Hormonal regulation of oligopeptide transporter Pept-1 in a human intestinal cell line

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Thamotharan, Manikkavasagar, Shahab Zare Bawani, Xiaodong Zhou, and Siamak A. Adibi. Hormonal regulation of oligopeptide transporter Pept-1 in a human intestinal cell line. Am. J. Physiol. 276 (Cell Physiol. 45): C821–C826, 1999.—The intestinal oligopeptide transporter (cloned as Pept-1) has major roles in protein nutrition and drug therapy. A key unstudied question is whether expression of Pept-1 is hormonally regulated. In this experiment, we investigated whether insulin has such a role. We used a human intestinal cell monolayer (Caco-2) as the in vitro model of human small intestine and glycylglutamine (Gly-Gln) as the model substrate for Pept-1. Results showed that addition of insulin at a physiological concentration (5 nM) to incubation medium greatly stimulates Gly-Gln uptake by Caco-2 cells. This stimulation was blocked when genistein, an inhibitor of tyrosine kinase, was added to incubation medium. Studies of the mechanism of insulin stimulation showed the following. 1) Stimulation occurred promptly (30–60 min) after exposure to insulin. 2) There was no significant change in the Michaelis-Menten constant of Gly-Gln transport, but there was a nearly twofold increase in its maximal velocity. 3) Insulin effect persisted even after Golgi apparatus, which is involved in trafficking of newly synthesized Pept-1, was dismantled. 4) However, there was complete elimination of insulin effect by disruption of microtubules involved in trafficking of preformed Pept-1. 5) Finally, with insulin treatment, there was no change in Pept-1 gene expression, but the amount of Pept-1 protein in the apical membrane was increased. In conclusion, the results show that insulin, when it binds to its receptor, stimulates Gly-Gln uptake by Caco-2 cells by increasing the membrane population of Pept-1. The mechanism appears to be increased translocation of this transporter from a preformed cytoplasmic pool.

Caco-2 cells; gene expression; glycylglutamine; insulin; microtubules

STUDIES IN HUMAN SMALL intestine have established the presence of an oligopeptide transporter that has major roles in assimilation of dietary proteins and absorption of peptidomimetic drugs such as β-lactam antibiotics (2). This transporter was recently cloned and designated Pept-1 (9). This cloning provides a novel opportunity to investigate whether the expression of the intestinal oligopeptide transporter is metabolically regulated and, if so, to determine the mechanism of its regulation.

In metabolic regulation, insulin is usually the key hormone serving as a mediator. Therefore, the question becomes whether insulin has any effect on expression of Pept-1 and, if so, what cellular and/or molecular mechanisms insulin uses to cause the effect. In this experiment, we investigated these questions in a human intestinal cell line (Caco-2 cells) so that we could investigate dipeptide transport in the absence and presence of insulin as the only hormone.

We used glycylglutamine (Gly-Gln) as a model dipeptide for transport by the apical membrane of Caco-2 cells. Gly-Gln currently serves as a stable source of glutamine for cells in culture and for patients needing nutritional support (15, 21). Our previous studies have validated the use of the Caco-2 cell monolayer as the in vitro model for studies of Gly-Gln transport in human intestine (2, 19). For example, these studies showed that Gly-Gln uptake by Caco-2 cells is largely as intact dipeptide and that the uptake is mediated by an oligopeptide transporter with functional and biological similarities to human Pept-1 (2, 19).

MATERIALS AND METHODS

Materials. The cell line Caco-2 was purchased from the American Type Culture Collection (Manassas, VA). Custom-synthesized [Gln-3,4-3H]Gly-Gln (49 Ci/mmol) was obtained from DuPont-NEN (Boston, MA). Cloned cDNA encoding Pept-1 was provided by Dr. Matthias A. Hediger (Brigham and Women’s Hospital, Harvard Medical School, Boston, MA). Other chemicals were purchased from Sigma Chemical (St. Louis, MO) or Bachem Bioscience (Philadelphia, PA).

