Characteristic and Localization of the Monitor Peptide Receptor

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The monitor peptide receptor in the small intestine was investigated. The receptor is on the intestinal mucosal cells [Biochem. J., in press]. The specific binding of the $^{125}$I labeled monitor peptide to dispersed rat small intestinal cells was inhibited by treatment with $p$-toluenesulfonyl-$l$-lysine chloromethyl ketone (TLCK), an affinity labeling reagent for trypsin. Soybean trypsin inhibitor (SBTI) did not inhibit the binding. Analysis with reduced SDS electrophoresis-autoradiography indicated that an affinity-cross-linked complex of the $^{125}$I labeled monitor peptide and the receptor was abolished by the TLCK treatment but was not affected by the presence of SBTI. Histochemical studies found a predominant binding of $^{125}$I labeled monitor peptide on cholecystokinin (CCK)-immunoreactive cells in tissue sections of the rat upper intestine. The result suggests that the monitor peptide receptor is on the surface of CCK-producing cells.

The monitor peptide, consisting of 61 amino acids, was isolated from rat bile-pancreatic juice as a stimulant for pancreatic enzyme secretion, by intraduodenal infusion and this stimulus was transmitted via CCK.$^{1-4}$ The monitor peptide also inhibited trypsin.$^{1}$ We have reported the specific binding of the monitor peptide to rat small intestinal mucosal cells and the presence of the monitor peptide reector on the cells.$^{5}$ For the specific binding of the monitor peptide to the cells, pH 8–9 was optimal, which was consistent with the physiological pH of the small intestine. The monitor peptide receptor is a protein. Analysis with SDS electrophoresis-autoradiography of the affinity-cross-linked complex consisting of $^{125}$I labeled monitor peptide and the receptor showed that the apparent molecular mass of the complex was about 53,000 and that of and reduced complex with 2-mercaptoethanol was about 33,000.

Monitor peptide mutants in which the trypsin inhibitory activity is abolished lose binding ability with the cells.$^{5}$ Thus the monitor peptide receptor was assumed to bind to the peptide in a trypsin-like manner. We therefore compared effects of TLCK, an affinity labeling reagent for trypsin,$^{6}$ and TPCCK, that for chymotrypsin,$^{7}$ on the specific binding between $^{125}$I labeled monitor peptide and the cells.

To find whether the monitor peptide receptor is directly expressed on CCK-producing cells is of importance. Thus we observed in single tissue sections both the binding sites of $^{125}$I labeled monitor peptide and CCK-immunoreactive cells to obtain direct evidence to support the presence of the monitor peptide receptor on CCK-producing cells in this study.

Materials and Methods

Materials. Male Wistar rats weighing 250–300 g were purchased from Oriental Bio (Kyoto, Japan). TCMS-199 medium and Triton X-100 and TLCK and TPCCK were from Nakalai Tesque (Kyoto, Japan). Soybean trypsin inhibitor (SBTI), collagenase, and deoxyribonuclease I were purchased from Sigma (St. Louis, MO, U.S.A.). Dispase was purchased from Godo Shusei Co. (Tokyo, Japan). Disuccinimidyl suberate was purchased from Pierce (Rockford, IL, U.S.A.). Other biochemical reagents were purchased from Wako Pure Chemical Co. Ltd. (Tokyo, Japan). $^{125}$I was obtained from DuPont/NEN Research Products.

Purification of the monitor peptide. The monitor peptide was purified by the method previously described.$^{9}$ Rat pancreatic juice was collected in citrate buffer, pH 2.5, containing 0.5 M NaCl. After heating (80°C, 30 min) and centrifugation at 3000 × g for 30 min at 4°C the supernatant was eluted by reverse-phase chromatography through Synchroprop RP-4 (Synchrom Inc., Indiana, U.S.A.), with 50% acetonitrile-0.05% TFA. After further fractionation by high-performance liquid chromatography using a C₄ column AP823 (YMC, Kyoto, Japan), purified monitor peptide was obtained.

