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Topical Review

Oligopeptide Transport by Epithelial Cells

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Introduction

This review focuses on the mechanism of peptide entry into and exit from vertebrate epithelial cells. The entry step across the apical membrane, a particularly interesting example of ion-coupled, electrogenic transport, is contrasted with the electroneutral exit step at the basolateral membrane. As in many reviews on other aspects of epithelial function, a keynote is this asymmetry between the processes occurring at the apical and basolateral membranes. However, in contrast to many epithelial transport processes for other solutes, the physiological interplay between transporter and hydrolytic activity determines the fate of peptide substrate.

Peptide Influx at the Apical Membrane is Proton Coupled

Early work on the intestinal uptake of intact peptides has been reviewed in depth and with scholarly care by Matthews (1991); although the historical development of this field is exceptionally interesting, it will not be addressed here. Studies on the cellular basis of peptide transport in vertebrate cells started only within the last decade, and without doubt the two major findings during this time have been the discoveries that peptide transport is coupled to the proton (not the sodium) electrochemical gradient (*see* Ganapathy & Leibach, 1983, 1985; Hoshi, 1985) and the very recent publication of papers reporting the cloning of genes encoding two families of peptide transporters (Fei et al., 1994; Dantzig et al., 1994, Hediger et al., 1995).

Two tissues have been studied in detail, and therefore most of the work reviewed here will be based on studies on the small intestine and renal proximal tubule. It is important to note that other tissues have become the focus of some recent studies; these will be mentioned at the end of this review. Molecular evidence for tissue specific expression in rabbit of one particular peptide transporter (PepT1) has been described very recently by Fei et al. (1994). The PepT1 mRNA was expressed at high levels in proximal small intestine, as shown by Northern blotting, and was also detectable, but at much lower levels, in renal cortex and medulla. An unexpected finding was expression in the liver at a higher level than in the kidney, and "weak expression in the brain."

In addition to cloning the human PepT1, Hediger et al. (1995) reported that they have isolated a second PepT isoform from the human kidney (PepT2), which is approximately 50% identical to PepT1. Detailed computer comparisons by Paulsen & Skurray (1994) of the PepT1 sequence with several sequences from bacterial genes, some of which are known to be involved with peptide transport, led to their suggesting that these proteins form a novel 'proton-dependent peptide transport' (POT) superfamily found in both eukaryotes and prokaryotes. Expression of another putative peptide transporter (HPT-1) has been studied immunohistochemically by Dantzig et al. (1994); they report the presence of antigen in human small intestine and colon, but absence from the kidney and liver. Strong staining for HPT-1 was also seen in the pancreatic ducts. [PepT1 and HPT-1 will be discussed fully below.]

Figure 1 shows a vesicle experiment indicating the dependence of peptide transport on the proton gradient in

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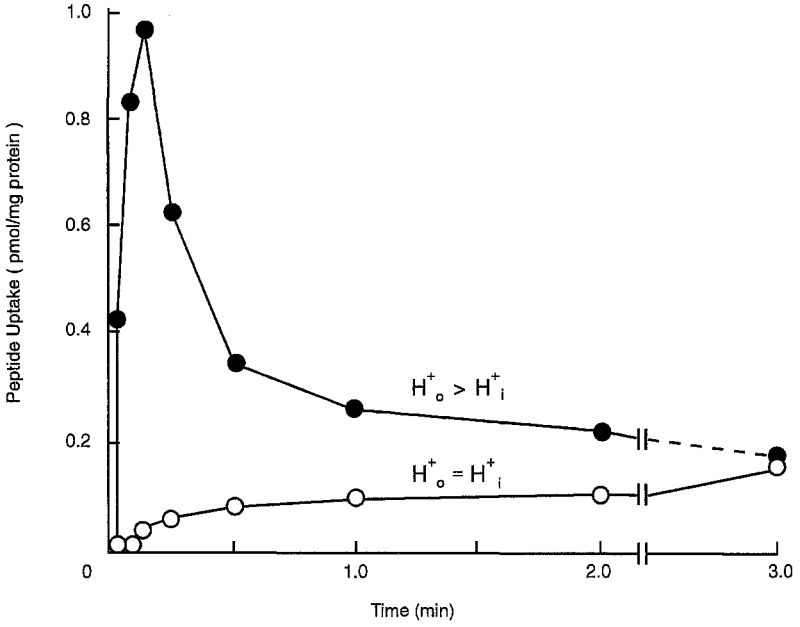

Fig. 1. An inwardly-directed proton gradient (filled circles) stimulates a transient overshoot of dipeptide (Gly-Gln, 0.1 μM) uptake into renal bbmv (pH_o = 6.7, pH_i = 8.3). When there is no proton gradient (open circles, $pH_o = pH_i$ = 8.3), there is no overshoot. Note that these experiments were performed in the absence of sodium. (Modified from Daniel et al., 1991).

brush-border membrane vesicles (bbmv) from rat kidney cortex.

This observation, which has now been repeated in a number of laboratories using different peptides as substrates, supports earlier observations on the electrogenic nature of peptide transport and explains why in these earlier studies (despite clear evidence of a depolarization with an associated rise in membrane conductance) the removal of sodium failed to alter the electrical response to peptides (Fig. 2). [As proposed by Ganapathy et al. (1984) and discussed by Matthews (1991) the involvement of the apically located Na^+/H^+ exchanger in the recycling of protons, and therefore also in the maintenance of the proton gradient, explains the apparent dependence on sodium observed in certain intact in vitro preparations.]

More recently, studies on peptide uptake at the apical membrane have focused on five aspects of transport which we will consider: the chemical features (e.g., the maximal size) of peptides which can be substrates for transport; the stoichiometry of proton-coupled peptide transport; and the question of whether peptide transport is subserved by a family of transporters. Finally, there has been considerable investigation of the structure-function relationship of peptide interaction with the transporter(s), by chemical modification of either the transporter and/or the substrate. Most recently, molecular biological techniques have allowed the sequences of PepT1 and HPT-1 to be elucidated, and this will greatly facilitate the structure-function studies. The fact that many molecules of therapeutic importance enter epithelia through peptide transporters adds pharmacological and pharmaceutical significance to work in this field. For example, most orally active antibiotics are carried by this transporter (*see* e.g., Bai et al., 1992) and also the anticancer agent bestatin (Hori et al., 1993, Takano et al., 1994). However, we will not attempt to review this literature systematically since for this field also the absence of molecular definition of the transporter(s) makes extrapolation to physiological substrates hazardous.

