Mammalian ion-coupled solute transporters

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Active transport of solutes into and out of cells proceeds via specialized transporters that utilize diverse energy-coupling mechanisms. Ion-coupled transporters link uphill solute transport to downhill electrochemical ion gradients. In mammals, these transporters are coupled to the co-transport of H^+, Na^+, Cl^- and/or to the countertransport of K^+ or OH^-. By contrast, ATP-dependent transporters are directly energized by the hydrolysis of ATP. The development of expression cloning approaches to select cDNA clones solely based on their capacity to induce transport function in *Xenopus* oocytes has led to the cloning of several ion-coupled transporter cDNAs and revealed new insights into structural designs, energy-coupling mechanisms and physiological relevance of the transporter proteins. Different types of mammalian ion-coupled transporters are illustrated by discussing transporters isolated in our own laboratory such as the Na^+/glucose co-transporters SGLT1 and SGLT2, the H^+-coupled oligopeptide transporters PepT1 and PepT2, and the Na^+- and K^+-dependent neuronal and epithelial high affinity glutamate transporter EAAC1. Most mammalian ion-coupled organic solute transporters studied so far can be grouped into the following transporter families: (1) the predominantly Na^+-coupled transporter family which includes the Na^+/glucose co-transporters SGLT1, SGLT2, SGLT3 (SAAT-pSGLT2) and the inositol transporter SMIT, (2) the Na^+- and Cl^--coupled transporter family which includes the neurotransmitter transporters of γ-amino-butyric acid (GABA), serotonin, dopamine, norepinephrine, glycine and proline as well as transporters of β-amino acids, (3) the Na^+- and K^+-dependent glutamate/neurotransmitter family which includes the high affinity glutamate transporters EAAC1, GLT-1, GLAST, EAAT4 and the neutral amino acid transporters ASCT1 and SATT1 reminiscent of system ASC and (4) the H^+-coupled oligopeptide transporter family which includes the intestinal H^+-dependent oligopeptide transporter PepT1.

Previous studies of ion-coupled organic solute transporters in mammalian cell membranes led to the identification of functionally distinct systems such as the acidic amino acid transport system X_{AG}^-, the Na^+-dependent neutral amino acid transport system A or the ubiquitous Na^+-dependent amino acid transport system ASC (Ala, Ser, Cys). This division has been recently challenged by the cloning and functional characterization of transporter cDNAs. The advances suggest that a more reasonable approach for classifying ion-coupled transporters is according to their coupling patterns to inorganic ions, in conjunction with sequence homology. Based on this consideration, the following families can be distinguished: (1) predominantly Na^+-coupled transporters such as the SGLT-family (Hediger & Rhoads, 1994), (2) the Na^+- and Cl^--coupled GABA/neurotransmitter transporter family (Kanner, 1993), (3) the Na^+- and K^+-dependent glutamate/neurotransmitter transporter family (Kanai *et al.* 1993) and (4) the H^+-coupled oligopeptide transporter family which is characterized by coupling to the co-transport of H^+ (Fei *et al.* 1994). This review focuses on the structure, function and physiological relevance of Na^+/glucose co-transporters, Na^+- and K^+-dependent high affinity glutamate transporters and the H^+-coupled oligopeptide transporter.

Na^+/glucose co-transporters

Active absorption of D-glucose across epithelial cells of the small intestine and the kidney proximal tubule is provided by Na^+/glucose co-transporters in the brush-border membranes (Fig. 1). Transport of each glucose molecule is coupled either to the co-transport of two Na^+ ions (SGLT1) or one Na^+ ion (SGLT2). Glucose thus accumulated inside cells then diffuses into the blood via facilitated glucose transporters such as GLUT2 and GLUT1. The Na^+/K^+-ATPase located in the basolateral membrane maintains the inwardly directed Na^+ electrochemical gradient required to energize uphill glucose transport into the cell. To date, three different Na^+/glucose co-transporter isoforms have been reported:

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The intestinal high affinity Na^+/glucose co-transporter SGLT1

Crane *et al.* (1976) were the first to report the solubilization and reconstitution into liposomes of the renal and intestinal Na^+/glucose co-transporters. Semenza and colleagues (Schmidt *et al.* 1983) isolated the rabbit small intestinal Na^+/glucose co-transporter as a 72 kDa polypeptide (by SDS-PAGE) to near homogeneity. Peerce & Clarke (1990) also purified the transporter. These efforts, however, did not result in the isolation of the transporter in a form suitable for amino acid sequencing. Hediger *et al.* (1987) subsequently cloned the transporter by functional expression in *Xenopus* oocytes. SGLT1 is a hydrophobic integral membrane protein with approximately twelve putative membrane-spanning domains (Fig. 2). Homologous clones were isolated for SGLT1 from rat, pig and human (see Hediger & Rhoads, 1994).

Tissue distribution: Based on high stringency Northern analysis of rat tissues, SGLT1 mRNA is strongly expressed in small intestine (duodenum, jejunum, ileum) and at lower levels in kidney, lung and liver (Lee *et al.* 1994). *In situ* hybridization of antisense rat SGLT1 cRNA probe to tissue sections of jejunum revealed a SGLT1 signal mainly over the cells lining the lower two-thirds of the small intestinal villi, diminishing progressively towards the villus tip (Lee *et al.* 1994, Burant *et al.* 1994). In contrast, Na^+/glucose co-transport activity in rabbit intestine has been shown to be confined to the upper two-thirds of jejunal villi (see Fig. 1*D* in Smith *et al.* 1992). This is consistent with the view that during differentiation, as enterocytes migrate from stem cells of the crypt to the villus over a time period of 2–4 days, SGLT1 mRNA is transcribed followed by translation and insertion of functional SGLT1 carriers.

