low depressions in 2% agar. Labeled cells were visualized on a Zeiss epifluorescence microscope with objectives ranging from ×2.5 to 40 at an excitation wavelength of 540 to 560 nm. Data were recorded onto a video optical disk recorder (OMDR) with a light-intensifying camera (Hamamatsu SIT) and image processor (Imaging Technology 151, Woburn, MA) and the VidIm software package (G. R. Belford, J. Stollberg, S. E. Fraser, unpublished data) (*16*).
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31. We thank R. G. Northcutt, M. Bronner-Fraser, S. Cohen-Cory, C. Gans, J. Shih, G. N. Serbedzija, and S. C. Smith for comments. Supported in part by NSF grant 8821241 and AAFD and RSCA grants from San Diego State University (to P.M.M.), USPHS grants HD25138 (to M. Bronner-Fraser) and HD26864 (to S.E.F.), and a fellowship from the Muscular Dystrophy Association (to A.C.).

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Association of Intestinal Peptide Transport with a Protein Related to the Cadherin Superfamily

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The first step in oral absorption of many medically important peptide-based drugs is mediated by an intestinal proton-dependent peptide transporter. This transporter facilitates the oral absorption of β-lactam antibiotics and angiotensin-converting enzyme inhibitors from the intestine into enterocytes lining the luminal wall. A monoclonal antibody that blocked uptake of cephalexin was used to identify and clone a gene that encodes an approximately 92-kilodalton membrane protein that was associated with the acquisition of peptide transport activity by transport-deficient cells. The amino acid sequence deduced from the complementary DNA sequence of the cloned gene indicated that this transport-associated protein shares several conserved structural elements with the cadherin superfamily of calcium-dependent, cell-cell adhesion proteins.

Several peptidyl mimetic drugs, such as β-lactam antibiotics and angiotensin-converting enzyme inhibitors, are readily absorbed orally in humans. These drugs along with di- and tripeptides share a transport mechanism that is a major route for the assimilation of peptide nutrients in the intestine (*1*). Substrates are cotransported with protons and concentrated initially within the intestinal enterocyte before exiting the serosal side of the enterocyte (*2*). Information about the transporter would be helpful in the rational design of new oral small peptidyl-like therapeutic drugs.

When confluent, the human colon adenocarcinoma Caco-2 cells differentiate and acquire intestinal enterocytic-like properties (*3*). These cells possess a proton-dependent peptide transporter that takes up β-lactams including cephalexin, cefaclor, and loracarbef (*4*). Properties of this transporter closely resemble those of the intestinal peptide transporter of other species (*4*). The biochemical purification of the rabbit intestinal peptide transporter revealed a ~127-kD integral membrane glycoprotein that exhibits transport activity when reconstituted into liposomes (*5*). The gene encoding this transporter, however, has not been identified.

To identify the human intestinal peptide transporter, monoclonal antibodies were prepared that blocked cephalexin uptake into Caco-2 cells (*6*), and monoclonal antibody (mAb) 13G6 was selected. Membrane proteins of Caco-2 cells were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotted with antibody (Fig. 1, lane 1). A single protein band with an apparent mass of 120 ± 10 kD was detected in Caco-2 membranes. Deglycosylation with endoglycosidase F before SDS-PAGE shifted the immunoreactive band to ~100 kD (*7*). A survey of membranes from several human cell lines indicated the presence of the 120 ± 10 kD protein in cells derived from the gastrointestinal (GI) tract but not in cell lines derived from other tissues (Fig. 1). Immunohistochemical staining of normal human tissues indicated the presence of antigen in tissues along the GI tract (jejunum, duodenum, ileum, and colon) and the pancreatic ducts and the absence of antigen on specimens examined from the kidney, lung, liver, brain, adrenal gland, and skin (Fig. 2).

A Caco-2 complementary DNA (cDNA) library constructed in λgt11 (*8*) was screened with mAb 13G6 for expression of the antigen. A ~750–base pair (bp) cDNA fragment was repeatedly recovered. The deduced amino acid sequence of the open reading frame (ORF) of this cDNA showed a protein with mass of ~35 kD, considerably smaller than the expected size of ~100 kD. Therefore, a new cDNA