Chemical Requirements of Peptide Substrates

Size

Both di- and tripeptides appear to be preferred substrates; tetrapeptides, if they interact at all, do so with a much lower affinity (Daniel et al., 1992; Fei et al., 1994, but *see* Kramer et al., 1994). Amino acids, which by definition lack a peptide bond, appear to be unable to interact with the transporter. Thus the oligopeptide transporter (the term dipeptide transporter not adequately describing the range of potential substrates) has the potential of interacting with respectively 400 naturally occurring dipeptides and 8,000 tripeptides (excluding D-isomers, *see below*). The size of potential substrates thus covers a fivefold range from 102 Da for Gly-Gly, to 576 Da for Trp-Trp-Trp. When the number of potential substrates for the oligopeptide transporter is compared to those for the known amino acid transporters, where each transport system for example often has a very restricted range of preferred substrates, then it becomes apparent that similarly for peptides there may be a number of different transporters.

Charge

Rather little systematic work has been done on the effects of the charge carried by the substrate on peptide

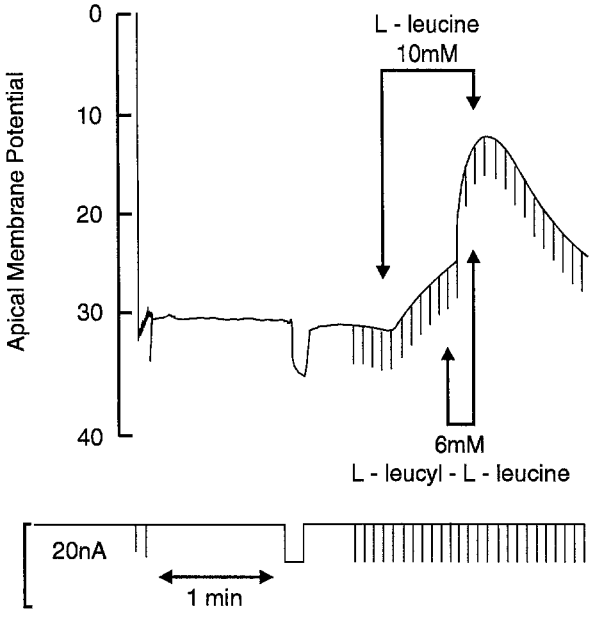

Fig. 2. Persistence in the absence of sodium of intestinal electrogenic peptide transport. The peptide-induced depolarization (unlike that caused by the amino acid) is extremely rapid in the absence of luminal sodium. (Modified from Boyd & Ward, 1982).

translocation. Wootton and Hazelwood (1989) (*see* Table) have studied the effect of a series of charged dipeptides on the uptake of the neutral peptide Gly-Pro into intestinal bbmv. Their study shows that the ability of dipeptides to interact with the Gly-Pro transporter is strongly affected both by the charge and the position of the charge on a competing dipeptide. In particular, dipeptides containing an anionic amino acid have markedly reduced inhibitory affinity as reflected by their high K_I; when a dipeptide with two anionic residues is used, its inhibitory affinity is more than a 100-fold lower than that seen with a neutral dipeptide. In contrast, cationic amino acid substitution in the inhibitor dipeptide is without substantial effect, particularly if the substitution is at the carboxyl terminus. However, the double cationic substitution does result in a dramatic fall in the affinity, and in this regard does not fit any simple model, perhaps indicating that there is only limited capacity of the Gly-Pro transporter to neutralize cationic charge on a substrate. It is important to note that these careful experiments on intestinal bbmv do not address the issue of whether the particular inhibitor dipeptides studied are in fact translocated through the Gly-Pro transporter. Furthermore, these results do not receive support from the more recent experiments of Daniel et al. (1992) using a different peptide on renal bbmv (*see below*).

Stereochemistry

Recent studies from a number of laboratories confirm that peptides composed solely of D-isomer amino acids

Table. Inhibition by dipeptides carrying different charges on the uptake of ^{14}C-Gly-Pro (0.16 mM) into small intestinal bbmv

Inhibitory peptide	K_I (mM)
Val-Val	0.62
Val-Glu^-	5.12
Val-Lys^+	0.64
Glu^--Val	11.2
Glu^--Glu^-	34.7
Glu^--Lys^+	5.2
Lys^+-Val	1.8
Lys^+-Lys^+	23.1

(Modified after Wootton & Hazelwood 1989).

are not translocated by the oligopeptide transporter (Boyd & Ward, 1982; Lister et al., 1994; *see* Kramer et al., 1994). Perhaps more interestingly there have been three studies in the last year which have confirmed a differential effect of D-substitution at the carboxyl from that at the amino terminal of the substrate dipeptide. Thus Lister et al. (1994), showed in intestine that of the stereochemically-mixed peptides composed of Phe and Ala, only D-Phe-L-Ala and D-Ala-L-Phe are translocated; in contrast L-Phe-D-Ala and L-Ala-D-Phe are not. Essentially identical conclusions were reached from similar experiments on peptide transport in lung by Boyd et al. (1993), and Morimoto et al. (1993) showed that Gly-D-Phe (unlike Gly-L-Phe) does not act as a substrate for transport across monolayers of rat cultured alveolar pneumocytes. Daniel et al. (1992) reached similar conclusions on the relative importance of carboxyl and amino terminal D-amino acid substitution using an inhibition assay of Gly-Gln uptake in kidney bbmv (*see below*). In addition, they showed that there was less marked stereospecificity at the amino terminal amino acid when this residue was hydrophobic. In the case of the carboxyl amino acid, substitution with a D-isomer always very substantially reduced inhibitory affinity, although peptides with a hydrophobic D-amino acid did show limited interaction with the transporter.

The Stoichiometry of Peptide/Proton Coupling Remains Controversial

In an important paper, Abe, Hoshi and Tajima (1987) studied the effects of Gly-Gly transport in toad isolated small intestine, measuring both the transepithelial flux of the dipeptide and the short-circuit current (which they showed to be due to hydrogen ion flux). From these measurements, they estimated the stoichiometry of the H^+-peptide cotransport to be 2:1 for this substrate in this tissue. This result remains the best (only) estimate of this important relationship in the intact tissue; however, *see below* for estimates of stoichiometry of the cloned

