transport is distinct from that of PAT1 (Boll *et al.*, 2002; Kennedy *et al.*, 2005). Half-maximal transport via PAT2 is observed at extracellular pH values as high as pH 7.4–9.3 (Boll *et al.*, 2002; Rubio-Aliaga *et al.*, 2004; Foltz *et al.*, 2004b; Kennedy *et al.*, 2005), suggesting that maximal transport is not likely to be altered greatly by

changes within the physiological pH range. Foltz *et al.* (2004b) investigated the pH dependence and membrane potential sensitivity of mouse PAT2 in some detail, and the greatest influence on PAT2 appears to be the effect of changes in membrane voltage where a large decrease in proton binding affinity is observed as the membrane is depolarized from -120 to -20 mV (Foltz *et al.*, 2004b). In addition, a decrease in substrate affinity was observed as extracellular pH was increased to neutral and alkaline pH values when membrane potential was held at -60 mV (Foltz *et al.*, 2004b). The extracellular pH in the proximal tubule can be as low as pH 6.7–6.8 (Vieira and Malnic, 1968), suggesting that the transporter may function relatively independently of pH at these values but will be modulated greatly by changes in membrane voltage (Foltz *et al.*, 2004b). As observed with PAT1, PAT2 can also function bidirectionally, and the direction of movement (influx or efflux) will depend upon the prevailing substrate concentration gradient, pH gradient and membrane potential (Rubio-Aliaga *et al.*, 2004; Kennedy *et al.*, 2005; Edwards *et al.*, 2011).

Influences on gene expression of the transporter(s)

SLC36A2

This is an area where there is relatively little information in the literature, although a few studies have identified regulated expression of PAT2. The POU domain transcription factor pou3f1 is required for normal differentiation of myelinating Schwann cells. The *PAT2* gene is one of six genes that are down-regulated in the sciatic nerves of pou3f1-null mice, suggesting a role for PAT2 in amino acid transport in differentiating Schwann cells (Bermingham *et al.*, 2002).

Developmental iminoglycinuria is common in infant mammals and is associated with hyperexcretion of glycine, proline and hydroxyproline. This failure to adequately reabsorb filtered amino acids in the renal proximal tubule is due to the delayed expression of a number of amino acid transporters including PAT2, XT2 (SLC6A18), B⁰AT1 (SLC6A19) and SIT1 (SLC6A20) (Vanslambrouck *et al.*, 2010). In developing mouse kidney, there is a 5.3-fold increase in *PAT2* mRNA expression (as determined by qPCR) between birth and day 7. The level of *PAT2* mRNA then decreases at day 14 (20% of the level at day 7) before recovering to adult levels (Vanslambrouck *et al.*, 2010).

Embryo–maternal communication is critical in the preparation of the uterus for implantation of the developing conceptus. In a study investigating endometrial tissues from non-pregnant and pregnant (pre-implantation) horses, *PAT2* was one of the most highly up-regulated genes (15.5-fold), suggesting that PAT2 has an important role to play in nutrient supply in early embryo development (Klein *et al.*, 2010).

SLC36A4

In rat brainstem, PAT4 mRNA levels decrease following either a 24 h starvation or a 3 day treatment with L-DOPA (Sundberg *et al.*, 2008).

Biochemistry and genetics

In all mammalian genomes investigated thus far, there are four SLC36-related genes. In each genome, the genes for PAT1 (SLC36A1), PAT2 (SLC36A2) and PAT3 (SLC36A3) are clustered together (e.g. on human chromosome 5q33.1), whereas the gene for PAT4 (SLC36A4) has a distinct location (human chromosome 11q21) (Boll *et al.*, 2003b; Chen *et al.*, 2003b; Bermingham and Pennington, 2004; Thwaites and Anderson, 2007a). The gene cluster, when considered alongside the observation that the coding region for each of the three genes *SLC36A1*, *SLC36A2* and *SLC36A3* is each contained within 10 exons, suggests that the transporter genes have diverged from a common ancestor (Boll *et al.*, 2003b; Chen *et al.*, 2003b).

