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PROTEOLYTIC PROCESSING IN A NON-LYSOSOMAL COMPARTMENT IS REQUIRED FOR TRANSCYTOSIS OF PROTEIN-POLYLYSINE CONJUGATES IN CULTURED MADIN-DARBY CANINE KIDNEY CELLS

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The transcytosis of horseradish peroxidase, as well as its poly(L-lys) and poly(D-lys) thioether conjugates, was investigated in Strain I Madin-Darby canine kidney (MDCK) cell monolayers grown on 0.4 μm pore size polycarbonate membranes in Costar Transwells. The 3 types of HRP had almost identical rates of transport during the first 2 hr of incubation. However, a significant increase of basal-to-apical transport was detected beginning at 3 hr only in Transwells containing the poly(L-lys) conjugate. This increase was inhibited by colchicine (2 μM) and by the Bowman-Birk protease inhibitor (0.1 mg/ml), but not by NH_4Cl (10 mM) or chloroquine (0.1 mM). The increase was abolished either by prior trypsinization of the conjugate or by incubation at 4°C. Ultrafiltration studies indicated that the transcytosed poly(L-lys) conjugate was smaller in size than the original conjugate. These results indicate that the conjugate was processed during transcytosis in a non-lysosomal proteolytic compartment, where its poly(L-lys) moiety was selectively degraded, allowing active peroxidase to be released into the apical medium.

In polarized epithelial cells, transcytosis controls the transport of membrane components (1) and of membrane-bound ligands (2,3) between the apical and basolateral surfaces. The process is of physiological importance for the transport of polypeptide hormones from the blood to their target tissues (4), and of pharmacological importance for the transport of drugs across endothelia, especially those that form the blood-brain barriers (5). Like endocytosis, transcytosis is initiated by vesiculation at the plasma membranes (6). Little is known, however, about the properties of vesicles engaged in transcytosis, while the various endocytotic compartments have been well characterized (7).

ABBREVIATIONS: HRP, Horseradish peroxidase; PLL, poly(L-lysine); HRP-S-PLL, Horseradish peroxidase-poly(L-lysine) conjugates with a thioether linkage; PDL, poly(D-lysine); HRP-S-PDL, Horseradish peroxidase-poly(D-lysine) conjugates with a thioether linkage; MDCK, Madin-Darby Canine Kidney Cells; BBI, Bowman-Birk protease inhibitor; TEER, Transepithelial electrical resistance; MEM, Eagle's minimum essential medium; FBS, Fetal bovine serum; PBS, Phosphate-buffered saline, pH 7; Sulfo-SMBP, sulfo-succinimidyl 4-(p-maleimidophenyl) butyrate; SPDP, N-succinimidyl 3-(2-pyridyldithio)propionate.

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In this report, we use a poly(L-lysine) (PLL) thioether-linked conjugate of horseradish peroxidase (HRP-S-PLL) as a probe to investigate transcytosis from the basolateral to the apical surface in cultured Madin-Darby canine kidney (MDCK) epithelial monolayers. We have shown previously that PLL conjugates are good models for the study of non-specific, adsorptive endocytosis due to the high affinity of the polycation to the cell membrane (8). The transcytosis of HRP-S-PLL described here, however, involves not only the internalization of the conjugate at the basolateral membrane, but also the release of the active HRP at the opposite side of the cell. One possible requirement for this release is the processing of the PLL moiety of the conjugate. By comparing HRP-S-PLL with its optical isomer containing the non-degradable poly(D-lysine), i.e. HRP-S-PDL, we obtained evidence for such a requirement by demonstrating the selective digestion of PLL during transcytosis and the release of active HRP at the apical surface of the epithelium.