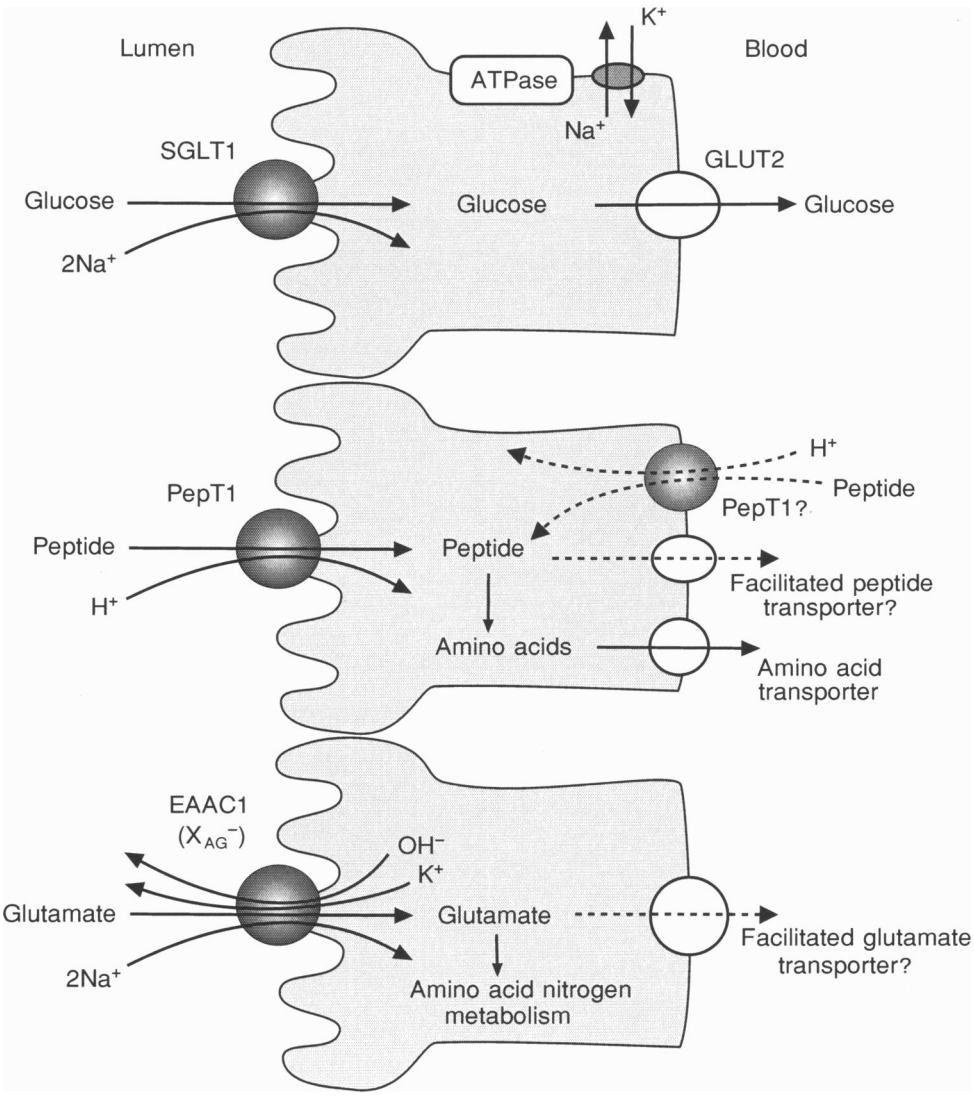

Fig. 1. Schematic model illustrating mechanisms of epithelial glucose, peptide and glutamate transport in the intestine. SGLT1 is the intestinal high affinity Na^+/glucose co-transporter, PepT1 the intestinal H^+/oligopeptide transporter, EAAC1 the neuronal and epithelial Na^+, K^+ and OH^--coupled high affinity glutamate transporter with characteristics reminiscent of system X^{AG}, and GLUT2 a facilitated glucose transporter.