The human PAT proteins are 476 (PAT1), 483 (PAT2), 470 (PAT3) and 504 (PAT4) amino acids in length. Over the aligned regions, the human PAT1 sequence shares with its paralogues approximately 73% identity (84% similarity) with PAT2, 67% identity (83% similarity) with PAT3 and 53% similarity (70% identity) with PAT4. A high degree of likeness (78–88% identity, 88–94% similarity) is observed between human, mouse and rat orthologues over the aligned regions.

PAT1 and PAT2 are both predicted to have 11 transmembrane domains, each with a cytosolic N-terminus. The C-terminus of each transport protein will have an extracellular location when expressed at the plasma membrane and an intravesicular location when expressed at the membranes of intracellular organelles such as lysosomes and endosomes.

SLC36A1

A number of amino acid residues within the PAT1 sequence at sites likely to be involved in either proton sensitivity (histidine residues) or post-translational modification by N-glycosylation (asparagine residues) were predicted by Boll *et al.* (2003b) and Bermingham and Pennington (2004). These predictions have been tested by Brandsch and colleagues using site-directed mutagenesis of human PAT1 (Metzner *et al.*, 2008; Dorn *et al.*, 2009a). Mutation of His⁵⁵, which is conserved in human PAT1–4, to either Ala or Asn resulted in loss of PAT1 activity, suggesting that His⁵⁵ is obligatory for PAT1 function (Metzner *et al.*, 2008). This histidine residue (His⁵⁵) is predicted to lie close to the intracellular surface of transmembrane domain 1 within the PAT1 protein, and it is proposed to be involved in binding and translocation of the H⁺ during H⁺/amino acid cotransport (Metzner *et al.*, 2008).

N-glycosylation can be important in correct membrane targeting of proteins. Following expression in *Xenopus* oocytes, PAT1 protein was found to be glycosylated at three sites, Asn¹⁷⁴ and Asn¹⁸³ in the second extracellular loop (between transmembrane domains 3 and 4) and Asn⁴⁷⁰ located towards the C-terminus (Dorn *et al.*, 2009a). Glycosylation was lost when the asparagine residues were substituted with glutamine, but there was no reduction in PAT1 transport in the mutants containing a single substitution. In the triple Asn^{174/183/470}Gln PAT1 mutant, transport was reduced to 29% of wild type. The similar decrease in plasma membrane PAT1 protein levels suggests that the loss of transport is due to decreased PAT1 abundance rather than any effect on transporter function *per se* (Dorn *et al.*, 2009a). Western blot

analysis of an N-terminally HA-tagged human PAT1 protein recovered from injected *Xenopus* oocytes identifies a single band of approximately 50 kDa that is reduced to approximately 45 kDa following PNGase F treatment (Dorn *et al.*, 2009a). These observations are broadly consistent with the earlier study by Wreden and colleagues, which demonstrated the presence of a number of bands in protein from rat PAT1-transfected HeLa cells that were absent from the control cells (Wreden *et al.*, 2003). A strong band was observed at approximately 55 kDa and a weaker band at approximately 45 kDa. In addition, in protein from rat PAT1-transfected HeLa cells, a smear is observed around 75 kDa (Wreden *et al.*, 2003), which suggests that PAT1 may undergo greater glycosylation when expressed in human cells compared to *Xenopus* oocytes.

Once resident within the membrane some membrane proteins are stabilized by disulphide bond formation. Dorn *et al.* (2009b) have demonstrated that the function, but not membrane localization, of human PAT1 is dependent upon the formation of a disulphide bridge between Cys¹⁸⁰ in the second extracellular loop (between transmembrane domains 3 and 4) and Cys³²⁹ in the fourth extracellular loop (between transmembrane domains 7 and 8). Formation of this bridge likely stabilizes the substrate binding site of PAT1 (Dorn *et al.*, 2009b).

Relatively little is known about the effects of natural mutations or single nucleotide polymorphisms on function of the members of the SLC36 family. However, a single change in the second exon of the horse *PAT1* gene produces a Thr⁶³Arg change in horse PAT1 (contained within transmembrane domain 1), which is responsible for a change in skin pigmentation known as the champagne dilution phenotype (Cook *et al.*, 2008). The effect on horse PAT1 function of the Thr⁶³Arg mutation is not known, although its location within transmembrane domain 1 suggests that it will lie close to the substrate binding pocket.