Cell culture. At approximately passage 30, Caco-2 cells were seeded in 200-ml flasks and passaged in DMEM supplemented with 1% nonessential amino acids, 10% FCS, 1,000 U/l penicillin, and 1 mg/l streptomycin (complete DMEM). Monolayers were detached with trypsin, resuspended in medium, and split 3:1. When the cells reached passages 50–70, they were plated onto 12-well cluster trays at a density of 63,000 cells/cm². Cells were cultured in complete DMEM; the medium was replaced every 2–3 days. Monolayers were detached with trypsin, resuspended in medium, and split 3:1. When the cells reached passages 50–70, they were plated onto 12-well cluster trays at a density of 63,000 cells/cm². Cells were cultured in complete DMEM; the medium was replaced every 2–3 days. Monolayers were kept at 37°C, 5% CO₂, and 90% relative humidity and were used for experiments 4 days postconfluence, which is equivalent to 12–15 days after seeding. Serum was withdrawn from 4-day-postconfluent monolayers for 24 h before each experiment.

Uptake studies. The culture medium was removed, and the monolayers were washed twice with an Earle’s balanced salt solution (equilibration buffer) containing 2 mM bicarbonate, 5 mM glucose, and 10 mM HEPES (pH 7.5). After the wash, the cells were incubated with the equilibration buffer for 15 min at 23°C. After this interval, the equilibration buffer was removed and 500 μl of transport buffer containing [Gln-3,4-3H]Gly-Gln were added per well. The transport buffer was the same as the equilibration buffer except that 10 mM HEPES was replaced with 10 mM MES (pH 6.0). The final concentration of Gly-Gln was 0.1 mM except in kinetic studies. The incubation time was 5 min. During this incubation, the plates were circularly and continuously shaken (25 rpm). Uptake

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into cells was terminated by gentle suction of the uptake medium, followed by two washes of the monolayer with ice-cold transport buffer (pH 6.0). In all experiments, uptake at 0°C was used to determine the nonspecific binding of Gly-Gln. This value was subtracted from all uptake values. Isotope was extracted from each well by solubilizing the monolayers in 500 µl 0.2% SDS in 0.2 N NaOH. Radioactivity was quantitated using aliquots of the resulting solution. Each value is expressed as the mean ± SE of three to six wells, and each experiment was repeated at least twice.

Transport kinetics. To examine the kinetics of peptide transport by Caco-2 cells, the initial rates of uptake were measured as a function of dipeptide concentration in the transport buffer. Uptake at 0°C was used to estimate the nonspecific binding. After correction of the total rate of uptake for the nonspecific binding, kinetic constants [Michaelis-Menten constant (Km) and maximal velocity (Vmax)] were derived by a nonlinear regression method with the Michaelis-Menten kinetic equation using GRAFIT (Sigma). To determine the number of systems involved in the uptake of Gly-Gln, the uptake rates were transformed according to the Eadie-Hofstee method.