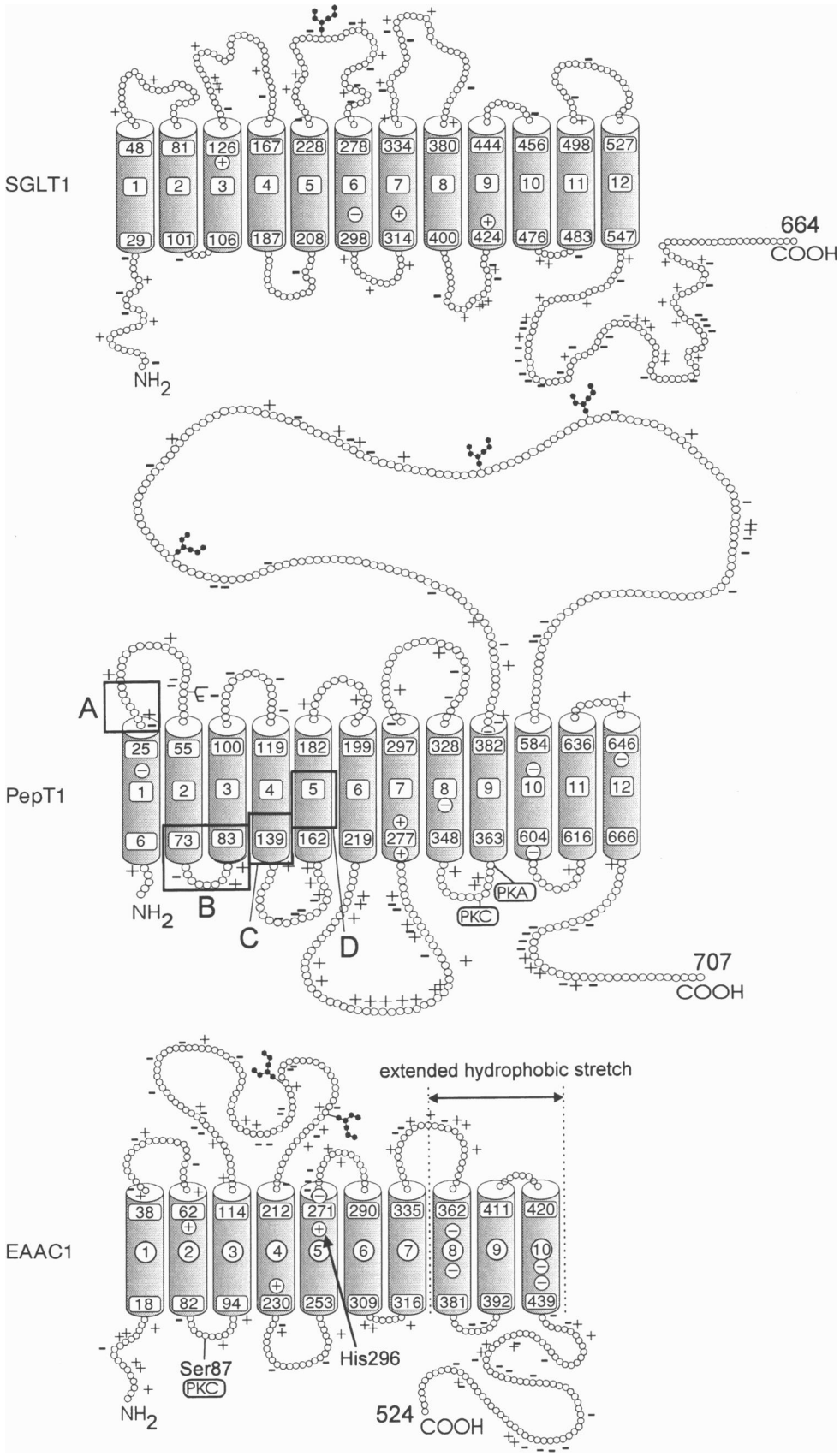

Fig. 2. Structural models of human Na^+/glucose co-transporter SGLT1, rabbit H^+/oligpopetide transporter PepT1 and rabbit Na^+-, K^+- and OH^--coupled glutamate transporter EAAC1. Potential N-glycoslylation sites are indicated by the hexagons. Potential phosphorylation sites PKC (protein kinase C) and PKA (cyclic AMP- and cyclic CMP-dependent protein kinases) are marked for PepT1. The putative PKC site at Ser 87 of EAAC1 (see Casado *et al.* 1993) is also indicated. Regions A, B, C, and D of PepT1 are conserved motifs. The topology of EAAC1 is uncertain within the extended hydrophobic stretch of EAAC1 (residues 357–439). His 296 in EAAC1 is presumed to be required for intrinsic activity of the transporter.

In kidney, SGLT1 mRNA is present predominantly in proximal tubule S3 segments (Lee *et al.* 1994). Whether SGLT1 in lung is expressed in alveolar and/or tracheal epithelia, however, has not yet been determined. In liver, SGLT1 may be present in the canalicular membrane of hepatocytes where it could serve to reabsorb glucose from the bile or alternatively in the sinusoidal membrane.

Function: SGLT1 from different species expressed in *Xenopus* oocytes mediated the Na^+-dependent and phlorizin-sensitive uptake of D-glucose and α-methyl-D-glucopyranoside (αMeGlc). The $K_{0.5}^{\alpha MeGlc}$ was 110 μM for rabbit SGLT1 (Ikeda *et al.* 1989), ~400 μM for rat SGLT1 (Lee *et al.* 1994) and ~800 μM for human SGLT1 (see Hediger & Rhoads, 1994). Rabbit SGLT1 substrates include a specific range of sugars with a selectivity of D-glucose > D-galactose > αMeGlc > 3-*O*-methylglycopyranoside (3OMeGlc) >>> L-glucose, 2-deoxyglucopyranoside (Ikeda *et al.* 1989). This order was consistent with the data of rat SGLT1: uptake of [^{14}C]αMeGlc (50 μM) by rat SGLT1 was strongly inhibited by excess (1 mM) D-glucose, αMeGlc, or D-galactose, and moderately by 3OMeGlc and the inhibitory potencies decreased in the order D-glucose > αMeGlc > D-galactose > 3OMeGlc. Interestingly, uridine (1 mM) resulted in a partial inhibition (36%) of αMeGlc uptake by rat SGLT1.

The stoichiometry of SGLT1-mediated transport in oocytes was accurately determined by combining [^{14}C]αMeGlc uptake studies with the two-electrode voltage clamp technique (Lee *et al.* 1994). In this procedure, the membrane potential was controlled and the Na^+ influx was measured as a current. Comparing the Na^+ influx and αMeGlc uptake rates revealed a Na^+ : glucose coupling ratio of 2 : 1 for rat and rabbit SGLT1.

Regulation: Studies on the dietary dependence of Na^+/glucose co-transporter expression, the regulation of expression in sheep intestine and dysregulation in experimental diabetes, indicate that SGLT1 regulation occurs primarily at the translational or post-translational levels and, at a lower level, at the transcriptional level (see Hediger & Rhoads, 1994, for review). Expression of SGLT1 in ruminants is particularly interesting because after weaning all dietary glucose is fermented in the rumen and no glucose reaches the small intestine. When the rumen develops, intestinal SGLT1 expression essentially disappears but remains inducible. Shirazi-Beechey and colleagues (1995) demonstrated that there is a dramatic downregulation of SGLT1 expression, occurring initially primarily at the transcriptional level. Later, in adult sheep, transcription is also apparently lost since SGLT1 mRNA levels become undetectable. This downregulation could be reversed by intubation of sheep intestine with D-glucose or glucose analogues. The finding that 2-deoxyglucose, a non-substrate of SGLT1, is able to up-regulate co-transporter activity indicates that binding to a luminal receptor may signal the induction of transport. In contrast to the induction in sheep, Na^+/glucose co-transport function in other mammals exhibited only small variations (2- to 3-fold) in response to the presence of dietary substrate, an induction ratio much lower than in bacteria which typically exhibit induction ratios of 100- to 1000-fold for sugar transport (Diamond, 1991). This low level of induction may have evolved as a result of the relative constancy of hexoses in any given diet.

The kidney cortical low affinity Na^+/glucose co-transporter SGLT2

SGLT2 cDNA (Hu14) was originally isolated by Hediger and colleagues from a human kidney cDNA library (Wells *et al.* 1992; Kanai *et al.* 1994*b*) using rabbit SGLT1 cDNA as a probe; rat SGLT2 was subsequently isolated. The SGLT2 amino acid sequences are ~60% identical to that of SGLT1 and the proteins have the same predicted secondary structure. The isolation and characterization of a cDNA encoding the kidney cortical low affinity high capacity Na^+/glucose co-transporter was of particular significance because this protein constitutes the major reabsorption pathway for D-glucose in kidney proximal tubule S1 segments.

Tissue distribution: High stringency Northern analysis of rat tissues indicated that SGLT2 mRNA expression is restricted to kidney cortex. The exact site of expression of SGLT2 mRNA in the kidney nephron was subsequently determined by *in situ* hybridization using ^{35}S-labelled antisense cRNA probes and paraformaldehyde-fixed rat kidney sections (Kanai *et al.* 1994*b*). A strong signal was obtained over tubules of S1 segments. In adjacent sections SGLT2-positive tubules were immunopositive for the low affinity facilitated glucose transporter GLUT2. This strongly indicates that both low affinity glucose transporters, the brush-border SGLT2 and the basolateral GLUT2, are co-expressed in epithelial cells of proximal tubule S1 segments where they mediate the transepithelial reabsorption of glucose. The tissue distribution of SGLT2, together with its other functional characteristics (see below), agree well with the data of the previously studied kidney cortical low affinity/high capacity Na^+/glucose co-transporters (Barfuss & Schafer, 1981; Turner & Moran, 1982).