SLC36A2

Less is known about the importance of specific amino residues in PAT2 function and localization. Western blot analysis of protein from both rat sciatic nerve (Bermingham *et al.*, 2002) and mouse brain (Rubio-Aliaga *et al.*, 2004) using PAT2-specific antisera identify a single band approximately 52–55 kDa in size. Although this band is consistent with the predicted size of the unglycosylated protein, no deglycosylation measurements have been made, so it is unclear as to whether the PAT2 protein is glycosylated or not. Examination of protein from mouse PAT2-transfected HEK293 cells reveals specific bands at both 52 and 45 kDa (Bermingham *et al.*, 2002), which could be interpreted as representing both glycosylated and unglycosylated forms of the protein. It could be that the relative abundance of the glycosylated (relative to the unglycosylated) form is much greater in normal tissues compared with transfected cells, which could reflect a different pattern of sub-cellular distribution of the PAT2 protein.

In patients with the inherited renal disorder iminoglycinuria (excess urinary proline, hydroxyproline and glycine), two changes in the human *PAT2* gene have been shown to produce altered proteins (Bröer *et al.*, 2008). A single mutation in the *PAT2* gene produces a PAT2 transport protein with a Gly⁸⁷Val substitution in transmembrane domain 2. This Gly⁸⁷Val PAT2 had reduced transport activity versus wild-type

PAT2 due to an increase in K_m values for both proline (from 140 to 390 μ M) and glycine (from 490 to 2350 μ M), whereas maximal transport capacity and membrane expression are unaffected (Bröer *et al.*, 2008). It is estimated that homozygosity for this mutation will occur at a frequency of 1:6900. In a separate pedigree, a splice donor site mutation in the first intron of the *PAT2* gene was identified, which produced a non-functional truncated protein (Bröer *et al.*, 2008). Homozygosity for this mutation will occur at approximately 1:62 500.

SLC36A4

A single study using a PAT4-specific antiserum has demonstrated the presence of several bands in protein from rat PAT4-transfected HeLa cells with the strongest band at approximately 55 kDa, consistent with the predicted size of the rat PAT4 protein (Wreden *et al.*, 2003).

Pathology and clinical significance

Transporter dysfunction and its impact

SLC36A1

The champagne dilution phenotype in horses is characterized by a dilution of hair colour intensity in both red and black pigments such that horse skin colour changes from chestnut to gold champagne, bay to amber champagne and black to classic champagne (Cook *et al.*, 2008). In the horses expressing the PAT1 variant, changes were observed in eye, skin and hair pigmentation (Cook *et al.*, 2008). Further investigation is required to identify the role of the PAT1 mutation in this change.

SLC36A2

It has been proposed that three transport systems are responsible predominantly for the reabsorption of glycine and imino acids in the renal proximal tubule (Lasley and Scriver, 1979), and that defects in one or more of these carriers would be responsible for iminoglycinuria (excess urinary proline, hydroxyproline and glycine) and/or hyperglycinuria (OMIM 138500) (isolated excess urinary glycine). The suggestion that these transport systems would include a common transport system as well as specific transport systems for glycine and imino acids seems consistent with current knowledge with PAT2 fulfilling the role of the common transporter, and the IMINO transporter SIT1 (SLC6A20) (Kowalczyk *et al.*, 2005; Takanaga *et al.*, 2005) and XT2 (SLC6A18) (Singer *et al.*, 2009) being the imino acid and glycine-specific carriers respectively. Either of the PAT2 mutations identified by Bröer *et al.* (2008) will lead to a reduction in reabsorption of amino acids in the renal proximal tubule and hyperexcretion of amino acids in urine (Bröer *et al.*, 2008). However, the inheritance pattern of mutations leading to production of either an iminoglycinuria or hyperglycinuria phenotype is complex. Iminoglycinuria is observed in an individual homozygous for the truncated (non-functional) form of PAT2. However, in individuals homozygous for the mutated Gly⁸⁷Val PAT2 (which has reduced function), only hyperglycinuria is observed, unless the individual also shows haploinsufficiency in the proline