$^{125}$I labeling of the monitor peptide. The peptide was labeled with $^{125}$I Chloramine T.$^{10}$ The radioactivity of the labeled peptide was 0.3 μCi/pmol.

Preparation of dispersed rat intestinal mucosal cells. Dispersed rat intestinal mucosal cells were obtained by the method used to prepare guinea-pig gastric mucosa.$^{11,12}$ At first scrapable mucosa was incubated in TCMS-199 medium containing collagenase, disperse, and deoxyribonuclease I. At this point dispersed cells were stored and the remaining coagulated cells were further incubated in 25 mM NaHCO₃ buffer, pH 7.4, containing 118 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, and 1 mM Na₂EDTA. The supernatants containing dispersed cells were pooled and centrifuged at 1000 × g for 30 min at 4°C. The cell pellet was suspended in 10 mL HEPES buffer, pH 8.0, containing 145 mM NaCl (basal buffer) and washed twice with the same buffer. Washed cells were suspended in the basal buffer containing 10% glycerol and stored in the freezer at −70°C until use. Freezing did not damage the cells with respect to binding ability with the monitor peptide.$^{9}$

Treatment with TLCK or TPCCK. Frozen cells were thawed, washed with basal buffer, resuspended in the basal buffer containing 0.6 mM TPCCK or TLCK, and incubated at 37°C for 30 min. The buffer was then removed by centrifugation at 15000 × g for 5 min at 37°C and the pellet was washed twice with basal buffer. These cells were used in the next binding assay.
Cells incubated without TLCK or TPCCK provided the control.

**Binding assay.** Cells (1 x 10⁶) were pipetted into microtubes and incubated with 125I labeled monitor peptide in 100 μl of basal buffer at 37°C for 1 h. Subsequently, 1 ml of ice-cold basal buffer was added to the microtubes, which were centrifuged at 1500 x g for 30 min at 4°C. After they were washed twice with basal buffer, the radioactivity in the cell pellet was measured in a gamma-counter. Nonspecific or total binding of 125I labeled monitor peptide to the cells was obtained in the presence or absence of a 1000-fold excess of unlabeled monitor peptide. Specific binding was calculated by subtracting the nonspecific binding value from that of the total binding. As shown previously, 23 under these assay conditions, 125I labeled monitor peptide was not internalized by the cells. Therefore, all radioactivity detected was due to 125I labeled monitor peptide binding to the cell surface.

**SDS polyacrylamide gel electrophoresis.** Treated cells were incubated with 125I labeled monitor peptide and treated in a similar manner to that for the binding assay. After they were washed, cells were suspended in 1 ml of basal buffer containing 0.4 mM disuccinimidyl suberate, a divalent crosslinker, and incubated for 1 h on ice. The buffer was removed by centrifugation at 1500 x g for 10 min at 4°C. The cell pellet was washed twice with 10 mM Tris buffer pH 7.1 containing 145 mM NaCl. The cell pellet was then solubilized with 50 μl 10 mM Tris buffer, pH 7.1 containing 145 mM NaCl, 1 mg/ml leupeptin, and 2% TritonX-100 for 1 h on ice, followed by centrifugation at 10,000 x g for 3 min at 4°C. The cell lysate supernatant was mixed with 20 μl SDS sample buffer containing 2-mercaptoethanol, boiled for 5 min, and electrophoresed on an SDS polyacrylamide gel. The bands were made visible by autoradiography using Fuji X-ray film RX after exposure at ~70°C for 48 h with the intensifying screen, Lighting Plus (Du Pont, DE, U.S.A.).