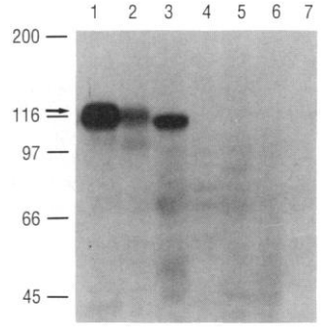

Fig. 1. Immunoblot analysis of membranes from human cell lines with mAb 13G6 to transporter. Membrane proteins (~100 μg per lane) were separated by 8% SDS-PAGE for ~14 hours (*21*). Gel was electroblotted onto nitrocellulose paper; blocked with 3% bovine serum albumin and 0.1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.01 M tris-HCl (pH 7.5); and reacted with purified mouse antibody to transporter (20 μg/ml) followed by incubation with goat antibody to mouse [^{125}I]immunoglobulin G (IgG) (0.3 μCi/ml). Lane 1, colon Caco-2; lane 2, colon HT-29; lane 3, colon COLO 320; lane 4, lymphoblast CCRF-CEM; lane 5, lymphoblast IM-9; lane 6, neuroblastoma SK-N-MC; lane 7, glioblastoma U-373. The arrow indicates the band for a 120 ± 10 kD protein, the putative transporter. Molecular masses are given to the left in kilodaltons.

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library was prepared in λgt11 from 2-day, post-confluent Caco-2 cells and was screened with the cloned ~750-bp fragment after labeling. This process produced a clone of 3345 bp containing a 2496-bp ORF designated *hpt-1* (9). The calculated mass of the encoded 832–amino acid putative transport protein was 92 kD, consistent with the apparent size of the deglycosylated transporter.

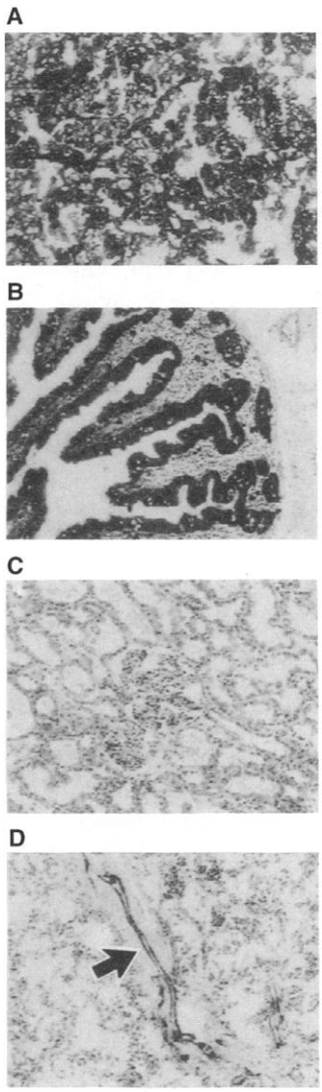

Fig. 2. Immunohistochemical analysis of antigen expression in human tissue specimens. (**A**) Two-week-old post-confluent Caco-2 cells or frozen tissue specimens (5-μm sections) were dried and fixed with formalin (*22*). Specimens were incubated with mAb 13G6 to transporter (5 μg/ml) or with an isotype-matched mAb of irrelevant specificity, followed by incubation with a secondary goat antibody to mouse IgG conjugated to horseradish peroxidase. Subsequently, samples were incubated with the chromogenic substrate diaminobenzidine (*22*). An irrelevant control antibody showed no appreciable staining of the same tissues (*23*). Other specimens shown are (**B**) jejunum, (**C**) kidney, and (**D**) pancreas. The arrow points to the staining of the pancreatic ducts. The staining of the jejunum is representative of staining of other GI tract specimens (*23*); a lack of staining of the kidney is representative of other antigen-negative specimens indicated in the text (*23*).

Protein database searches (*10*) revealed similarity of HPT-1 to members of the cadherin superfamily of calcium-dependent, cell-cell adhesion proteins (Fig. 3). Although HPT-1 is only 20 to 30% identical to individual cadherins, it retains several features conserved in most members of this family. In contrast, the cadherins are 50 to 70% identical to one another and share a common basic structure with an extracellular region containing at least two internal repeats, a transmembrane domain, and a highly conserved intracellular region (*11*, *12*). The HPT-1 protein contains two hydrophobic regions, one at the NH_2-terminus characteristic of signal peptides and one near the COOH-terminus (amino acids 778 to 809) that may form a transmembrane domain. The extracellular region of HPT-1 contains three internal repeats located approximately between amino acids 60 to 700. This region also contains three copies of the four and five amino acid repeats LDRE and DXNDN typically repeated in the cadherins three to four times. The spacing of these repeated elements in HPT-1 is similar to that seen in the cell-cell adhesion proteins (Fig. 3). In addition, the region preceding the putative hydrophobic transmembrane domain contains four Cys residues in an analogous position to that of conserved Cys residues in the cadherins. Seven potential sites for *N*-linked glycosylation are present in the putative extracellular region that may result in the shift in apparent molecular mass after enzymatic deglycosylation. In sharp contrast to the cadherins, HPT-1 lacks a cytoplasmic domain corresponding to the most highly conserved region found in the cadherin superfamily. This intracellular domain is required for the cadherin cell-cell adhesion function (*13*) and may be responsible for their binding to actin-based cytoskeletons and other cytoplasmic proteins (*12*).