Function: Expression of SGLT2 from rat in *Xenopus* oocytes stimulated Na^+-dependent and phlorizin-sensitive low affinity glucose transport 4·5-fold (G. You, Y. Kanai, W.-S. Lee & M.A. Hediger, manuscript in preparation). SGLT2 from human stimulated Na^+-dependent transport of D-glucose 2·0- to 3·5-fold with a $K_{0.5}$ for αMeGlc of ~1·6 mM, consistent with Na^+-dependent low affinity glucose co-transport (Kanai *et al.* 1994*b*). D-galactose was not a substrate of SGLT2, in agreement with inhibition studies of Turner & Moran using brush-border membrane vesicles from rabbit kidney outer cortex (Turner & Moran, 1982). SGLT2-mediated transport of [^{14}C]αMeGlc (1 mM) in *Xenopus* oocytes expressing SGLT2 was inhibited by phlorizin with an IC_{50} of ~1 μM. Uptake studies of [^{14}C]uridine revealed that nucleotides are not transported

by SGLT2. Hybrid depletion of rat kidney superficial cortex mRNA before injection into oocytes with an antisense oligonucleotide corresponding to the sequence of the rat SGLT2 start codon region suppressed the α-MeGLc uptake stimulated by kidney RNA > 95% (G. You & M.A. Hediger, unpublished data). This strongly indicates that the uptake stimulated by rat cortical mRNA is due to the expression of SGLT2 mRNA.

Another characteristic of the low affinity high capacity system previously identified by Turner & Moran (1982) is that the $K_{0.5}^{Na^+}$ is above 200 mM. Consistent with this finding, our data indicated that the $K_{0.5}^{Na^+}$ of SGLT2 expressed in oocytes is between 250 and 300 mM (Kanai *et al.* 1994).

One further important characteristic of the kidney cortical low affinity Na^+/glucose co-transporter is the Na^+ : glucose coupling ratio of 1 : 1. Since Hill plot analysis was found not to be appropriate for studying the stoichiometry of Na^+/glucose co-transporters we determined the stoichiometry of SGLT2 by comparing the Na^+ and sugar fluxes. This experiment clearly revealed a Na^+ : glucose coupling ratio of 1 : 1 for both rat and human SGLT2.

Wright and colleagues (Mackenzie *et al.* 1994) suggested that SAAT1-pSGLT2 (herein referred to as SGLT3) from porcine LLC-PK_1 cells is the previously studied cortical low affinity Na^+/glucose co-transporter (Barfuss & Schafer, 1981; Turner & Moran, 1982) and that human SGLT2 is the human version of the rabbit nucleoside transporter SNST1 (Pajor & Wright, 1992). However, the previously determined tissue distribution of SGLT3 (see below), its low $K_{0.5}^{Na^+}$ as well as the lack of nucleoside transport activity of human and rat SGLT2 are inconsistent with this proposal. The low nucleoside uptake of SNST1 and its close homology to SGLT2 rather suggest that SNST1 represents the low affinity Na^+/glucose co-transporter SGLT2 from rabbit kidney proximal tubule S1 segments.

An argument which could be raised against SGLT2 as a low affinity high capacity system in kidney cortex is that for a high capacity system, SGLT2 does not induce a high level of stimulation of αMeGlc uptake compared with the high affinity system SGLT1, when expressed in *Xenopus* oocytes. The high capacity feature of SGLT2, however, can be well explained when considering the following:

1. Based on a Na^+ : glucose stoichiometry of 1 : 1, the Na^+ drive of SGLT2, is 140 times lower than that of SGLT1 which has a coupling ratio of 2 : 1 (see Hediger & Rhoads, 1994). This explains in part the lower uptakes obtained for SGLT2 compared with SGLT1.

2. At an extracellular Na^+ concentration significantly below $K_{0.5}^{Na^+}$, Na^+-binding of SGLT2 may become rate limiting. Reducing the extracellular Na^+ concentration from 140 mM (the prevalent luminal Na^+ concentration of proximal tubules) to 100 mM (the maximal concentration which can be used in oocytes) may therefore cause Na^+-binding to become rate limiting. This would be in analogy to studies by Parent *et al.* on SGLT1 (Parent *et al.* 1992) which clearly revealed that Na^+ binding becomes rate limiting at Na^+ concentrations below 10 mM, which is one-third of the $K_{0.5}^{Na^+}$ of ~30 mM.

3. Factors such as a lower turnover rate or a lower density of functional carriers in the oocyte's plasma membrane may furthermore contribute to the low V_{max} observed for SGLT2.

4. Even though V_{max} of SGLT2 in oocytes may appear to be low, a high level of expression in kidney cortex could produce a high V_{max}. The strong *in situ* hybridization signal we observed for SGLT2 mRNA in proximal tubule S1 segments supports the view that SGLT2 is highly expressed in rat kidney cortex (Kanai *et al.* 1994*b*).

The low affinity Na^+/glucose co-transporter SGLT3 (SAAT-pSGLT2)

As discussed above, recent studies by Wright and colleagues revealed that pig SAAT1 isolated from LLC-PK_1 cells by Lever and colleagues which was originally reported to correspond to system A is in fact a low affinity Na^+/glucose co-transporter (Mackenzie *et al.* 1994). The amino acid sequence of this transporter herein referred to as SGLT3 is ~60% identical to that of the low affinity Na^+/glucose co-transporter SGLT2. SGLT3 mediated low affinity Na^+/glucose co-transport when expressed in *Xenopus* oocytes with a $K_{0.5}^{\alpha MeGlc}$ of ~2 mM. It appears unlikely, however, that SGLT3 is the kidney cortical low affinity Na^+/glucose co-transporter for the following reasons: (1) the low $K_{0.5}^{Na^+}$ of ~7 mM is inconsistent with the $K_{0.5}^{Na^+}$ of 228 mM determined by Turner & Moran (1982) for proximal tubule S1 segments of rabbit kidney. There would have to be an unusual species variation to explain this discrepancy. (2) The previously reported tissue distribution of SGLT3 (SAAT1) also does not appear to match that of the cortical low affinity glucose transporter (Barfuss & Schafer, 1981) since expression of SAAT1 was reported to be strong in intestine, spleen, liver and muscle and at a significantly lower level in kidney (Kong *et al.* 1993). It is therefore possible that SGLT3 represents an additional low affinity glucose transporter expressed in some tissues.

The mammalian H^+-coupled transporter of oligopeptides and peptide-derived antibiotics (PepT1)

Intestinal peptide transport plays a central role in the absorption of protein digestion end-products (Fig. 1). Transport studies of intestine, kidney, placenta and cultured cells revealed the existence of specific peptide plasma membrane transport proteins (for review see Ganapathy *et al.* 1994). Earlier studies indicated that the small intestine absorbs dietary protein predominantly as small peptides rather than single amino acids. This conclusion was based on ion-exchange chromatography which revealed that absorbed peptides were mainly di-, tri- and tetrapeptides. While many ion-coupled solute

transporters in mammalian cells are driven by an inwardly directed electrochemical Na^+ gradient, oligopeptide transporters of intestinal brush-border membrane vesicles and CaCo-2 cells are energized by the proton-motive force (Thwaites *et al.* 1993).