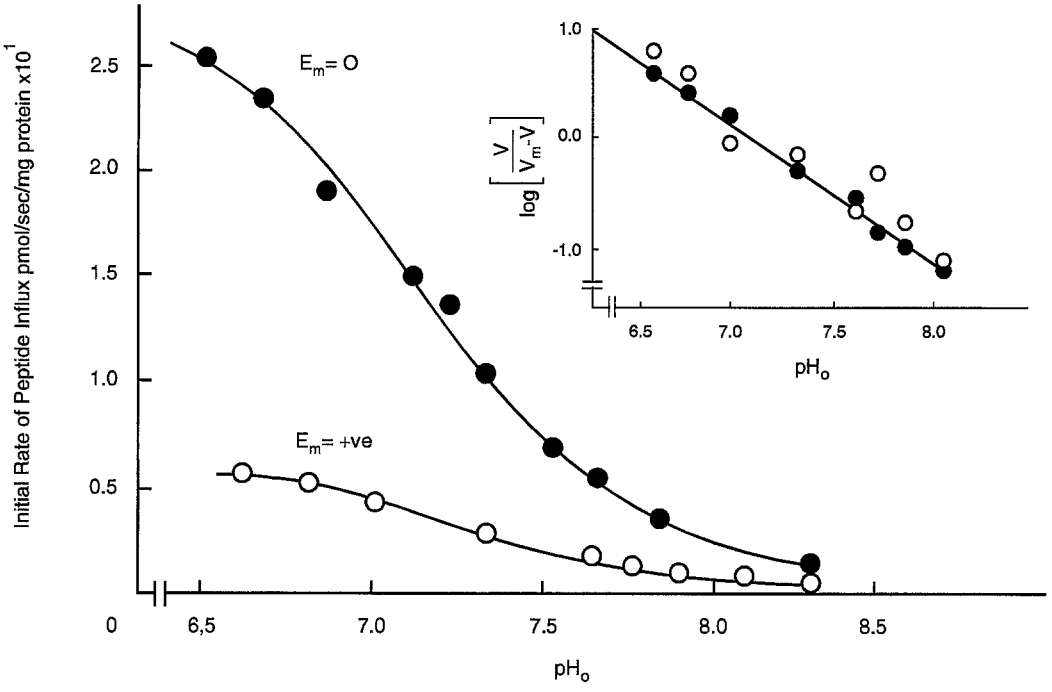

Fig. 3. External pH dependence of dipeptide (Gly-Gln, 0.1 μM) influx into renal bbmv with $E_m = 0$ (upper line) or inside positive (lower line). The inset shows the Hill plot for both conditions, which can be fitted with a single line of slope 1.21 ± 0.10, suggesting for this neutral peptide a stoichiometry of proton:peptide coupling of 1:1 at either potential. (Modified from Daniel et al., 1991).

PepT1 when expressed in *Xenopus* oocytes. Thwaites et al. (1993*a*) arrive at a similar figure to Abe et al. (1987) for Gly-Sar transport in cultured Caco-2 cells). In contrast, in rat kidney brush-border membrane vesicles the stoichiometry for Gly-Gln has been estimated indirectly by Daniel et al. (1991) to be 1:1 (Fig. 3). Their estimate for the Hill coefficient of the relationship between flux and external pH is not significantly different from one, as shown in the figure insert, both under voltage clamp (Em = 0) and in vesicles which are not voltage-clamped. In the absence of further information, it is not at present possible to decide whether these differences in stoichiometry are methodological or reflect the properties of different transporter molecules in the two tissues. The issue is complicated by the recent finding that in kidney there are at least two systems present in the brush border for peptide transport. It is not clear whether this is also true in intestine, and, if so, which system was predominantly responsible for the findings of Abe and colleagues. Interestingly, analysis of the electrical responses induced by Gly-Gly in intestine, and comparison with the electrical responses to other di- and tripeptides, suggest that in this tissue there is one family of neutral peptides with the same maximal response, and therefore presumed same stoichiometry, as Gly-Gly; a second family including Leu-Leu, Phe-Phe, Phe-Leu, Gly-Lys, and Glu-Glu had a maximal electrical effect that appears to be precisely half that produced by Gly-Gly transport. This might suggest for this second family a net movement of only one positive charge per peptide molecule translocated; however, since some of the second family of peptides carry a net charge at physiological pH, this finding cannot be explained in all cases by a simple 1:1 stoichiometry. Nevertheless, for one particular peptide, carnosine (β-Ala-L-His), there is strong evidence for such a 1:1 stoichiometry in intestine (Himukai & Hoshi, 1985), and indeed in the study of Abe et al. (1987) this peptide similarly showed a maximal electrical effect precisely half that seen with Gly-Gly. The properties of intestinal carnosine transport were also shown to be different from the transport of other neutral dipeptides (e.g., Leu-Leu) by Boyd & Ward (1981); a similar conclusion is reached in kidney by Skopicki et al. (1991) and Daniel et al. (1992).

Most recently, expression of PepT1 in *Xenopus* oocytes allowed Fei et al. (1994) to examine the stoichiometry of peptide coupling for this particular transporter. They conclude that for Gly-Sar the stoichiometry is 1:1, and, assuming this ratio, the authors show that the calculated proton electrochemical gradient is not insufficient to account for the accumulation of peptide.

A Family of Peptide Transporters?

Daniel, Morse and Adibi (1991) have provided convincing evidence that the transport of a neutral dipeptide (Gly-Gln) in rat renal bbmv is by more than a single

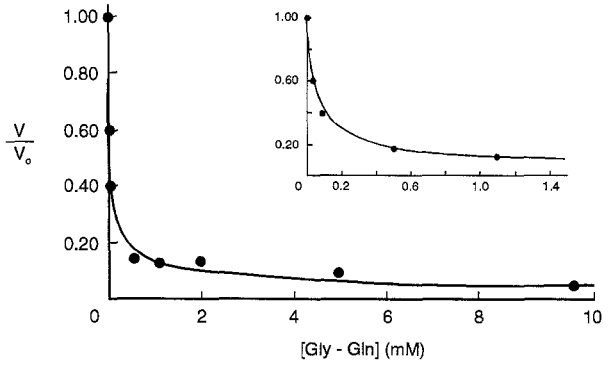

Fig. 4. Kinetics of self-inhibition of tracer Gly-Gln uptake into renal bbmv, showing evidence for two transport systems. The fractional inhibition (V/V_o) of ^{3}H-Gly-Gln (0.1 μM) influx by unlabelled peptide (0–10 mM, insert 0–1 mM) is shown. Superimposed on the data (Daniel et al., 1991) is the best fit for the sum of two saturable transport systems with kinetic constants of respectively 0.044 and 2.6 mM, giving a calculated value (*see* Devés et al., 1991) of the ratio of the permeabilities (V_{max}/K_m) of the two transporters of 0.13 ± 0.01.