The *hpt-1* ORF was subcloned into the mammalian expression vector pRc/RSV and used to transfect Chinese hamster ovary cells (CHO) (*14*). Immunoblot analysis (Fig. 4A) indicated the presence of an immunoreactive 120 ± 10 kD protein in membranes from transfectant clone 9 that co-migrated with the immunoreactive protein observed in Caco-2 membranes and that was absent in the transfectant control membranes (Fig. 4A). Transfectant clone 9 possessed ~5% of the immunoreactive protein present in Caco-2 cells. Cephalexin uptake by these transfectants was compared in 25 independent experiments measured in triplicate over ~1.5 years; transfectant clone 9 consistently showed two- to three-fold higher uptake than the transfectant control. After an 18-day growth period (Fig. 4B), cephalexin uptake by clone 9 was 2.7-fold higher than the transfectant control (2.36 ± 0.16 and 0.89 ± 0.14 nmol per milligram of protein per 2 hours, respectively; $P < 0.001$). Drug uptake by transfectant clone 9 was inhibited significantly by a 25-fold excess of unlabeled cephalexin. When these cells were incubated at pH 7.5 or pH-clamped at pH 6.0 with KCl and nigericin, uptake was inhibited significantly, indicating the necessity of an inwardly directed proton gradient for uptake (*4*). Furthermore, the presence of the sulfhydryl reagents, dithiothreitol and phenazine methosulfate, inhibited uptake as expected, on the basis of the presence of Cys residues predicted from the gene sequence. In contrast, these conditions had little to no effect on cephalexin uptake by the control transfectant, indicating that uptake was due to simple diffusion in CHO cells lacking the *hpt-1* gene. Moreover, the incubation of Caco-2 cells in the same conditions gave an identical inhibition pattern of uptake to the transfectant clone 9, indicating that the properties of the peptide transporter were unaltered when expressed in CHO cells (*4*, *7*). Uptake of the dipeptide bestatin (Fig. 4C) was found to be significantly higher in clone 9 than in the transfectant control (twofold, $P < 0.001$). Bestatin uptake was

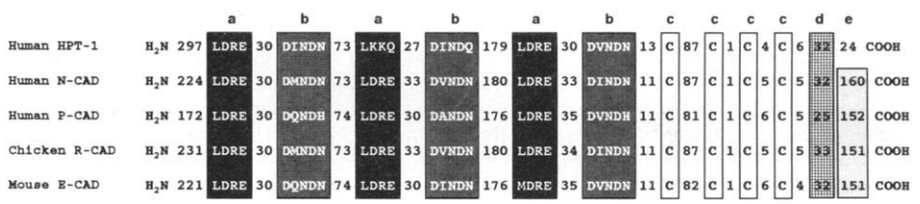

Fig. 3. Domain structures and partial amino acid sequences of HPT-1 and the cadherins. The putative human peptide transporter (HPT-1), human neural cadherin (human N-CAD) (*24*), human placental cadherin (human P-CAD) (*25*), chicken retinal cadherin (chicken R-CAD), (*26*), and mouse epithelial cadherin (mouse E-CAD) (*27*) are aligned with the NH_2-termini to the left and the COOH-termini to the right. Shown are the conserved LDRE repeats (a), DXNDN repeats (b), cysteines (c), hydrophobic transmembrane segments (d), and the conserved cadherin cytoplasmic domains (e). The number of amino acid residues separating the domains is shown (*28*).

inhibited significantly by 35 mM cephalexin in transfectant clone 9 but not in the transfectant control. Moreover, bestatin uptake into transfectant clone 9 was inhibited by 50 mM glycycl-D-proline and by pH clamping. These results are quite similar to those reported for bestatin uptake by *Xenopus laevis* oocytes that express Caco-2 mRNA (*15*). Cephalexin and bestatin uptake by clone 9 was dependent on the extracellular pH, and each inhibited the other's uptake (Fig. 4D), consistent with uptake by the intestinal apical peptide transporter of Caco-2 cells (*16*). Furthermore, the incubation of clone 9 with mAb 13G6 (15 μg/ml) reduced cephalexin uptake to the level of that measured in the presence of bestatin (*7*). The 2.7-fold increase in cephalexin uptake observed for transfectant clone 9 is in the same range that has been reported for some other cloned transporters (*17*).