Structure: Despite extensive efforts to purify the rabbit intestinal oligopeptide transporter by affinity chromatography of photoaffinity labelled proteosomes (Kramer *et al.* 1992), the structure of oligopeptide transporters remained elusive until recently. By expression cloning we have isolated a peptide tranporter cDNA based on the ability of cDNA clones to induce uptake of the ^{14}C-labelled dipeptide Gly-Sar into *Xenopus* oocytes (Fei *et al.* 1994).

The cDNA encodes a novel 707 amino acid residue peptide transporter, called PepT1. A human homologue, HPepT1 was isolated from intestine which has 81% amino acid sequence identity with rabbit PepT1 (Liang *et al.* 1995). PepT1 mRNA is present in small intestine, liver and at a lower level in kidney.

Most mammalian co-transporters including the members of the SGLT-family and the GABA transporter family are intrinsic membrane proteins that span the membrane 10–12 times. Likewise, the amino acid sequence of rabbit and human PepT1 predicts a membrane protein with twelve membrane-spanning regions (Fig. 2). However, the presence of an unusually large hydrophilic loop with several N-glycosylation sites and the low sequence similarity to other reported sequences makes this protein distinct from other mammalian protein families.

The PepT1 amino acid sequence does not have significant homology to other known mammalian sequence. However, it shows weak (25% identity) similarity to the H^+-dependent nitrate transporter CHL1 from plants (*Arabidopsis thaliana*) (Tsay *et al.* 1993) and a peptide permease from *Saccharomyces cerevisiae* (Perry *et al.* 1994). Recently additional sequence similarities were found (Paulsen & Skurray, 1994) with the di-/tripeptide transporter DtpT from *Lactococcus lactis* and the putative protein YhiP of *E. coli*. Other unidentified open reading frames from rice and a region downstream of the cadA, B genes from *E. coli* were also identified. All these proteins have two highly conserved regions (regions B and D) located between the transmembrane segments 2 and 3 and within transmembrane segment 5 (Fig. 2). Furthermore, a stretch of seven residues (ERFSYYG, region A in Fig. 2) of PepT1 is conserved with the yeast peptide permease but not with the nitrate transporter. Another stretch of six amino acids (ALGTGG, region C) is conserved with the plant nitrate transporter CHL1 but not with the yeast permease. The conservation of these motifs suggests that they may play important structural or functional roles. Site-directed mutagenesis of PepT1 regions conserved with the yeast permease should help to define more precisely the mechanisms which underlie peptide translocation.

A human protein called HPT-1 expressed in intestine and pancreas but not in kidney has also been identified using a monoclonal antibody that blocked cephalexin uptake into CaCo-2 cells (Dantzig *et al.* 1994). Although HPT-1 was proposed to be an oligopeptide transporter the following information indicates that it is not a transporter: The HPT-1 cDNA encodes a protein with a single putative transmembrane domain. As discussed above, membrane transport proteins typically contain 10–12 transmembrane domains and to our knowledge there is no report which unambigously demonstrated that a protein with a single transmembrane domain functions as a tranporter. HPT-1 is related to the cadherin cell adhesion protein superfamily which further distinguishes its structure from known membrane transport proteins. Moreover, expression in CHO cells resulted in only low levels of uptake of transport substrates whereas expression of PepT1 in *Xenopus* oocyte induced strong uptake of dipeptides. These data suggest that HPT-1 is not the actual H^+-coupled oligopeptide transporter but that it somehow modulates endogenous oligopeptide transport in CaCo-2 cells.

Function: Since PepT1-mediated transport is electrogenic, the substrate range of PepT1 expressed in oocytes could be easily determined by two-electrode voltage clamp experiments (Fei *et al.* 1994). Large inward currents were observed when substrates such as dipetides, tripeptides and β-lactam antibiotics were applied. Small peptides containing either neutral, basic or acidic amino acids were transport substrates. Peptides larger than tetra-peptides did not produce significant inward currents.

The affinities among the substrates varied substantially. PepT1 appears to have a preference for peptides containing bulky aliphatic side chains. For instance, the $K_{0.5}$ values of the dipeptides Gly-Leu and Gly-Gly are 137 μM and 2·5 mM, respectively. β-Lactam antibiotics such as cyclacillin, cephadroxil and cephalexin are also transported by PepT1 which is consistent with previous studies of intestinal peptide transport.

An interesting question is how the unusually broad substrate specificity which appears to include any di- or tripeptide and many peptide-derived drugs is achieved by a single transport protein. What structural attribute of the PepT1 binding site allows such a broad substrate range? Is it shared with the antigen binding site of the peptide transporters Tap1 and Tap2 of the MHC class I complex (Momburg *et al.* 1994)? In the latter protein, recognition of a broad range of small peptides, primarily 9-mers, is achieved by the requirement that the peptide contains specific amino acid residues at certain positions. Extensive structure–function studies will be required to address these questions.

Utilizing the proton-motive force to energize uphill solute transport

Lucas (1983) demonstrated using pH microelectrodes that the unstirred water layer lining the surface of brush-border membranes is slightly acidic. The pH in this layer varied between 5·5 and 6·0, whereas the intracellular pH of

intestinal epithelia cells is approximately 7·0. Hence there is a significant H^+ electrochemical gradient across the plasma membrane to drive uphill transport of small peptides via PepT1 into enterocytes. Consistent with the pH of the unstirred luminal layer of enterocytes, studies of PepT1 in *Xenopus* oocytes revealed that peptide transport reached a maximum at an extracellular pH of 5·5. Uptake studies of ^{14}C-labelled Gly-Sar and measurements of intracellular pH using pH microelectrodes impaled into oocytes revealed that transport is associated with intracellular acidification (Fei *et al.* 1994). Our stoichiometric studies revealed that each Gly-Sar molecule is co-transported with one H^+. Given a 1 : 1 stoichiometry and assuming that pH_o of the unstirred water layer in the intestinal lumen is 5·5, that the intracellular pH_i is 7·0 and the membrane potential is −60 mV, PepT1 can concentrate neutral oligopeptides up to 300-fold across brush-border membranes.

Since dipeptides carrying a negative net charge such as Ala-Asp also induced inwardly directed currents in PepT1 cRNA injected oocytes this strongly indicates that acidic peptides are translocated with additional protons and that the 1 : 1 coupling ratio for transport does not generally hold true for all peptides. It will be interesting to determine whether transport of basic peptides by PepT1 is also coupled to the co-transport of H^+.