Western blot analyses. Apical membrane vesicles from Caco-2 cells were prepared as described previously for preparation of brush-border membrane vesicles from human enterocytes (12). In brief, Caco-2 cells were homogenized in buffer [in mM: 60 mannitol, 0.1 phenylmethylsulfonyl fluoride, 10 EDTA, and 12 Tris (pH 7.4)] in a blender for 2 min. Magnesium chloride was added to a final concentration of 10 mM, and the mixture was allowed to stand for 15 min (step 1). The suspension was centrifuged at 3,000 g for 15 min, and the resulting supernatant was centrifuged at 27,000 g for 30 min (step 2). The pellet from the high-speed spin was resuspended in 35 ml of the above buffer using a Potter-Elvehjem homogenizer. Steps 1 and 2 were repeated on this homogenate, and the resulting pellet was resuspended in the above buffer by repeated passage through an 18-gauge needle. The protein concentration of the membrane suspension was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Identical amounts of apical membrane proteins (100 µg) from control and insulin-treated Caco-2 cells were suspended in SDS buffer [4% wt/vol SDS, 0.125 M Tris·HCl (pH 6.8), 20% vol/vol glycerol, and 0.125 wt/vol 2-mercaptoethanol]. Samples were subjected to 10% SDS-PAGE in a Laemmli system (8). Resolved proteins were transferred to nitrocellulose membranes and subjected to immunoblot analyses. The membranes were incubated with polyclonal antibody (1:1,000) raised against Pept-1 protein. For preparation of antibody, a synthetic peptide (Glu-Asn-Pro-Tyr-Ser-Ser-Leu-Glu-Pro-Val-Ser-Gln-Thr-Asn-Met) corresponding to the 15 carboxy-terminal amino acids of Pept-1 was used as epitope. Antibody was generated by immunization of rabbits with the epitope, and specificity of the antibody was confirmed by Western blot analysis with antibody that had been preabsorbed with epitope. We (19) previously validated the use of this antibody for the Western analysis of Pept-1. After incubation with Pept-1 antibody, the membranes were washed and incubated with the second antibody (peroxidase-conjugated goat anti-rabbit IgG, 1:2,000) as previously described (4, 13). Pept-1 protein in Caco-2 apical membrane was detected with an enhanced chemiluminescence Western blotting system (ECL Plus, Amersham Life Science, Arlington Heights, IL). The intensity of bands was quantitated using Image PC (Scion, Frederick, MD). Preliminary studies showed linearity of the Western blot assay from 25 to 200 µg of Caco-2 apical membrane protein. The correlation coefficient between the amount of protein and ECL image intensity was 0.967.

Northern blot analyses. For Northern blot analyses, 5 µg of poly(A)+ RNA (isolated by using a miniribocep isolation kit from Collaborative Research, Becton Dickinson, Bedford, MA) from Caco-2 cells were resolved by electrophoresis in 0.9% agarose gels containing formaldehyde and transferred onto Nytran membranes (Schleicher & Schuell, Keene, NH) by capillary action. After transfer, mRNA was immobilized by radiation with ultraviolet light (UV cross-linker, Stratagene, La Jolla, CA). Then the membranes were prehybridized overnight at 42°C in prehybridization solution [50% deionized formamide, 0.25 mM Na2HPO4 (pH 7.2), 0.25 mM NaCl, 1 mM EDTA, 100 µg/ml heat-denatured herring sperm DNA, 7% SDS, and 0.1% sodium pyrophosphate]. Specific 32P-labeled cDNA probes (Pept-1 or β-actin) were made by random primer technique using an oligolabeling kit (Amersham Pharmacia Biotech, Piscataway, NJ) and were added to fresh aliquots of the prehybridization solution. Hybridization was performed at 42°C for 24–72 h. To remove the unbound probe, membranes were washed twice for 20 min in each of the following buffers: 1) 0.5× SSC (sodium chloride-sodium citrate buffer) and 0.1% SDS at 42°C, 2) 25 mM Na2HPO4 (pH 7.2), 1 mM EDTA, and 0.1% SDS at 42°C, and 3) 25 mM Na2HPO4 (pH 7.2), 1 mM EDTA, and 1% SDS at 42°C. Hybridization signals were visualized by autoradiography with Biomax MS film (Kodak, Rochester, NY) for 48–72 h at −70°C. Densitometric analyses of the autoradiographs were carried out using Image PC. Membranes initially hybridized to Pept-1 were subsequently hybridized to β-actin to normalize for differences in mRNA loading between wells. Preliminary experiments showed that the abundance of β-actin mRNA was not affected by the addition of insulin to the culture medium.

Statistics. Each rate of uptake is the mean ± SE of determinations in three to six monolayers. Preliminary studies showed that the SD of the radiotracer method is ~10% of the mean of three replicates. Significance was tested with paired and unpaired tests and ANOVA, as appropriate.

RESULTS

Effect of insulin. To measure Gly-Gln transport, Caco-2 cells were incubated for 5 min with 0.1 mM Gly-Gln at pH 6.0, which is the optimum pH for uptake of Gly-Gln by Caco-2 cells (2). The incubation conditions were based on the results of preliminary experiments that showed that the rate of uptake was linear with regard to time of incubation and concentration of Gly-Gln.