Because transporters generally contain multiple transmembrane domains, an unexpected feature of HPT-1 is the presence of only one putative membrane-spanning region. In this regard, HPT-1 is similar to two recently described dibasic and neutral amino acid transporters (*18*). The presence of a single transmembrane domain in HPT-1 may indicate that the protein self-aggregates to form a fully functional intestinal peptide transporter or may associate with an as yet unidentified membrane protein to form a multimeric protein that exhibits transport activity. The Cys residues in the extracellular region may also be involved in aggregation.

The expression of the HPT-1 antigen along the GI tract is consistent with reports that the peptide transporter resides in these tissues (*19*). The lack of antigen expression in kidney tissue is consistent with reports that the intestinal and renal peptide transporters are distinct (*20*). The observation that the antigen is expressed along the pancreatic ducts suggests a potential new tissue site for the transport carrier.

Further characterization of the peptide transport protein will ultimately lead to a molecular understanding of the substrate binding site of the transport carrier. This information should greatly facilitate the rational design of new therapeutics for the treatment of human diseases that are orally absorbed by the human intestinal peptide transporter.

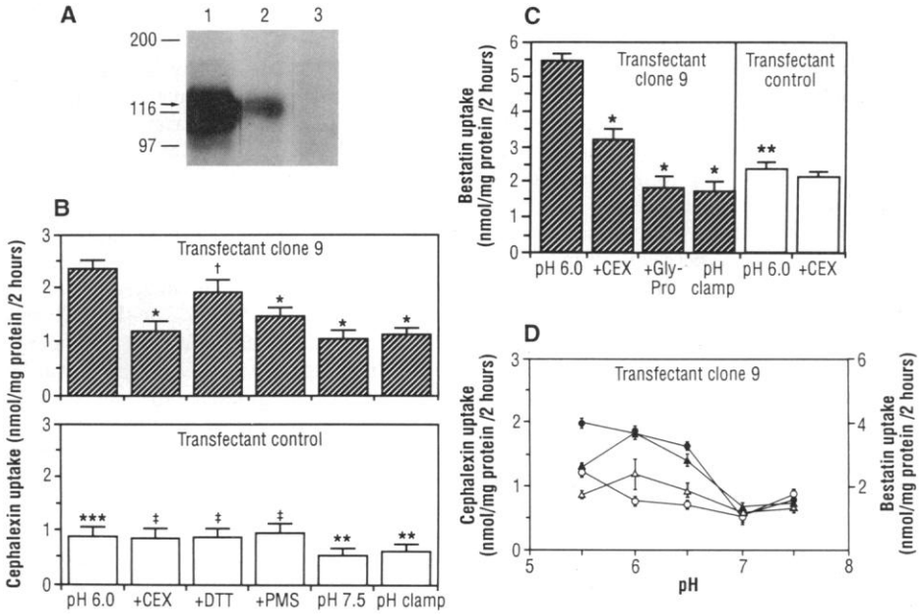

Fig. 4. Analysis of transfectants of CHO cells. (**A**) Immunoblots of membranes of Caco-2, CHO clone 9, and the CHO control. Membrane proteins (150 μg per lane) were separated by 8% SDS-PAGE and immunoblotted as described in the legend to Fig. 1. Lane 1, Caco-2 cells; lane 2, transfectant clone 9; lane 3, transfectant control. Molecular masses are given to the left in kilodaltons. (**B**) Cephalexin uptake by transfectants. Transfectants (~2.5 × 10^4 cells per well) were grown for 18 days in collagen-coated Costar (Cambridge, Massachusettts) 24-well plates. Uptake of 1 mM [^{14}C]cepha- lexin (Eli Lilly and Company, Indianapolis, Indiana) was measured for 2 hours at 37°C in sodium-free flux buffer (pH 6.0) (*4*). Drug uptake was measured in the absence or presence of 25 mM cephalexin (CEX), 10 mM dithiothreitol (DTT), or 5 mM phenazine methosulfate (PMS). Cells were pH-clamped at pH 6.0 with nigericin (10 μg/ml) and 135 mM KCl (which replaced choline chloride in the buffer) 10 min before and during the transport assay (*29*). Values represent the mean ± SE of 6 to 15 determinations and were determined in three independent experiments. Data were analyzed by a Student's *t* test: (single asterisk, double asterisk). They were significantly different ($P < 0.005$, $P \leq 0.05$, respectively) from uptake measured in pH 6.0 flux buffer in the absence of inhibitor for the same cell line. Dagger, $P = 0.12$; double dagger, not significantly different ($P > 0.5$). The 2.7-fold higher uptake in pH 6.0 flux buffer by clone 9 than by the control transfectant was highly significantly different on the basis of a *t* test (triple asterisk, $P < 0.001$). (**C**) Bestatin uptake by transfectants. Bestatin (1 mM) uptake was measured as described above and detected by high-performance liquid chromatography (*30*). Transfectants were 15 to 18 days old. Uptake was measured for 2 hours in sodium-free flux buffer (pH 6.0) in the absence or presence of 35 mM cephalexin or 50 mM glycycl-D-proline (Gly-Pro), or pH-clamped. Values represent the mean ± SE of six to nine determinations and represent three independent experiments. The single asterisk denotes that uptake was significantly different by *t* test ($P < 0.001$) from uptake measured at pH 6.0 for the same cell line. (**D**) Effect of extracellular pH on uptake. The uptake of 1 mM [^{14}C]cephalexin (filled circles) or 1 mM bestatin (filled triangles) by 13- to 18-day-old clone 9 cells was measured in the absence or presence of 15 mM bestatin (open circles) or 35 mM cephalexin (open triangles), respectively. The pH of the flux buffer was varied from 5.5 to 7.5 (*4*). Values are the mean ± SE of triplicate points. Curves are representative of two to three independent experiments. Uptake measured in the presence of the indicated inhibitor was significantly different by *t* test ($P < 0.05$) than uptake measured in the absence of inhibitor at pH 5.5, 6.0, and 6.5.