**Histochemical study.** Male Wistar rats weighing 250-300 g were anesthetized with sodium pentobarbital. The abdominal wall was incised, then the pancreatic duct at the distal terminal was ligated and a 2 cm long duodenal pouch was made. The proximal part of the pouch was cannulated with a polyethylene tube for injection of fluids, the distal part being for drainage. After an infusion of warm physiological saline containing SBTI (100 μg/ml, 10 ml) for 10 min to remove trypsin, saline containing 125I labeled monitor peptide (2.4 μg/ml, 0.25 ml) was infused and stored in the pouch for 15 min. The solution was thoroughly washed out with saline. The animals were rapidly perfused through the aorta with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The duodenum was removed from the perfusion-fixed animals and immersed in the same fixative for an additional 6h. Cryostat sections, 15 μm thick, were prepared and processed for immunostaining for CCK by the avidin-biotin peroxidase complex (ABC) method using a Biotin-streptavidin Immunostaining Kit (BioGeneX Lab., Dublun, U.S.A.). The antisera was produced in a rabbit using porcine CCK 33 (I-27)NH₂ as the antigen and proven to be specific for the 6-12 sequence of CCK 33. 23 After the sites of antigen-antibody reaction were made visible with diaminobenzidine, the tissue sections were dried with a graded series of ethanol. Glass slides with mounted sections were dipped in NTB2 nuclear track emulsion (Kodak), air-dried, and exposed for 3 weeks at room temperature. After development with Kodak D-19 developer, they were then dehydrated with ethanol and mounted with Eukitt (O. Kippler, Freiburg, Germany).

**Results and Discussion**

The effects of TLCK upon the binding of 125I labeled monitor peptide to dispersed small intestinal mucosal cells

Dispersed intestinal mucosal cells were incubated with TLCK or TPCCK. TLCK is an affinity labeling reagent for trypsin and TPCCK is for chymotrypsin. As shown in Fig. 1, incubation with TLCK considerably inhibited binding of the 125I labeled monitor peptide to the cells, but TPCCK did not affect it at all. This showed that the monitor peptide receptor on the intestinal mucosal cells was sensitive to TLCK not TPCCK.

Figure 2 represents a reduced complex consisting of 125I labeled monitor peptide and the monitor peptide receptor.

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**Fig. 1. The Effects of Affinity Labeling Reagents on the Binding of 125I Labeled Monitor Peptide to the Dispersed Rat Intestinal Mucosal Cells.**

Cells, 1 x 10⁶, were incubated in basal buffer (see Materials and Methods) containing 0.6 mM TLCK or 0.6 mM TPCCK for 30 min at 37°C. Control cells were incubated with basal buffer. The cells were then washed with basal buffer, and incubated with 0.2 pmol 125I labeled monitor peptide for 1 h at 37°C. The specific binding is shown as percentage of the control. Under these conditions, the nonspecific value was below 10% of the total binding. Bars correspond to the averaged value from three experiments, and error bars represent standard deviation.

**Fig. 2. Autoradiography of Complexes Consisting of 125I Labeled Monitor Peptide and Its Receptor.**

Dispersed rat intestinal mucosal cells, 5.0 x 10⁶, were incubated with the basal buffer in the presence or the absence of 0.6 mM TLCK and washed with the basal buffer. The cells were incubated with 7.6 pmol 125I labeled monitor peptide in basal buffer for 1 h at 37°C, in the presence or absence of the additives. The cells were crosslinked with 125I labeled monitor peptide using the 0.4 mM disuccinimidyl suberate. The experimental conditions were as described under Materials and Methods. Lane A, incubated in the absence of TLCK and incubated in the absence of the additives (positive control); lane B, incubated in the absence of the TLCK, then incubated in the presence of 3.8 nmol unlabeled monitor peptide (negative control); lane C, incubated in the absence of TLCK then incubated in the presence of 3.8 nmol SBTI; lane D, incubated in the presence of TLCK then incubated in the absence of the additives. All samples were reduced with 2-mercaptoethanol before the electrophoresis. This autoradiograph is representative of the three separate experiments. Molecular mass standards are shown on the right. k on the right means k dalton.