To investigate whether insulin has any effect on Gly-Gln transport, Caco-2 cells were preincubated for 30–120 min with 5 nM insulin. An approximately twofold stimulation of Gly-Gln uptake occurred after 60 min of preincubation with insulin (Fig. 1). Longer preincubation (90–120 min) or an increase in the insulin concentration did not further increase Gly-Gln transport. Therefore, for the following studies, Caco-2 cells were preincubated for 60–120 min with 5 nM insulin.

The following experiment was performed to investigate whether the stimulation in dipeptide transport requires binding of insulin to its receptor, which has been shown to be present in the membrane of Caco-2 cells (3, 10). Insulin action is mediated through the
insulin receptor, a transmembrane glycoprotein with intrinsic protein tyrosine kinase activity (17). When insulin binds to its receptor, the tyrosine kinase becomes activated. This activation can be blocked by genistein (11). Therefore, we added genistein before adding insulin to determine whether this inhibitor would block the stimulatory effect of insulin on Gly-Gln transport. The results showed that genistein had no effect on Gly-Gln transport by Caco-2 cells but completely blocked the stimulatory effect of insulin on this transport (Fig. 2).

Mechanism of insulin effect. Insulin might increase Gly-Gln uptake by increasing its affinity for the oligopeptide transporter or by activating a dormant transporter. To investigate these possibilities, we determined the effect of insulin on the kinetics of Gly-Gln uptake by Caco-2 cells (Fig. 3). Eadie-Hofstee plots of the data showed the presence of a single transport system in both control and insulin-treated cells. Furthermore, kinetic analysis of this system showed that insulin significantly \( P < 0.01 \) increased the \( V_{\text{max}} \) \( (3.53 \pm 0.61 \text{ vs. } 6.31 \pm 0.5 \text{ mmol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}) \) but had no significant effect on the \( K_m \) \( (1.49 \pm 0.55 \text{ vs. } 1.36 \pm 0.33 \text{ mM}) \). These data eliminated the possibilities of involvement of more than one system in dipeptide transport by the insulin-treated cells and a change in the affinity of Gly-Gln for the oligopeptide transporter.

The increase in \( V_{\text{max}} \) suggested that insulin increased the population of the peptide transporter in the apical membrane of Caco-2 cells. To investigate this suggestion, we used Western analyses to determine the amount of Pept-1 protein in the apical membrane of control and
insulin-treated cells. The results are shown in Fig. 4. As previously found by us (19) and others (5), the observed molecular weight of Pept-1 in Caco-2 cells was higher than its predicted value (9). This difference has been attributed to glycosylation of Pept-1 (5), which has several sites for this posttranscriptional modification. As is apparent from Fig. 4A, insulin treatment increased the amount of Pept-1 protein in the apical membrane of Caco-2 cells. This was confirmed by densitometric analyses (Fig. 4B) of immunoblots (P < 0.01).

To investigate whether the mechanism of increased Pept-1 protein was pretranslational, we determined the gene expression of Pept-1 in control and insulin-treated cells. Quantitative and qualitative analyses of Northern blots showed that abundance of Pept-1 mRNA was not affected by insulin treatment (Fig. 5).

Another possible mechanism is that insulin increases the membrane population of Pept-1 by a direct effect on its translation. To investigate this possibility, we determined whether insulin stimulates Gly-Gln transport in Caco-2 cells treated with brefeldin. Stimulation in transport was used as evidence of increased membrane population of Pept-1. In a previous study (19), we showed that the brefeldin treatment of Caco-2 cells selectively dismantles their Golgi apparatus, which is required for the processing of newly synthesized Pept-1 protein for insertion into plasma membrane. As shown in Fig. 6, brefeldin treatment did not affect Gly-Gln transport either in the control or in insulin-treated Caco-2 cells.