Are there additional oligopeptide transporters in the intestine? Several groups suggested that apical and basolateral epithelial peptide transporters have distinct functional properties. Inui and colleagues suggested that basolateral membranes in intestine and kidney contain a facilitated oligopeptide transporter (Fig. 1). This conclusion was based on difference in substrate specificity and pH dependence of the uptake of the ^{14}C-labelleld anti-cancer drug bestatin across apical and basolateral membranes (Saito & Inui, 1993). However, these differences could also be due to the different environment provided by apical and basolateral membranes, to different transporter densities at the apical and basolateral membranes or to a different activation state of a single transporter, e.g. by phosphorylation. The interpretaion of the data of Inui and colleagues is further complicated by their observation that, in spite of being a dipeptide, bestatin is transported via not only the H^+ oligopeptide transporter but also by the H^+/organic cation antiport system in rat renal brush-border membranes (Hori *et al.* 1993).

It is also of interest that under two-electrode voltage clamp conditions bestatin did not evoke a significant inward current in *Xenopus* oocytes injected with PepT1 cRNA, which was specific for PepT1 expression. Thus uptake of bestatin in the intestine may be mediated by a different transport system. Alternatively, bestatin transport by PepT1 may have been masked in oocytes by endogenous electrogenic bestatin transport transport which was observed in control oocytes.

To define whether PepT1 is the only intestinal peptide transporter we performed hybrid depletion studies. Hybrid depletion of rabbit small intestine mRNA before injection into *Xenopus* oocytes with an antisense oligonucleotide corresponding to the 5′-end coding region of PepT1 cDNA resulted in complete suppression of transport activity of the dipeptide Gly-Sar (Fei *et al.* 1994). Based on these studies, PepT1 appears to be the major Gly-Sar transporter in the intestine. The result, however, does not completely rule out a low level of expression of a facilitated basolateral peptide transporter (Fig. 1) as suggested by Inui and colleagues because this would produce only little uptake in mRNA-injected oocytes compared with PepT1 mRNA. In addition, we only examined the dipeptide Gly-Sar so additional peptide transporters with distinct substrate specificities may be present.

Finally, we have isolated a second PepT1 isoform from human kidney (PepT2) which is ~50% identical to PepT1 (Liu *et al.* 1995). Its tissue distribution and substrate specificity are currently being investigated.

Regulation: PepT1 from rabbit has a putative protein kinase C site (PKC) and a cyclic AMP-dependent phosphorylation site (PKA) (Fig. 2). Recent studies of Leibach and colleagues (Brandsch *et al.* 1994) of the H^+/oligopeptide transporter in the human cell line CaCo-2 revealed that activation of PKC decreases the maximal transport rate (V_{max}) of the transporter whereas the apparent affinity (K_m) remained unchanged. Consistent with this, the PKC of rabbit PepT1 is conserved in human PepT1 (HPepT1) and HPepT1 has an additional putative PKC site close to the C-terminus. The PKA site of rabbit PepT1, however, is not conserved in HPepT1.

Zinc transport: Even though zinc could be absorbed through brush-border membranes via a zinc-specific pathway not yet identified, recent studies suggest that zinc can also be transported across the plasma membrane via a peptide transporter system. Uptake studies into pig small intestine brush-border membrane vesicle preparations showed that zinc can associate with small peptides such as Gly-Gly-His to shuttle across the membrane (Tacnet *et al.* 1993). Zn^{2+} transport via PepT1 has not yet been evaluated.

The high affinity glutamate transporter EAAC1

Glutamate transport plays an important role in cellular amino acid nitrogen metabolism and in the fast excitatory synaptic transmission of the central nervous system (see Kanai *et al.* 1993, for review). In the small intestine and the proximal tubule of the kidney, glutamate transporters are involved in transepithelial glutamate transport (Fig. 1).

Glutamate is the predominant excitatory neurotransmitter of the mammalian central nervous system (Fig. 3). An important component of the glutamatergic transmission is the rapid removal of released glutamate from the synaptic cleft. High affinity glutamate transporters are thought to play an important role in terminating the postsynaptic action of glutamate. Transporters located in the pre- or postsynaptic

membranes are predicted to directly remove glutamate from the synaptic cleft. Transporters located in the plasma membrane of glial cells maintain the extracellular glutamate concentration at approximately 1 μM and establish a diffusion gradient which favours the movement of glutamate out of the synaptic cleft.

The neuronal and epithelial high affinity glutamate transporter EAAC1

A Na^+-dependent high affinity glutamate transport system X_{AG}^- has been previously identified in small intestine, kidney, liver and brain (see Kanai *et al.* 1993). To isolate a cDNA encoding a high affinity glutamate transporter, Kanai & Hediger (1992) screened a rabbit intestinal cDNA library for the ability to induce [^{14}C]glutamate uptake in *Xenopus* oocytes. A cDNA was isolated which encodes a 524 amino acid protein, referred to as EAAC1 (Fig. 2). This protein has approximately ten membrane-spanning domains. However, due to the presence of a large hydrophobic stretch near the C-terminus alternative models with a different number of membrane-spanning regions can be constructed.

The membrane model of EAAC1 shown in Fig. 2 shows several positively and negatively charged residues within membrane-spanning domains. Recently, Kanner and colleagues showed that His 326 of GLT-1 which corresponds to His 296 (see Fig. 2) is required for the intrinsic activity of the transporter (Zhang *et al.* 1994). However, whether this residue is involved in the coupling to Na^+, K^+ and OH^- (see below) or in glutamate translocation remains to be elucidated.

Distribution of EAAC1. Northern analysis of rabbit and rat tissues revealed that EAAC1 is strongly expressed in intestine, and at a lower level in kidney, liver and heart (Kanai & Hediger, 1992). In addition, EAAC1 was also found to be strongly expressed in brain. An EAAC1 cDNA