transporter SIT1, where a single nucleotide polymorphism in *SLC6A20* produces a threonine to methionine change (Thr¹⁹⁹Met) in the fifth transmembrane domain of the SIT1 protein (Bröer *et al.*, 2008). This Thr¹⁹⁹Met SIT1 protein has a much reduced transport capacity compared with the wild-type protein (Bröer *et al.*, 2008). Iminoglycinuria produced by a combination of mutations in PAT2 and SIT1 is estimated to occur at a combined frequency of 1:92 600. In both iminoglycinuria and hyperglycinuria individuals, additional mutations were also described in both XT2 (SLC6A18) and B⁰AT1 (SLC6A19), but no mutations were found in PAT1 (Bröer *et al.*, 2008). The complexity of these disorders suggests that other pedigrees may have different inheritance patterns involving a variety of SLC36 and SLC6 transporters, which also emphasizes the overlap in substrate specificity and function of these carriers. Clearly, the transport of any drug via these mutated SLC36 and SLC6 carriers will be reduced, which, in turn, will alter renal reabsorption and the pharmacokinetics of each drug compound.

Indirect influence of disease and drugs on transporter function

An area of low pH (pH 6.1–6.8) is present adjacent to the luminal surface of the mammalian small intestine (McEwan *et al.*, 1988; Daniel *et al.*, 1989), and this low pH environment (the acid microclimate) provides the pH gradient that is utilized by absorptive transporters such as the amino acid transporter PAT1. Thus, any pathophysiological or pharmacological modulation (alkalinization) of this environment will indirectly affect the efficiency of H⁺-coupled transporters involved in nutrient and drug absorption. The acid microclimate is alkalinized in coeliac disease and following exposure to enterotoxins, cyclic nucleotides and phorbol esters (Lucas *et al.*, 1978; McEwan *et al.*, 1988; Shimada and Hoshi, 1988). In studies using human intestinal epithelial Caco-2 cell monolayers, H⁺-coupled nutrient and drug transport (e.g. PAT1) is reduced indirectly by a reduction in NHE3 activity following exposure to amiloride-related drugs, cyclic nucleotides, phorbol esters and phosphodiesterase inhibitors found in diet (caffeine, theophylline) and those delivered orally such as pentoxifylline (used to treat intermittent claudication) (Anderson *et al.*, 2004; Anderson and Thwaites, 2005; 2007; 2010; Thwaites and Anderson, 2007a,b).

Conclusion

It seems likely that the historical view that most drug molecules are transported passively across biological membranes by simple diffusion is incorrect. Rather, many drug molecules are transported across cell membranes in the human body via movement through a diverse range of carrier proteins each possessing a unique substrate selectivity and mode of action (Hagenbuch and Meier, 2003; Mizuno *et al.*, 2003; Schinkel and Jonker, 2003; Koepsell *et al.*, 2007; Thwaites and Anderson, 2007b; Dobson and Kell, 2008; Rubio-Aliaga and Daniel, 2008; Anderson and Thwaites, 2010; Giacomini *et al.*, 2010). Many of these mammalian transporters are members of the SLC families of proteins (a list of which can be found at the HGNC Database at: <http://www.bioparadigms.org>) (Hediger

et al., 2004). The relative concentration of any drug in any tissue will depend in part on the rates of influx and efflux mediated by various SLC and ABC transporters and the relative levels of expression of each transport protein in each membrane. To aid rational drug design and delivery, knowledge of the substrate specificity, ion-coupling and tissue and membrane distribution of each solute transporter is required. In this review, we summarize the current state of knowledge relating to the SLC36 family of amino acid transporters. Although relatively little is known about SLC36 transporter function, in comparison with some other SLC families, it is clear that members of SLC36 have great potential as routes for drug delivery in the small intestine and reabsorption in the kidney of a variety of amino acid derivatives and analogues, including neuromodulatory agents, anti-bacterials, anti-hyperglycaemics and anti-cancer agents.

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Conflict of interest

The authors state no conflicts of interest.

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