MATERIALS AND METHODS

Strain I MDCK cell line used in this investigation was a gift from Dr. M. J. Cho, the Upjohn Co. Transwells (2.45 cm dia., 0.4 μm pore size) were obtained from Costar, Cambridge, MA. Horseradish peroxidase (HRP), Rz = 3.0, poly(D-lysine) HBr, Mr: 54,500 (PDL), poly(L-lysine) HBr, Mr: 52,000 (PLL), were purchased from Sigma Chemical Co., St. Louis, MO. Protein cross-linking reagents, Sulfo-SMPB and SPDP, were products of Pierce, North Chicago, IL. Trypsin and cell culture medium were from GIBCO, Grand Island, NY. Purified Bowman-Birk protease inhibitor (BBI) was a gift from Dr. Ann R. Kennedy, University of Pennsylvania School of Medicine.

Cell cultures

MDCK (Strain I) cells were routinely grown in Eagel's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and non-essential amino acids as described (9). In transcytosis studies, MDCK cells were seeded in Transwells with 1 X 10^5 cells/well. The amount of FBS in the medium was gradually decreased when the transepithelial electrical resistance (TEER), measured with a Epithelial Voltohmmeter, EVOM (World Precision Instruments, West Haven, CT), reached 1,000 ohm/cm^2. The confluent monolayers showed a TEER about 2,000 ohm/cm^2, and were maintained in medium containing 1% FBS. For endocytosis studies, MDCK cells were grown in medium with 10% FBS in T-25 tissue culture flasks (Corning).

Preparation of HRP-S-PLL and HRP-S-PDL

HRP was conjugated to either PLL or PDL through a stable thioether linkage. Briefly, HRP (10 mg) was modified with 1.5 mg of sulfo-SMPB in 1 ml of PBS for 1 hr at 25°C. After extensive dialysis, the modified HRP was mixed with an equal amount of either PLL or PDL, which was freshly modified with SPDP, reduced by dithiothreitol, and subsequently purified by Sephadex G-50 gel filtration. The reaction mixture was stirred at 4°C for 16 hr, and the conjugates were purified with a Sephadex G-100 column (1.5X70 cm). The HRP and polylysine contents in the conjugates were determined by A403 nm (10) and the trypan blue precipitation method (11), respectively. The molar ratio of HRP to the poly(amino acid) was approximately 1 in both HRP-S-PLL and HRP-S-PDL. About 60% of the enzyme activity of HRP was retained in both conjugates.

Measurement of basal to apical transcytosis of HRP and its conjugates

Confluent MDCK monolayers in Transwells were treated in fresh medium containing 1% FBS. Either HRP or its conjugates were added to the medium in the basal

compartment to give a concentration of 3 μg HRP/ml. In some experiments, [^{14}C] sucrose was also added simultaneously to the basal medium at 1 μCi/ml to monitor the integrity of the tight junction. At various time intervals, 0.1 ml aliquot of medium was pipeted from the apical compartment, and HRP activity was measured immediately by the o-dianisidine colorimetric method. In the study of effects by different agents and in low temperatures, cells were preincubated with the treatment for 30 min before the addition of HRP or its conjugates, except in the colchicine treatment where cells were preincubated with 2 μM colchicine for 2 hr. In the study of trypsinized HRP-S-PLL, the conjugate was pretreated with 0.025% trypsin in phosphate-buffered saline, pH 7 (PBS) at 37°C for 5 min before the addition to the basal medium in the Transwell. The final trypsin concentration in those wells was about 4 μg/ml and its effect on transcytosis is negligible. The HRP enzyme activity in the trypsinized HRP-S-PLL was about 20% higher than that in the original conjugate.

Endocytosis and intracellular degradation of HRP in MDCK cells

Confluent MDCK monolayers in culture flasks were incubated with 1 mg/ml of HRP in medium containing 10% FBS. For the treatment with reagents, cells were preincubated with NH_4Cl (10 mM) or BBI (0.1 mg/ml) for 30 min before the addition of HRP. Monolayers were exposed to HRP for 1 hr, washed extensively with cold PBS to remove residual HRP. Half of the flasks were then treated with trypsin/EDTA to detach cells. After centrifugation, cells were washed twice each with 5 ml cold PBS and the final cell pellets were dissolved in 0.5 ml of 0.05% Triton X-100. HRP activity in the cell extracts was measured with the o-dianisidine method, and was set as the initial activity for the study of lysosomal HRP degradation. The other half of the flasks were reincubated in 10% FBS medium containing the reagent of the treatment for an additional 5 hr and were then processed to measure cell-associated HRP activity as described above.