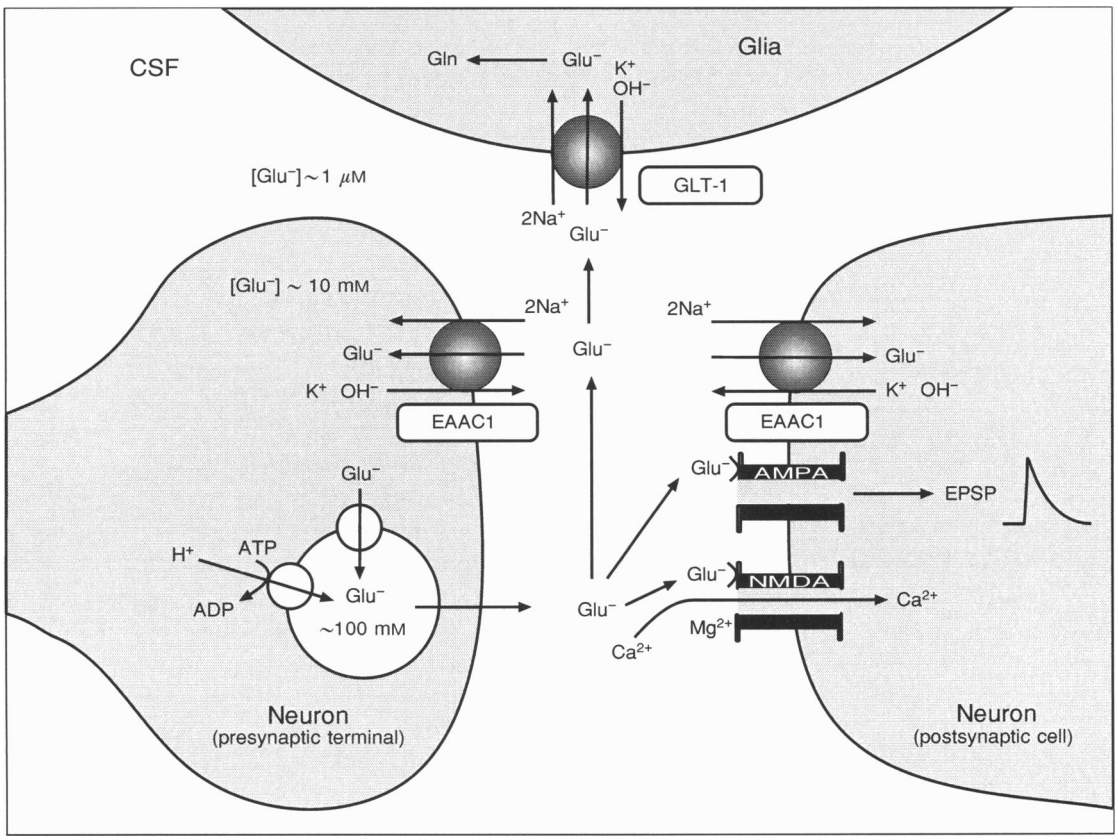

Fig. 3. Glutamate mobilization at glutamatergic synapses. Glutamate stored in synaptic vesicles is released by fusion of vesicles to the presynaptic membrane. Subsequent stimulation of postsynaptic AMPA receptors by glutamate opens associated ion channels and induces an excitatory postsynaptic potential (EPSP). The NMDA receptor-associated channel does not conduct ions because of the voltage-dependent blockade of the channel by Mg^{2+}. Release of the Mg^{2+} block by depolarization of the postsynaptic membrane leads to Ca^{2+} influx through the associated channel, which triggers the initiation stage of long-term potentiation (LTP) of synaptic transmission. Glutamate is thought to be removed from the synaptic cleft through reuptake back into presynaptic terminal and diffusion out of the synaptic cleft down the glutamate concentration gradient. Reuptake presumably proceeds via the high affinity glutamate transporter EAAC1. Recent studies by Rothstein and colleagues (1994) further suggest that EAAC1 may also act as a postsynaptic carrier. Further studies are required to confirm the expression of EAAC1 in pre- and postsynaptic membranes. Diffusion of glutamate out of the synaptic cleft is created by the glial high affinity glutamate transporter which corresponds to GLT-1. The amino acid sequence identity between GLT-1 and EAAC1 is 51%. Glutamate in glial cells is metabolized to glutamine by glutamine synthase, and also α-ketoglutarate by glutamate dehydrogenase or glutamate oxaloacetate transaminase. Glia are believed to supply the nerve terminal with glutamine and α-ketoglutarate which can serve as precursors of glutamate synthesis.

clone from a rat cerebral cortex cDNA library was subsequently isolated and the tissue distribution and localization in rat brain was determined by *in situ* hybridization (Y. Kanai *et al.* in preparation). EAAC1 mRNA expression was found to be expressed in neurons throughout the central nervous system. Expression was particularly strong in pyramidal cells of the cerebral cortex, pyramidal cells of the hippocampus, mitral cells of the olfactory bulb and cells of the thalamus. In general, the data suggest that EAAC1 is expressed in glutamatergic neurons throughout the central nervous system. In addition, EAAC1 mRNA was also detected in some non-glutamatergic neurons such as GABAergic cerebellar Purkinje cells and cholinergic α-motoneurons of the spinal cord. We propose that EAAC1 in Purkinje cells may provide glutamate as a precursor for GABA synthesis. In other non-glutamatergic neurons, glutamate uptake by EAAC1 may have a metabolic function. Figure 3 hypothetically shows EAAC1 in the pre- as well as postsynaptic cell of a glutamatergic synapse. The concept is that EAAC1 is a presynaptic uptake carrier originated from functional assays using synaptosomal preparations (see Kanner & Schuldiner, 1987). The possibility that EAAC1 may also act as a postsynaptic carrier was raised by immunocytochemical data (Rothstein *et al.* 1994). However, the exact subcellular localization of EAAC1 in glutamatergic neurons requires further studies.

Function and stoichiometry. The transport kinetics, substrate and stereospecificity, ion dependency and electrogenic properties of EAAC1 all agree with the properties described in the past for high affinity glutamate transport. The $K_{0.5}$ for EAAC1 from rabbit was ~12 μM (Kanai & Hediger, 1992). Whole-cell clamp studies of glutamate uptake in salamander retinal glia demonstrated that transport is electrogenic and coupled to the co-transport of two Na^+ ions and the countertransport of one K^+ and one OH^- ion (Bouvier *et al.* 1992). Hence one positive charge is translocated with each glutamate molecule. Consistent with this, we found that L-glutamate induces a strong inward current in oocytes injected with EAAC1 cRNA. We have found the stoichiometry of the salamander transporter holds true for EAAC1 expressed in *Xenopus* oocytes (Y. Kanai *et al.* manuscript in preparation). First we demonstrated that transport of EAAC1 is completely dependent on extracellular Na^+ (but not Cl^-), in contrast to the Na^+- and Cl^--coupled neurotransmitter transporters such as the GABA transporter GAT-1. Estimation of the Na^+ : glutamate stoichiometry of rabbit EAAC1 by measuring the Na^+ dependence of the glutamate-evoked current revealed a sigmoidal dependence of this current on extracellular Na^+ concentration. Interestingly, the Hill coefficient of this sigmoidal function was approximately 1 at 1 mM glutamate and increased to > 2 with decreasing glutamate concentrations (near or below $K_{0.5}^{\text{glutamate}}$). Subsequent studies comparing the initial rates of the [^{22}Na]- and [^{14}C]glutamate uptakes, however, demonstrated that two Na^+ ions are co-transported with each glutamate molecule at both 1 mM and 200 μM glutamate (Y. Kanai & M.A. Hediger, manuscript in preparation). A likely explanation for these apparently conflicting observations is that EAAC1 has two Na^+-binding sites (see Fig. 4) with different affinities and that at low extracellular L-glutamate (near or below $K_{0.5}$), the apparent affinity of the second Na^+ binding site is reduced.