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from 24 positive pooled supernatants (~20% inhibition or greater) were assayed for the inhibition of drug uptake; two reduced uptake by 60 to 90%. Hybridomas were subcloned by limiting dilution and fluorescence-activated cell sorting [P. Marder *et al.*, *Cytometry* **11**, 498 (1990)]. Monoclonal antibody 13G6, an IgG1, was purified from ascites fluid with the use of protein A [P. L. Ey *et al.*, *Immunochemistry* **15**, 429 (1978)]. The hybridoma (HB 11272) is deposited in the American Type Culture Collection (ATCC) (Rockville, MD).
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31. We thank S. W. Lacey for providing the initial Caco-2 cDNA library; T. Bennett, S. Burgett, B. Glover, and J. Young for technical assistance; and Ethicon, Inc. (Somerville, NJ) for kindly supplying the collagen dispersion used for the cell culture.

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Association of Poly(CA)·Poly(TG) DNA Fragments into Four-Stranded Complexes Bound by HMG1 and 2

Claire Gaillard and Francois Strauss*

The tandemly repeated DNA sequence poly(CA)·poly(TG) is found in tracts up to 60 base pairs long, dispersed at thousands of sites throughout the genomes of eukaryotes. Double-stranded DNA fragments containing such sequences associated spontaneously with each other in vitro, in the absence of protein, forming stable four-stranded structures that were detected by gel electrophoresis and electron microscopy. These structures were recognized specifically by the nuclear nonhistone high mobility group (HMG) proteins 1 and 2 as evidenced by gel retardation. Such sequence-specific complexes might be involved in vivo in recombination or other processes requiring specific association of two double-stranded DNA molecules.

Tandemly repeated sequences of one to six nucleotides, known as microsatellites, are very common in eukaryotic genomes. Among the most frequently found is poly(CA)·poly(TG), which is present in mammals in approximately 10^5 tracts, each up to 60 base pairs (bp) long (*1*). Although the function of these sequences is still unknown, they are of interest for several reasons. First, their length at any given site in the genome varies, and this length polymorphism has been a useful source of genetic markers for genome mapping (*2*). Second, they enhance recombination between DNA molecules, as observed in eukaryotes (*3*) and bacteria (*4*). Third, their physical conformation changes readily from the orthodox right-handed B-DNA to left-handed Z-DNA with ionic conditions (*5*) or negative supercoiling (*6*), providing a useful model to study the function of Z-DNA.

We observed that DNA fragments containing poly(CA)·poly(TG) formed stable multimeric complexes in vitro (Fig. 1). By gel electrophoresis, a 120-bp double-stranded DNA fragment containing a 60-bp tract of poly(CA)·poly(TG) showed, in addition to the intense band corresponding to the starting DNA fragment, a ladder of slowly migrating weak bands (Fig. 1A, lane 1). No protein and no reagent known to bind DNA had been added to the sample, however. When DNA from the predominant band was electroeluted and immediately loaded on a new gel, no band ladder was observed (lane 3). But the ladder reappeared when the same sample was analyzed again on a gel a few days later (*7*). In the group of lower bands, two were readily identified as the single strands of the DNA fragment (lane 2, bands labeled CA and TG);

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