system. Their evidence is kinetic, and is shown (replotted) in Fig. 4. They observed that the relationship between flux and concentration is not readily fitted to a single Michaelis-Menten relationship; it is, however, adequately described by the sum of two such terms. Daniel et al. (1991) characterized these as reflecting respectively the contributions of high affinity (HA) and low affinity (LA) transporters. The K_ms for the two systems are approximately 40 μM and 2.5 mM, and thus differ by more than 50-fold; in contrast, the V_{max} for the LA system is only 10-fold greater than that of the HA system. Thus at tracer concentrations of peptide, more than 80% of the flux will be through the HA system (*see* Fig. 4), whereas at high concentrations of peptide, >80% of the flux will be through the LA system. When the substrate concentration is 0.5 mM, the rate of entry of peptide through the two systems will be approximately equal. Because at present there is no way of differentially inhibiting either of the two systems, further analysis of the properties of the LA transporter is problematic. Daniel et al. (1991) have studied the effects both of pH gradients and of membrane potential on Gly-Gln uptake at low (0.1 μM) and higher (1 mM) concentration; they have analysed this data, corrected for diffusion, by fitting it to the sum of two Michaelis-Menten equations (i.e., they have sought to derive from the data unique estimates of four unknown kinetic constants, $K_{m(HA)}$, $K_{m(LA)}$, $V_{max(HA)}$, and $V_{max(LA)}$). This analysis will probably give reasonable estimates for the HA system since when the substrate concentration is very low the two constants for this system dominate. However, at higher substrate concentration there is greater uncertainty. An additional problem occurs when the transmembrane pH gradient is abolished; under these conditions it is no longer possible to fractionate the flux kinetically, since a single saturable relation between flux and substrate concentration is sufficient. Daniel et al. (1991) (interpret this as showing that only one system (LA) is active under these conditions; however, in the absence of a specific inhibitor for either the HA or the LA system it is not possible to distinguish this possibility from the alternative, namely that the ratio of V_{max}/K_m of the two systems under these conditions are sufficiently close to preclude experimental separation (cf. Devés et al., 1992; Eleno et al., 1994). In view of the interesting recent observation that ampicillin is not a substrate for the HA system (Daniel & Adibi, 1993), it could be rewarding to reexamine the kinetics of transport of Gly-Gln in the presence and absence of a high concentration of ampicillin. An analogous experimental approach was adopted by Devés et al. (1993), who showed that the kinetics for two separate routes of lysine transport could be analysed rigorously using *N*-ethylmaleimide (NEM) to inhibit one pathway preferentially.

In view of the greater certainty of the properties of the HA system, we will confine our comments to the transport (influx) of Gly-Gln through this system. System HA shows an affinity for its substrate which is substantially increased by imposing a steeper proton gradient (out > in) across the membrane; in contrast affinity is not substantially altered by changes of membrane potential. The V_{max} of system HA is increased both by an increased proton gradient and by hyperpolarization of the vesicle membrane. (While the effect on V_{max} is supported by the studies of Temple et al. (1994) and Boll et al. (1994) and by the evidence provided in Fig. 4f of Fei et al. (1994), it is notable that the latter authors state that "transport of Gly-Sar by PepT1 . . . is almost completely independent of [membrane potential] V_m.") Taken together, these findings suggest that the binding site of the peptide substrate on the HA transporter is external to the membrane field, and that this transporter in its unloaded form carries a net negative charge in its transmembrane domains (cf. Eleno et al., 1994), a prediction confirmed by Fei et al. The Hill plot for the HA system suggests a stoichiometry of 1:1 (*see above*). In their most recent contribution, Daniel and Adibi (1993) implicitly identify system HA as "the oligopeptide/H^+ symporter." The LA system, which is capable of operating in the absence of a proton gradient and shows less discrimination with respect to substrate structure, is described as "a second transport system," and may be identical (or related) to the 127 kDa glycoprotein originally isolated by photoaffinity labeling from both intestinal and renal brush-border membranes by Kramer and colleagues (*see* Kramer et al., 1994).

In contrast to the detailed studies on the kinetics of peptide transport in the kidney, there is no convincing evidence for kinetic heterogeneity in small intestine. Indeed, the important recent work of Fei et al. (1994) indicates that PepT1 is likely to be the major (if not the

only) transporter responsible for the translocation of Gly-Sar in enterocytes, since injection of unfractionated small intestinal mRNA which had been hybrid depleted with an antisense oligonucleotide to the 5′ end of the coding region of PepT1 cDNA failed to increase uptake above control levels in *Xenopus* oocytes. Although this work allows a clear conclusion to be reached in the case of Gly-Sar, it is important to note that it does not exclude the possible existence of other peptide transporters for other substrates.

Studies on the Peptide Transporter Protein

The work of Kramer and colleagues with photolabeled derivatives of β-lactam antibiotics (mentioned above) has lead to the isolation and reconstitution of a 127 kDa glycoprotein from rabbit small intestine, which has been suggested to be the LA transport system. These studies have resulted in the elucidation of certain features of the protein. Most interestingly, by the use of chemical modification of certain specific amino acid residues, Kramer's group have shown that: (i) histidine residue(s) are involved, as demonstrated by the inhibition of transport seen when the membranes are pre-incubated with diethylpyrocarbonate (DEPC); and (ii) acetylation of tyrosine residues by *N*-acetylimidazole (N-AI) has an inhibitory effect on cephalexin uptake into rabbit intestinal bbmv. (Due to the substrate concentrations used in these studies (e.g., 2 mM), analogy with the kidney (*see above*) would suggest that approximately 85% of cephalexin transport would be by the LA system.) The relative specificity of these inhibitors was shown by their lack of effect on other transporters in these membranes (e.g., Na^+/alanine, Na^+/glucose, and Na^+/taurocholate). In contrast, a range of other protein modifying agents, such as those which affect cysteine residues (e.g., NEM, PCMBS), vicinal cysteines (e.g., PAO), and amino groups (e.g., NBD-chloride) did not inhibit cephalexin transport, although these agents had marked effects on the other transporters. It seems possible that these and other reagents may have differential inhibitory effects on the HA and LA transport systems that could usefully be exploited experimentally.

Until more information is known about the molecular structure of this peptide transporter, the characteristics of the substrate binding site can be inferred only from chemical modification studies such as these. For example, the inhibition seen on *N*-carboxyethylation of the imidazole ring of the histidine residue(s) of the transporter in intestinal bbmv has lead Kramer to postulate that the α-amino group of a transportable substrate binds to a histidine residue of a transport protein. Such residues may also be involved in the proton binding needed for stimulation of substrate translocation through proton-peptide cotransport. Similarly, chemical modification of the transporter with N-AI suggests that a tyrosine residue is involved in substrate binding; this hypothesis receives support from the observation that in the presence of substrate the rate of inactivation by N-AI is reduced. Such differences in the detailed behaviour of intestinal and renal oligopeptide/antibiotic transporters with respect to their sensitivity to chemical modification adds weight to the notion that the transporter molecules in these two tissues are not identical. The isolation by Kramer and colleagues using photo-affinity labeling of candidate peptide transporters of similar molecular weight suggests that they are, nevertheless, likely to be closely related. The simplest explanation is that a family of transporter isoforms is differentially expressed in the two tissues.