Consistent with the K^+ counter transport model, the glutamate-evoked current was substantially suppressed when the extracellular K^+ concentration was increased from 2 to 50 mM (Kanai & Hediger, 1992). We also compared the glutamate-evoked depolarization of the membrane potential of EAAC1 cRNA-injected oocytes with the intracellular pH using an electrode filled with a hydrogen-selective ionophore (Y. Kanai *et al.* manuscript in preparation). Bath-applied glutamate (1 mM) depolarized the membrane potential from −60 to 0 mV and decreased the intracellular pH from 7·5 to 7·3. This finding is consistent with the OH^- countertransport model. These studies indicate that the stoichiometry determined by Attwell and colleagues holds true for EAAC1 (Bouvier *et al.* 1992).

In brain, this coupling pattern provides the accumulative power required to terminate the neurotransmitter action of glutamate and to keep its extracellular concentration below neurotoxic levels (< 1 μM). Based on this stoichiometry, EAAC1 can concentrate glutamate more than 10 000-fold across plasma membranes.

Hypothetical transport model of EAAC1: We have constructed a transport model of EAAC1 (Fig. 4) based on the coupling of EAAC1 to inorganic ions and based on our preliminary electrophysiological data (see also Kanai *et al.* 1994*a*). The model includes ordered transport kinetics. We predict that the transporter has a cation binding site for

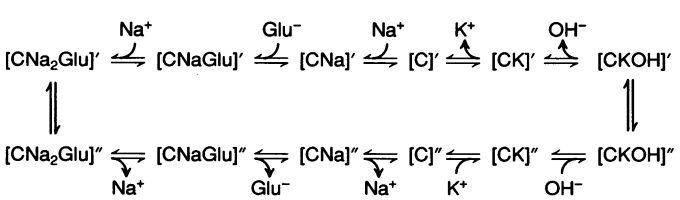

Fig. 4. Hypothetical kinetic model of EAAC1.

either two Na^+ ions or one K^+ ion and an anion binding site for either one glutamate$^-$ ion or one OH^- ion. We further propose that the cation binding site has two Na^+-binding sites with different affinities for Na^+ (see stoichiometry above). After binding of the first Na^+, glutamate binds, followed by binding of the second Na^+. This would be analogous to the model for the Na^+/glucose co-transporter proposed by Bennet & Kimmich (1992). Binding of two Na^+ and one glutamate is followed by translocation of the substrates and their release to the cytoplasm. K^+ and OH^- then bind to the cation and anion binding sites, respectively, and the transporter recycles to the original state, releasing K^+ and OH^- to the extracellular side.

Countertransport of K^+ and OH^- may serve to increase the rate of recycling of the transporter (Heinz *et al.* 1988). Recycling of the empty Na^+/glucose co-transporter is thought to be rate limiting. To terminate glutamate's neurotransmitter action at the synaptic cleft, glutamate transporters must have a high turnover rate. The required speed may be achieved by the electroneutral coupling of the recycling step to K^+ and OH^- countertransport.

Regulation: Activation of protein kinase C (PKC) at presynaptic terminals was proposed to be involved in the maintenance of long-term potentiation (LTP) of synaptic transmission (Linden & Routtenberg, 1989). EAAC1 has several putative PKC sites but only one is conserved with rat GLT-1 and rat GLAST: Ser 87. Recent studies by Kanner and colleagues (Casado *et al.* 1993) revealed that a mutation of this site which corresponds to Ser 113 in GLT-1 to Asn abolishes stimulation of transport by phorbol esters. Thus direct phosphorylation at the corresponding serine residue in HEAAC1 (Ser 87) may modulate its function.

The EAAC-family: Three additional cDNAs which encode related high affinity glutamate transporters have been reported. These are GLT-1 (Pines *et al.* 1992), GLAST (Storck *et al.* 1992), and EAAT4 (Fairman *et al.* 1994).

These proteins show 51–55% amino acid sequence identities to rabbit EAAC1. In contrast to EAAC1, they are predominantly brain specific although some expression in peripheral tissues has also been reported for GLT-1 and GLAST (Y. Kanai *et al.* unpublished data; Arriza *et al.* 1994). GLT-1, which is expressed in astrocytes, is ideally suited to maintain the low extracellular glutamate concentration of ~1 μM below neurotoxic levels. GLAST may be expressed in specialized neurons and some glial cells but its function remains unknown. EAAT4 is most abundant in cerebellum. The apparent $K_{0.5}$ values for all these transporters are between 1 and 30 μM at resting potential and these values seem to depend significantly on the expression system.

Recently, cDNAs encoding two other mammalian members of the EAAC1-family were reported: the neutral amino acid transporter ASCT1 with properties of system ASC (Arriza *et al.* 1993) and a second system ASC-like neutral amino acid transporter, SATT1 (Shafqat *et al.* 1993). EAAC1 also has significant homology to the H^+-coupled GLTP glutamate transporters of *E. coli*, *B. stearothermophilus* and *B. caldotenax*, and to the DCTA dicarboxylate transporter of *Rhizobium meliloti* with sequence identities ranging between 27 and 32% (see Kanai *et al.* 1993, for review). There is no homology between members of the EAAC1 family and those of the Na^+- and Cl^--dependent GABA/neurotransmitter transporter family.

Concluding remarks and future perspectives

The recent progress in the molecular physiology of ion-coupled mammalian solute transporters has provided new information on how uphill solute transport is linked to downhill electrochemical ion gradients and on how transporters fulfil their functions in their specific environment. These advances further revealed that ion-coupled mammalian transporters can be divided into distinct protein families based on the coupling of the transporters to inorganic ions and based on sequence alignment. The finding that most transporters are composed of ten or twelve transmembrane domains suggests that these protein families evolved from common ancestors, and that they arose in response to evolutionary pressure to satisfy specific requirements. It will be interesting to learn to what extent basic transport mechanisms such as the coupling to inorganic ions have been conserved during evolution. Important future goals will be to advance our understanding of how transporters are regulated, e.g. in response to changes of the physiological environment, to further develop a detailed knowledge of the different transport mechanisms, and to elucidate and to compare the tertiary structures of these proteins.

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