Finally, we investigated whether the mechanism of increased membrane population of Pept-1 is an increase in their translocation from a preformed cytoplasmic pool. To investigate this possibility, we determined whether insulin stimulates Gly-Gln transport of Caco-2 cells treated with colchicine. Previous studies have shown that treatment of cells (including Caco-2 cells) with colchicine results in depolymerization of microtubules, which disrupts the translocation of proteins targeted for membrane insertion (1, 14, 22). As shown in Fig. 7, colchicine had no effect on the uptake of Gly-Gln by the control Caco-2 cell monolayers. However, when colchicine was added 20 min before the addition of insulin, it completely abolished the stimulatory effect of insulin on Gly-Gln uptake.

DISCUSSION

The results of the present study bring a new dimension to the field of peptide transport by providing the first evidence for its stimulation by a hormone. The stimulation was shown in Caco-2 cells treated with a physiological concentration of insulin. This observation raises a question about the specificity of the insulin effect. In other words, is the transport of other nutrients also increased by insulin treatment of Caco-2 cells? Among the various nutrients, glucose would be the most likely candidate for this effect. However, a

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**Fig. 4.** Effect of insulin on amount of Pept-1 protein in apical membrane. Culture medium of treated cells was enriched with insulin (5 nM) 2 h before protein measurement. Apical membrane proteins (100 µg) of control and insulin-treated Caco-2 cells were subjected to Western blot analyses. For each analysis, apical membranes were prepared from cells grown in 3–5 culture bottles, each with an area of 175 cm². A: lane 1, control cells; lane 2, insulin-treated cells. B: quantitative densitometric analyses of Western blots for Pept-1 protein. Values are means ± SE of 6 analyses, expressed as percent control. KD, kilodaltons.

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**Fig. 5.** Effect of insulin on abundance of mRNA encoding Pept-1. Culture medium of treated cells was enriched with insulin (5 nM) 2 h before mRNA measurement. A: mRNA was extracted from control and insulin-treated Caco-2 cells and was subjected to Northern blot analysis using 32P-labeled cDNAs encoding Pept-1 and β-actin. Lane 1, control cells; lane 2, insulin-treated cells. B: densitometric analyses of Northern blots. Level of mRNA in each sample was normalized to abundance of β-actin mRNA, which was not affected by insulin treatment. Values are means ± SE of 4 analyses, expressed as percent control.

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**Fig. 6.** Effect of insulin on abundance of mRNA encoding Pept-1. Culture medium of treated cells was enriched with insulin (5 nM) 2 h before mRNA measurement. A: mRNA was extracted from control and insulin-treated Caco-2 cells and was subjected to Northern blot analysis using 32P-labeled cDNAs encoding Pept-1 and β-actin. Lane 1, control cells; lane 2, insulin-treated cells. B: densitometric analyses of Northern blots. Level of mRNA in each sample was normalized to abundance of β-actin mRNA, which was not affected by insulin treatment. Values are means ± SE of 4 analyses, expressed as percent control.
previous study (10) showed that insulin treatment does not affect glucose transport by Caco-2 cells. Therefore, the effect of insulin on dipeptide transport appears to be specific.

The present study also provides novel information about the mechanism of a regulatory action of insulin regarding a nutrient transport by an intestinal cell. It shows that insulin increases the membrane population of oligopeptide transporter by increasing its translocation from a preformed cytoplasmic pool. The evidence for this is based on 1) increased \( V_{\text{max}} \) of dipeptide uptake by the apical membrane of insulin-treated Caco-2 cells and 2) increased amount of Pept-1 in the apical membrane fraction isolated from these cells.

Our previous studies in Caco-2 cells have suggested that the alteration in the gene expression does not affect glucose transport by Caco-2 cells. Therefore, the effect of insulin on dipeptide transport appears to be specific.

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port in the intestine. However, a previous study from our laboratory, performed over two decades ago, found that diabetes has no effect on dipeptide absorption (18). Although this study needs to be repeated with the tools currently available, it is possible that other factors besides insulin may regulate dipeptide transport in the intestine of diabetic rats. Clearly, the present study needs to be followed by further studies on physiology and biology of hormonal regulation of dipeptide transport.

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REFERENCES