In view of their similar tissue distribution, substrate specificity and molecular size, it seems possible that the protein HPT-1 (*see below*) may be a candidate for the transporter purified biochemically by Kramer et al. (1994).

The Molecular Basis of Oligopeptide Transport

Expression Cloning of PepT1

The paper of Fei et al. (1994) marked the advent of molecular biology to the field of peptide transport. In yet another triumph for expression cloning, the authors of this paper isolated a rabbit intestinal cDNA which when expressed in *Xenopus* oocytes, induced uptake of labeled dipeptide (Gly-Sar). The protein encoded by this gene was designated PepT1, and showed weak homology to certain proton-coupled prokaryote transporters. The full length clone coded for a protein of 707 amino acids, which was not dissimilar to the estimated molecular weight (60kDa) of the endoglycosidase H treated protein on reducing gels.

Using the same technique, Boll et al. (1994) reported the cloning of a gene, which, when expressed in *Xenopus* oocytes, resulted in pH-dependent transport of the amino-cephalosporin cefadroxil. Although the functional studies had suggested otherwise, when sequenced the coding section of this gene was shown to be identical to PepT1, although there were some differences in the 5′ and 3′ untranslated regions. The differences in the pH dependence of transport and effects of membrane potential seen by Boll et al. (1994) were presumably as a consequence of differences in the experimental protocols.

The Membrane Model of PepT1

A striking feature of the protein was the presence of 12 hydrophobic sequences each of sufficient length to span the lipid bilayer (Fig. 5). The authors propose, on the basis both of potential N-glycosylation sites ($Asn_{50, 439, 498, 513}$) and potential sites for phosphorylation (by PKA and PKC, of Thr_{362} and Ser_{357} respectively) that the amino and carboxy terminals are intra-

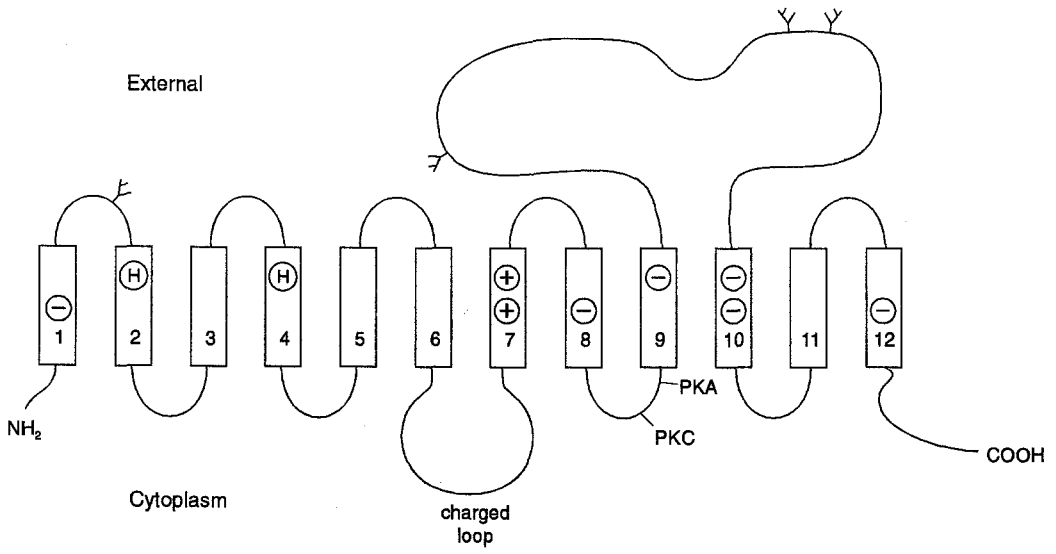

Fig. 5. Membrane model of PepT1 (modified after Fei et al., 1994). H = histidine residue; PKA and PKC represent proposed sites of phosphorylation by protein kinases A and C respectively; TM charged residues and potential sites of glycosylation are also shown.

cellular. Thus the model proposed by the authors for the primary structure of PepT1 has a very short amino terminal followed by 12 transmembrane (TM) domains connected by loops by varying length. The loop between TM9 and 10 (extracellular) is unusually large, containing 202 residues and three of the four potential N-glycosylation sites. Another notable loop is that connecting TMs 6 and 7 (intracellular), which has a high density of cationic amino acids (>25%) suggesting interaction with another (intracellular) protein.

Given the role of the protein as a transporter, and in view of its exceptionally large range of substrates, both with respect to size and charge, the nature of the substrate binding site and the TMs will be especially interesting. Although the membrane model of PepT1 does not directly address substrate binding, taken together with kinetic studies (Daniel et al., 1992; Temple et al., 1994) which show voltage dependence of K_m, and therefore by inference of substrate binding, we suggest that residues within the TMs will contribute to the peptide binding process. Moreover, the effects of external proton concentration on the kinetics imply that among the residues which contribute to the substrate binding site will be ones which are titrated by changes in extracellular pH over the range 5.5 to 7.4. Together, these two observations predict that histidine residues within the TMs are candidates for contributing to substrate binding. Interestingly there are two such residues (His_{57} and His_{121}) towards the outer ends of TMs 2 and 4 respectively. It will be of considerable interest to see if these histidine residues are conserved in other members of the PepT family (e.g., PepT2) as their sequences are elucidated. Additionally, given the effects of membrane potential on the V_{max} of peptide uptake, it is predicted that the part of the protein which lies within the membrane field should carry an overall net negative charge. It is thus interesting that the model of Fei et al. (1994) predicts a net negative charge when all charged amino acids in the predicted TMs are considered (of –2 to –4, depending on the degree of protonation of the TM histidine residues).

A further notable feature of the TMs is the prevalence of helix-breaking amino acids (Pro and Gly). These are present in all except TM12, with an average of two per TM. By acting as 'hinges', such residues may allow sufficient flexibility within the ensemble of the TMs to be the structural feature responsible for the unusually diverse range and size of transported substrates (as discussed earlier).

A Potential Subunit for a Peptide Transporter?