RESULTS

A comparison of transcytosis of HRP, HPR-S-PDL and HRP-S-PLL in MDCK cell monolayers is shown in Fig. 1. The 3 forms of HRP showed very similar basal to

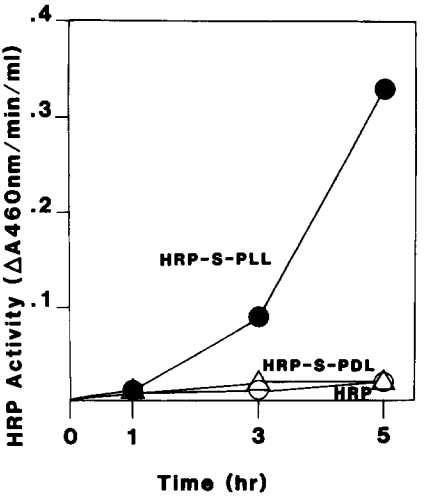

Fig. 1 Transcellular transport of HRP and its conjugates in MDCK cell monolayers
Confluent MDCK monolayers in Transwells were treated at the basal compartment with 3 μg/ml of HRP, either as the free enzyme (o), or as PLL (•) or PDL (Δ) conjugate. At various time intervals, 0.1 ml aliquot of the medium in the apical compartment was pipeted and the HRP activity was assayed immediately.

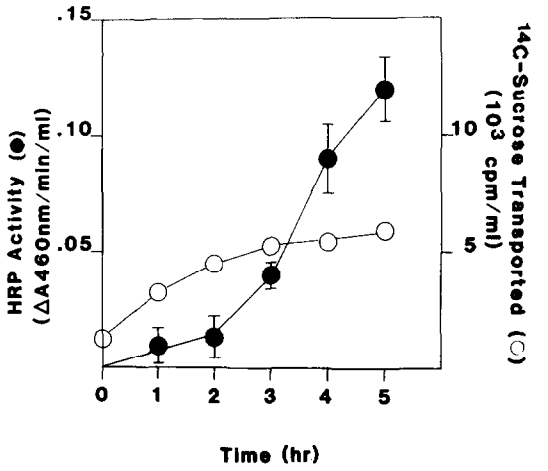

Fig. 2 Comparison of the transcellular transports of HRP-S-PLL and [^{14}C] sucrose in MDCK cell monolayers HRP-S-PLL (3 μg/ml) and [^{14}C]sucrose (1 μCi/ml) were added simultaneously to the basal compartment of Transwells with confluent MDCK cell monolayers. At various time intervals, 2 aliquots of 0.1 ml medium from the apical compartment were pipeted; one for HRP activity assay (●) and the other for ^{14}C-radioactivity measurement (o). Fresh medium (0.2 ml) was added to the apical compartment after each sampling to compensate the decrease of volume and to maintain the same level of medium in the apical and basal compartments. Bars indicate the standard deviations (n=3) in HRP assays.

apical transport in the first 2 hr of incubation. After 2 hr, however, the rate of HRP release into the apical medium began to increase in the HRP-S-PLL containing Transwells, while it remained very low and linear in the Transwells containing HRP or HRP-S-PDL. After 5 hr the HRP activity in the apical medium was about 10-fold higher for HRP-S-PLL than for the other two markers (Fig.1).

The transcytosis of HRP-S-PLL was measured simultaneously with [^{14}C]sucrose transport in the same Transwell units. As shown in Fig. 2, [^{14}C]sucrose transport was very low with a rate about 80 nl/hr/cm^2, and did not increase after 3 hr when the transport rate of HRP-S-PLL was drastically raised (Fig. 2). This result suggests that the high HRP-S-PLL transport was not due to a leakage of the cell junction, nor to a general increase in either paracellular or transcellular fluid transport process. Transepithelial electrical resistance (TEER) measurements in Transwells before and after the 5-hr incubation with HRP-S-PLL were 2167 ± 58 and 1946 ± 403 ohm/cm^2, respectively. The small decrease in TEER had no effect on the Transcellular transport of either [^{14}C]sucrose or HRP.