A band of about 33,000 was detected in the positive control (lane A), incubated without additions, and it was not detected in the negative control (lane B), incubated with a 500-fold excess of unlabeled monitor peptide. The 33,000 band was not affected by SBTI (lane C) but it was significantly attenuated by treatment of the cells with TLCK.
The autoradiograph showed that the monitor peptide receptor would be sensitive to TLCK, an affinity labeling reagent for trypsin. Thus the monitor peptide receptor seemed to have an affinity site for its ligand resembling that of trypsin. The trypsin inhibitory activity of the monitor peptide seemed to be a prerequisite for its binding to its receptor on the cell surface. The inhibition by TLCK suggests a competitive relationship in the in vivo small intestinal lumen, between trypsin and the monitor peptide receptor with regard to the binding the monitor peptide.

A possible physiological function of the monitor peptide was proposed by Iwai et al. During the postabsorption phase, the monitor peptide is easily hydrolyzed by proteases. If excess food protein comes to the lumen at a postprandial phase for example, the proteases would be so occupied that a lot of the monitor peptide would survive intact. Intact monitor peptide stimulates the release of CCK from the small intestine. According to this hypothesis, food proteins, even if they were trypsin inhibitor, would not directly cause the release of CCK and the succeeding pancreatic enzyme secretion. Pancreatic enzyme secretion is not induced by SBTI, bovine trypsin inhibitor, egg white trypsin inhibitor, or milk whey protein. It was induced only by the monitor peptide when intraduodenally infused in vivo. Furthermore the efflux of calcium ions from the intestinal mucosal cells, an indication of the CCK release, was not induced by SBTI, egg white trypsin inhibitor, or the synthetic trypsin inhibitor FOY-305, but was by the monitor peptide in vitro. Figure 2 (lane C) shows that the monitor peptide receptor, unlike trypsin, would not bind any trypsin inhibitor, consistent with previous results. The monitor peptide receptor had higher ligand specificity than trypsin, probably due to a different molecular structure than that of the affinity site. To explain the difference between the monitor peptide and the other proteins, especially trypsin inhibitors, with respect to releasing CCK from the small intestine, this high ligand specificity of the monitor peptide seemed to be of considerable importance. When considering this physiological function of the monitor peptide we assumed the presence of the monitor peptide receptor on the CCK producing cell. However, direct evidence to demonstrate this was not obtained. The monitor peptide evoked pancreatic enzyme secretion only when it was infused into the upper intestine in vivo and the Scatchard plot of the specific binding of labeled monitor peptide with the dispersed intestinal mucosal cells suggested that the monitor peptide receptor was inclined to be distributed more in the upper intestine than in the lower. Those results coincided with the CCK-producing cells being distributed more in the upper than lower intestine. However, this only indirectly indicated the presence of the monitor peptide receptor on CCK-producing cells. We then did histochemical studies to identify the relationship between the distribution of the monitor peptide receptors and CCK-producing cells.

**Histochemical study**

We compared the distribution of the CCK-immunoreactive cells and the scatter of the dot-like silver grains due to the labeled monitor peptide in cryostat sections of the rat upper intestine. Before exposure to labeled monitor peptide in vivo, the intestine was washed with saline containing SBTI to exclude the trypsin activity. The autoradiograph shows more numerous dot-like silver grains due to labeled monitor peptide on the CCK-immunoreactive cells compared with the surrounding cells (Fig. 3). This showed that labeled monitor peptide bound to the CCK-immunoreactive cells much more than to the other epithelial cells in vivo. This fact suggested the presence of the monitor peptide receptor expressed on the cell bodies of CCK-immunoreactive cells. We thus assumed that the CCK-producing cells express the monitor peptide receptor and that the signals release CCK into the blood would be transmitted via this receptor. This supports the assumption described above more directly. If the monitor peptide receptor is on the cell surface of the CCK-producing cells, it would be a reasonable and convenient site at which to implement the physiological functions of the monitor peptide. However, the grains showing the binding sites of labeled monitor peptide were dispersed all over the CCK-immunoreactive cells, not restricted to the luminal surface of the cells, so we cannot define the subcellular localization of the binding sites. To do so successive and detailed sampling of the upper intestine after exposure to labeled monitor peptide will be necessary.

**References**