Published almost simultaneously to the sequence of PepT1, Dantzig et al. (1994) reported the properties of a 120 ± 10KDa protein (HPT-1, a putative human peptide transporter), which has been isolated immunologically from the human tumour cell line Caco-2. Remarkably, this 832 amino acid protein (predicted to be 92KDa when deglycosylated) had but a single TM, and showed strong sequence homology (in the large extracellular domain) to the cadherin superfamily of calcium-dependent adhesion proteins. When expressed in CHO cells, the uptake of peptides (cephalexin and bestatin) was consistently 2- to 3-fold higher than in the transfectant control. This is a modest increase when compared to the 63-fold increase in uptake into PepT1-expressing oocytes, suggesting that HPT-1 may not be a complete peptide transporter. The sequence of the single TM domain of HPT-1 is ILLT-

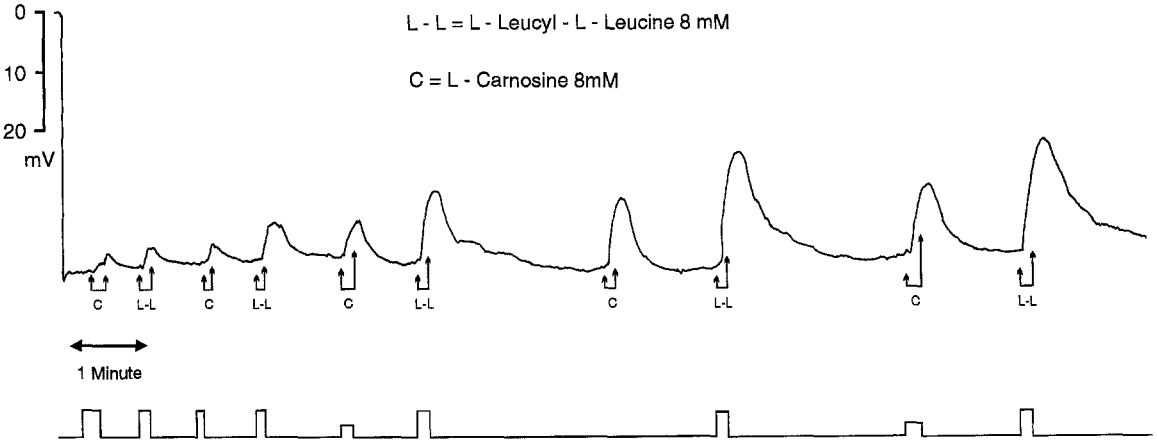

Fig. 6. Intracellular electrical response of intestinal brush-border membrane to the repetitive application of dipeptide (carnosine, C; Leu-Leu, L-L). *See* text for explanation. (Boyd and Ward, unpublished).

TLLVIGIILAVVFI (residues 791 to 808); three points of interest arise from this sequence. Firstly, this TM sequence is not homologous to any of the 12 TMs of PepT1. Secondly, the absence of any charged residues confirms that the HPT-1 protein alone cannot be responsible for the voltage-dependent kinetics of peptide transport (as discussed above). Finally, Gly_{800} is not conserved in other members of the cadherin family, which is compatible with HPT-1 being able to "associate with [another] membrane protein to form a multimeric protein that exhibits transport activity" (Dantzig et al., 1994). HPT-1 is similar in having a single TM to two other recently described proteins which when expressed induced transport activity (for dibasic and neutral amino acids, Wells & Hediger, 1992, Bertran et al., 1992); it seems probable that all three will be confirmed in having regulatory functions on the actual transporter in question.

Is Peptide Transport Regulated?

There is relatively little information available on the regulation of peptide transport. In the intestine during starvation there are differential long-term effects on the rates of peptide and amino acid uptake; the literature on this is reviewed by Matthews (1991). It is not clear whether the observed increase in the rate of peptide uptake and concomitant decrease in the rate of amino acid absorption is due to direct effects on the transporter proteins, or is a consequence of reduced luminal and increased cytosolic peptidase activity.

In the short term, unpublished data of C. Boyd and M. Ward (Fig. 6) suggest that in *Necturus* over a period of approximately 15 min, the intracellular electrical response to applying either L-Leu-L-Leu (L-L) or L-Carnosine (C) (both at 8mM) to small intestine *in situ* can in certain circumstances increase following repetitive exposure to the peptides. This suggests that with saturating concentrations of peptide, the augmented electrical response is due to an increase in the number of functional peptide transporters in the apical membrane. An increase in the number of functional transporters could either be brought about by insertion of more transporters from an intracellular pool, or by activation of existing transporters. An obvious way in which this latter possibility could occur is by altered covalent modification of the transport protein; Brandsch et al. (1994) have suggested that protein kinase C may modulate peptide transport, with phosphorylation reducing the V_{max}, presumably by phosphorylation of Ser_{357} of the PepT1 sequence. Analogous studies on a variety of amino acid transporters have shown that hormones can produce such effects in a wide range of epithelial tissues (Kilberg, 1987). The potential role of HPT-1 as a modifier of transport activity has been discussed above.

Peptide Efflux Across the Basolateral Membrane of Epithelial Cells Involves a Different Mechanism

In general, the transport properties of the basolateral membrane of epithelial cells are less well characterized, and this is also true for oligopeptide transport. Recently, three experimental models have been used to investigate the exit step in transepitheial peptide transport: intestinal basolateral membrane vesicles, monolayers of Caco-2 cells, and uptake across the contraluminal membrane of the kidney proximal tubule.

Studies on the transport of Gly-Pro into basolateral membrane vesicles (bmv) prepared from rabbit small intestine by Dyer et al. (1990) revealed the presence of a transport system for this peptide. Uptake into the bmv was stimulated by an inwardly directed proton gradient; this stimulation was abolished in the presence of the protonophores CCCP or TTFB. Although these data suggest the presence of a peptide-proton symport, it is strikingly different (*cf.* Fig. 2) to the apical oligopeptide transporter in that it is electroneutral. This is clearly