The effects of temperature, weak bases, microtubule inhibitors and protease inhibitors on the transcytosis of HRP-S-PLL were also investigated. As shown in Table 1, the basal-to-apical transcytosis of HRP-S-PLL was almost totally abolished at 4°C, markedly inhibited by BBI and partially inhibited by colchicine, while weak bases (NH_4Cl and chloroquine) at concentrations known to increase endosomal and lysosomal pH, had no inhibitory effect. The lysosomal

TABLE 1. EFFECTS OF DIFFERENT TREATMENTS ON THE TRANSCELLULAR TRANSPORT OF HRP-S-PLL IN MDCK CELL MONOLAYERS

Treatment	HRP activity in apical medium[a] % of control ± S.D.(n=3)
None	100.0
Chloroquine, 0.1 mM	90.1 ± 12.9
NH_4Cl, 10 mM	83.3 ± 8.6
Colchicine, 2 μM	65.6 ± 3.8
BBI, 0.1 mg/ml[b]	27.4 ± 3.8
Trypsinization[b]	8.5 ± 5.5
4°C	4.8 ± 2.2

[a] HRP activity in apical medium after a 5-hr incubation with the addition of 3 μg/ml of HRP-S-PLL in the basal compartment.
[b] HRP-S-PLL was pretreated with 0.025% trypsin in PBS at 37°C for 5 min before the addition to the basal medium in the Transwell.

degradation of endocytosed HRP in MDCK cells was inhibited by NH_4Cl, but not by BBI (Table 2).

The medium collected from the apical compartment after 5-hr incubation was measured for the HRP activity in Centricon-100 filtration units (Amicon). As shown in Table 3, neither HRP-S-PLL nor HRP-S-PDL conjugates passed through the filter. By contrast, more than 1/3 of the HRP-activity in the apical medium of the Transwell containing HRP-S-PLL passed through the Centricon-100 units. This result indicates that the PLL moiety in HRP-S-PLL had been selectively degraded during the transcytotic process. The degradation of the PLL moiety of HRP-S-PLL is consistent with the observation that ultrafiltration of trypsinized HRP-S-PLL yielded a filtrate containing 77% of the HRP activity, compared to 3% for trypsinized HRP-S-PDL (Table 3).

DISCUSSION

HRP-S-PLL can be transported across MDCK cell monolayers from the basolateral to the apical surface under conditions where the tightness of

TABLE 2. EFFECTS OF AMMONIUM CHLORIDE AND BOWMAN-BIRK INHIBITOR ON THE DEGRADATION OF ENDOCYTOSED HRP IN MDCK CELLS

Treatment	Cell-associated HRP activity (ΔA460nm/min) Initial	After 5 hr	% HRP degraded
Control	0.165	0.065	60.6 (100)
BBI, 0.1 mg/ml	0.128	0.057	54.9 (91)
NH_4Cl, 10 mM	0.138	0.108	21.8 (36)

Confluent MDCK cells in T-25 flasks were incubated with 1 mg/ml HRP for 1 hr at 37°C, and the cell-associated HRP was measured and treated as the initial activity. Cells were than reincubated in HRP-free medium and the cell-associated HRP was measured again after 5 hr to determine the degradation of endocytosed HRP. Cell-associated HRP activities are averages of measurements from duplicate flasks with an identical cell number per flask. The detailed procedure is described in the text.