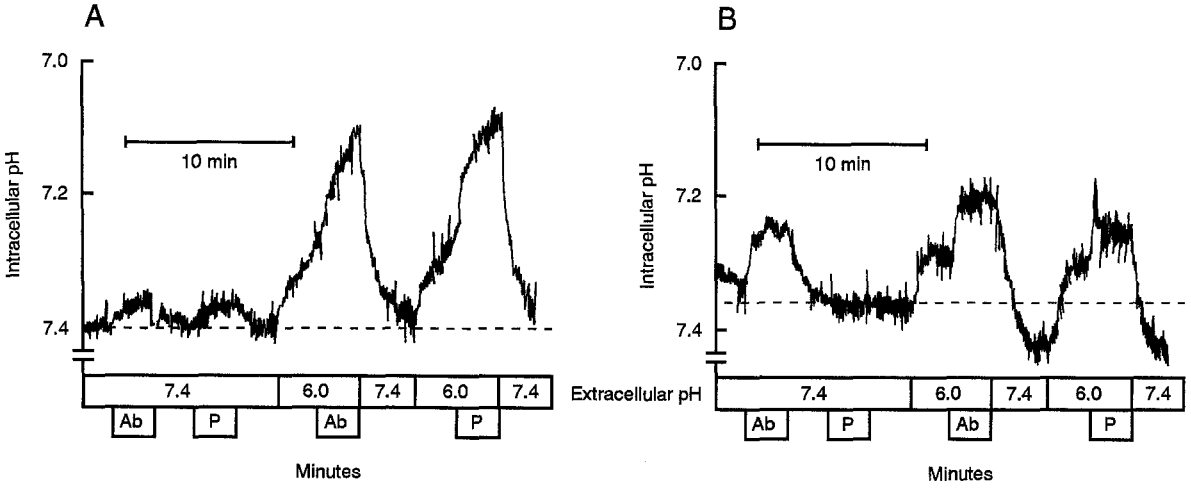

Fig. 7. Intracellular pH in Caco-2 monolayers exposed to the peptide Gly-Sar (P) or the antibiotic cephalexin (Ab) at either the apical (*A*) or the basal (*B*) surface. External pH was varied as shown. (Redrawn from Thwaites et al., 1993*b*).

shown in the paper of Dyer et al. (1990) when they measured the initial rates of 10 mM Gly-Pro uptake into the vesicles from external media containing 100 mM sodium thiocyanate or sodium gluconate. Due to the relative permeabilities of these anions (thiocyanate >> gluconate), if the transport of Gly-Pro resulted in a movement of net positive charge into the vesicles (as is the case in bbmv), then a faster initial rate of peptide transport would be expected in the presence of thiocyanate. However, there was no difference in the initial rates under either of these conditions, suggesting an electroneutral process.

Thwaites et al. (1993*b*) studied the transport of peptides and antibiotics across monolayers of cultured Caco-2 cells (a human intestinal epithelial cell line) grown on semipermeable filters. A combination of techniques was used, including measurement of the flux of radio-labeled substrate, the transepithelial electrical events associated with peptide transport, and intracellular pH using the pH-sensitive dye BCECF. As can be seen in figure 7*A*, when either 20 mM Gly-Sar (*P*) or cephalexin (*Ab*) was added to the apical surface of the monolayers, in the absence of an inwardly directed pH gradient there was a small intracellular acidification. When the experiments was repeated in the presence of an inwardly directed proton gradient, then the observed fall of intracellular pH was larger. These results are consistent with there being peptide-proton symport at the apical membrane of these cells, for which the antibiotic is also a substrate. If the experiment is performed with substrate at the basal surface of the monolayer (Fig. 7*B*), then only cephalexin is capable of producing intracellular acidification in the absence of a proton gradient. In the presence of an inwardly directed proton gradient, then both Gly-Sar and cephalexin produced an intracellular acidification, although of a much smaller magnitude than that observed at the apical membrane. Of particular note in Fig. 7*B* is the transient intracellular *alkalinization* observed on the simultaneous removal of cephalexin and the proton gradient; this strongly suggests that the basal transporter can be reversed by the exit of accumulated cephalexin from the cells. This effect is not seen in the apical membrane, and this, along with the apparent electroneutrality of the transport process shown by Dyer et al. (1990) and the inability of Kramer (1994) to localize the 127 kDa glycoprotein immunocytochemically to the basolateral membrane of rabbit intestine or kidney, necessitates the proposal of a different mechanism for peptide exit across the basolateral membrane. The studies of Inui et al. (1992) and Saito and Inui (1993) show the mechanisms of cephalosporin and bestatin uptake by the dipeptide transport system at the apical and basal surfaces of Caco-2 cells to differ in both their pH dependence and inhibitor sensitivity. Again, the authors' findings suggest that the processes responsible for the entry of these peptides at the two faces of the epithelium are different.

A Model for Transepithelial Peptide Transport

Figure 8 shows a model for the transepithelial transport of an oligopeptide based on the traditional models for transepithelial organic solute transport, in which we try to account for all the findings mentioned so far based on the transport of a peptide with a neutral pK (e.g., 7.0). While in the lumen, where the pH is approximately 6.5 due to the operation of the Na/H exchanger and the effects of unstirred layers, the peptide is predominantly in its zwitterionic state. It crosses the apical membrane via the electrogenic proton-peptide transporter which has been widely described (e.g., Ganapathy & Leibach, 1983). Due to charge translocation associated with pep-

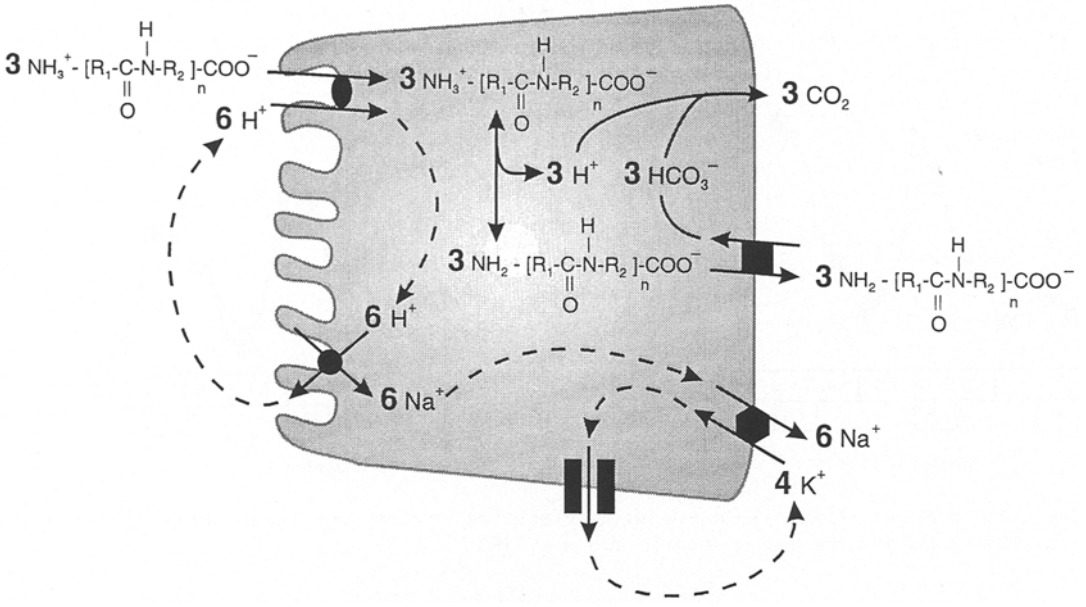

Fig. 8. Model of transport systems involved in transepithelial peptide translocation.

tide transport, this transporter is not reversed when the substrate and proton gradient are removed (Fig. 7*a*). On entering the cell, the peptide is exposed to an environment with a pH of 7.2, so that a sufficient proportion of the peptide will now be in an anionic state to cross the basolateral membrane, coupled to the entry of a bicarbonate ion (equivalent to the exit of a proton), i.e., in an electroneutral fashion. This proposed exit step is consistent with an electroneutral process (*cf.* Dyer et al., 1990), transporter reversibility and proton-dependence (*cf.* Thwaites et al., 1993*a,b*). Removal of this species will permit further net delivery of anionic peptide according to its reaction equilibrium. In order to maintain a steady-state intracellular pH, the cell extrudes protons at the apical membrane in exchange for sodium ions, which are pumped out of the cell by the basolaterally located Na^+/K^+-ATPase. The potassium brought in by the pump leaves via basolateral K^+ channels, thus maintaining the steady-state cell potential. It is additionally proposed that basally applied Gly-Sar fails to elicit intracellular acidification in the absence of an inwardly-directed proton gradient because it is a low affinity substrate for this transporter (e.g., Fei et al., 1994).

The hypothesis that peptides may cross the basolateral membrane via an anion transporter has received support from recent studies on the in vivo microperfused rat kidney by Ullrich et al. (1993). These authors studied the affinity of a range of aromatic peptides and cephalosporin antibiotics for the organic anion (p-aminohippurate, PAH) and the organic cation (*N'*-methylnicotinamide, NMeN, or tetra-ethylammonium, TEA) transporter. All of the peptides and all but one of the antibiotics tested had pK values around neutrality, and had a markedly higher affinity for the PAH transporter over that for the NMeN transporter (the apparent K_Is were at least tenfold higher for the PAH transporter). Cephaloridine was the only cephalosporin to have a higher K_I for the NMeN transporter than for the PAH transporter (2.1 *vs.* >12.5 mM). As the pK of this antibiotic is 3.4, at pH 7.4 it would be predominantly in the positively charged form, and therefore would be expected to be translocated by the cation transporter. It would be of interest to know whether basal entry of this substrate would produce an intracellular alkalinization in Caco-2 cells.

Peptide Transport Across the Intact Epithelium

Figure 8 suggests a plausible model for the transepithelial transport of an intact oligopeptide. The evidence for this model is largely derived from studies discussed above, and is most appropriate for the small intestine. Nevertheless it may be extended to other tissues, in particular the proximal tubule. What is not indicated in Fig. 8 is the role of peptidases, both intra- and extracellular. It seems highly likely that under physiological circumstances the rate of intracellular peptide hydrolysis is sufficiently fast to ensure that only minimal amounts of intact peptide appear in the vascular compartment, in spite of the very rapid rate of entry of peptide into the epithelial cell. This implies that the route of exit used by the intact peptide is very much slower than is either the apical entry step or, for most peptides, intracellular hydrolysis. The other important point which Fig. 8 omits is the contentious issue of the physiological significance of peptide delivery across the epithelium *via* a paracellular

pathway. This is covered in detail in a recent review by Gardner (1994). Thus, after a meal, peptides resistant to hydrolysis will be found in the portal vein, but the vast majority of absorbed α-amino nitrogen will appear as amino acids, despite being taken up into enterocytes from the intestinal lumen as oligopeptide as shown by e.g., Sykes et al. (1995).

Peptide Transport in Other Epithelia

Recent work suggests that transport of small peptides is found in epithelia such as placenta, lung, blood-brain barrier, and distal nephron. However, for all of these tissues peptide transport has been identified using functional assays; the relationship between these processes and those summarized in Fig. 8 is thus often not clear. In the placenta, Vatish et al. (1992) have confirmed the earlier findings of Ganapathy et al. (1985) that oligopeptides are transported intact into isolated bbmv. However, the uptake of the dipeptide L-Trp-L-Tyr was electroneutral and not stimulated by an inwardly directed pH gradient. Placental oligopeptide transport (for which a similar system was identified at both the apical and basal surfaces) may thus involve anion-exchange at both surfaces, and this tissue may lack the distinct apical system found in intestine and kidney.

The lung is a fore-gut derivative, and it is therefore perhaps not surprising that Boyd et al. (1993) and Helliwell et al. (1994) have found evidence for the intact transepithelial transport in rat lung of a dipeptide (D-Phe-L-Ala) and a tripeptide (L-Ala-D-Phe-L-Ala), both of which are hydrolysis-resistant. "Moreover, Meredith & Boyd (1995) showed uptake of these peptides into bbmv prepared from rat lung. This process was electrogenic and stimulated by an inwardly directed pH gradient". This process was electrogenic, and more recent studies (*unpublished data*) show that uptake into bbmv is stimulated by an inwardly directed pH gradient. Due to the relatively high substrate concentrations used in these studies, the possibility of LA and HA systems analogous to those described in the kidney cannot be discounted. Very recently, Morimoto et al. (1993) have confirmed that type II cells from rat lung, when cultured as monolayers, are able to translocate dipeptides; this supports the preliminary finding of Meredith and Boyd (1992) who used acutely isolated type II cells. The absence of secreted pancreatic proteases from the lumen of lung alveoli, and the different cytosolic peptidases in this tissue, suggest that the lung may be a site allowing effective delivery of small, therapeutically active peptides. The experiments of Morimoto et al. showed that intact L-L peptide can cross a monolayer of alveolar type II pneumocytes. In an *in situ* perfused rat lung however, Helliwell et al. (1994) failed to observe transepithelial transport of intact L-L peptide, although the mixed isomer di- and tripeptides were measured in the vascular perfusate. Taken together, these experiments suggest that peptidases in the pulmonary vascular bed make a significant contribution to peptide hydrolysis in the intact lung.

Peptide Transporters Involved in Antigen Presentation

Rather remarkably, a family of intracellular (ER) peptide transporters in antigen presenting cells has recently been shown to be critically involved in the cellular immune response (*see* e.g. Neefjes et al., 1993; Sheppard et al., 1993). Antigen presentation also occurs in epithelial cells (*see* e.g., Gonnella & Wilmore, 1993); however, the processes occurring in HLA-linked class I antigen presentation appear to be fundamentally different from those discussed in this review, since these intracellular peptide transporters in the "immune" system are driven by primary active transport involving ATP hydrolysis (the cloned transporters turning out to be members of the ATP-binding cassette (ABC) superfamily of membrane proteins). Additionally, these transporters appear to be able to translocate larger peptide substrates than those described here. Although there is no sequence homology apparent between PepT1 and Tap-1 (the antigen presenting transporter), the TMs of both proteins are characterized by high abundance of proline, glycine and alanine residues. Future work on the molecular basis of peptide transport by site-directed mutagenesis will now allow the functional role of specific residues in these two important families of transport proteins to be elucidated.

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