TABLE 3. ULTRAFILTRATION OF HRP CONJUGATES[a]

HRP CONJUGATE	%HRP ACTIVITY IN FILTRATE
Free HRP	97.9 ± 11.3
HRP-S-PDL	4.0 ± 0.3
Trypsinized HRP-S-PDL[b]	3.4 ± 0.5
HRP-S-PLL	4.0 ± 0.7
Trypsinized HRP-S-PLL[b]	77.7 ± 9.2
Transcytosed HRP-S-PLL[c]	37.3 ± 5.0

a. Centricon-100 filter units (Amicon) were used.
b. Conjugates were incubated with 0.025% trypsin at 37°C for 5 min.
c. Apical medium collected after 5 hr incubation of MDCK monolayers in Transwells with 3 μg/ml of HRP-S-PLL in the basal medium.

epithelial junctions is established by an unaltered TEER and by an unaltered permeability to [^{14}C]sucrose (Fig. 1 and 2). This transport, therefore, must be due to transcytosis. The transcytosis of HRP-S-PLL differs in its kinetics from that of unconjugated HRP and HRP-S-PDL, since it alone increases sharply after a lag of 2 hr (Fig. 1). During the first 2 hr, both PLL and PDL conjugates were transported with identical, but low rates. This initial transport rate suggests that there is an immediate but inefficient transport of the conjugates from the basolateral to the apical membranes, which probably involves the shedding of the intact conjugate complexed with some membrane components at the apical surface.

The striking increase in transport that begins at 3 hr must be associated with a partial digestion of the PLL component of HRP-S-PLL, since no such increase is observed when PLL in the conjugate is replaced by its nondegradable isomer, PDL. The PDL conjugate has a similar charge affinity for cell membranes, a similar initial rate of endocytosis, and differs from PLL only by its resistance to cellular proteolytic enzymes (12). That processing of PLL occurs during transcytosis is also indicated by the finding that the transcytosed HRP-S-PLL is of smaller molecular size than the intact conjugate, as shown by the ultrafiltration data of Table 3. Trypsinization of the conjugate prior to the addition to the Transwell prevents its efficient transport (Table 1), indicating that the proteolytic processing of PLL is an intracellular event. Processing, however, does not appear to occur in lysosomes, since on the one hand it was not inhibited by lysosomotropic amines such as NH_4Cl and chloroquine (Table 1), which inhibit the lysosomal degradation of HRP (Table 2)(13), and on the other hand it was markedly decreased by a peptide protease inhibitor (BBI, Mr 8,000) (Table 1) that does not affect the lysosomal degradation of endocytosed HRP (Table 2). It thus appears that the attached PLL can alter the traffic of HRP away from lysosomes towards a proteolytic compartment associated with transcytosis.

Unlike unconjugated HRP, HRP-S-PLL can be expected to bind strongly and non-specifically to the basal surface of MDCK cells. Since membrane-bound PLL is not released at acid pH (14), there is no reason to expect its release in lysosomes or endosomes. It appears more plausible to assume that it will instead participate in the membrane sorting and redistribution that follows the internalization of plasma membranes. It can be postulated that proteolysis of membrane-bound PLL occurs at some point during this membrane reshuffling. At this time the only result that further characterizes this proteolytic process is its sensitivity to BBI, an inhibitor of trypsin- and chymotrysin-like proteases (15), and its insensitivity to NH_4Cl and chloroquine. Proteolytic degradation of the PLL component of HRP-S-PLL can be expected to ultimately release HRP from its membrane-bound state and allow it to be transported as a soluble enzyme to an exocytotic or transcytotic vesicle. This may explain the requirement for PLL proteolysis. The 2 hr lag that precedes the effective phase of transcytosis may correspond to the time required for PLL degradation and HRP release.

It is not known whether proteolysis of membrane-bound PLL is associated with a specific organelle and occurs at a specific intracellular site. Neither is it known whether the processed conjugate reaches a specific transcytotic compartment or joins the exocytotic pathway of secreted proteins. In this regards, it is of interest to recall that (a) secreted proteins often undergo proteolytic processing which is specific for lysine and arginine peptide bonds (16) and occurs in a non-acidic compartment (17); and (b) the kinetics of such processing can be of the order of 2 hr (16).

In summary, our results demonstrate that a PLL-specific proteolytic process occurs during the transcytosis of HRP-S-PLL from basolateral to apical surface in MDCK cells, and that this process takes place at sites other than lysosomes. The role of proteolysis in transcytosis and the use of degradable carriers for transepithelial drug transport are currently under investigation in our laboratory.

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