# Transepithelial Transport of Tyramine Across Filter-Grown MDCK Cells via a Poly(D-lysine) Carrier

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*Received October 1, 1993; accepted April 26, 1994*

In order to investigate the advantage of using membrane-adsorptive carriers to mediate drug transport across epithelial tissue, we have prepared disulfide- and thioether-linked conjugates of tyramine (tyn) as a model drug to a cationic, nondegradable carrier, poly(D-lysine) (PDL). The transport properties were evaluated using microporous filter-grown Madin-Darby canine kidney (MDCK, strain I) epithelial cells, and we have determined that: (a) the [$^{125}$I]tyn-SS-PDL conjugate predominantly transported [$^{125}$I]tyn in the apical-to-basal direction (20-fold greater transport vs. basal-to-apical); (b) [$^{125}$I]tyn-SS-PDL elicits a 10-fold greater degree of [$^{125}$I]tyn transport than [$^{125}$I]tyn-S-PDL, thus demonstrating the importance of the reducible disulfide linkage for [$^{125}$I]tyn transport to occur; (c) $^{125}$I-radioactivity recovered in the basal media was determined to be 95% [$^{125}$I]tyn-containing small molecules, thus demonstrating the release of [$^{125}$I]tyn from its PDL carrier; (d) the apical addition of an anionic species, heparin, completely blocks apical-to-basal transport of [$^{125}$I]tyn, indicating the importance of PDL-mediated binding to the apical membrane for transport to occur; (e) apical-to-basal transport proceeds via non-lysosomal pathways, as lysosomal inactivation by $NH_4Cl$ exposure does not inhibit transport, and (f) the addition of a membrane-impermeable inhibitor of disulfide reduction, bis-dithionitrobenzoic acid (DTNB), to the apical media inhibits transport by ~70%, indicating the importance of apically-localized disulfide reducing reactions for transport of [$^{125}$I]tyn. Pulse-chase studies indicate that there is no paracellular leakage of conjugate, and the ratio of recycled:membrane-associated: transported [$^{125}$I]tyn fragment following chase is 4:2:1.

KEY WORDS: poly(D-lysine); tyramine; transepithelial transport; MDCK epithelial cells.

## INTRODUCTION

Drug-carrier conjugate systems designed to accomplish increased transepithelial transport of a particular drug entity involve the utilization of either large or small molecular carriers covalently bound to either small drug molecules or larger, biologically, active proteins/peptides. Macromolecular carriers, such as those which serve as receptor-binding ligands, are generally able to achieve a high degree of selectivity with respect to delivery of drug-carrier conjugate to the targeted area. Examples of this system include a bioadhesive tomato lectin-coated microsphere (1) and an antibody-peptide conjugate (2). Certain macromolecular carriers which non-specifically enhance the adsorption of drug-carrier conjugate to cell membranes include the highly cationic polylysines (3–6) and other charged species such as wheatgerm agglutinin lectin (7). Transepithelial *transcytosis* of drug-carrier conjugates, which combines endocytosis of the drug-carrier conjugate from one side of a polarized epithelial cell, intracellular processing/trafficking of the endocytotic vesicle, and finally exocytosis of the drug released from its carrier at the opposite side of the cell, has been shown to be possible using both receptor-binding carriers (8) and adsorptive carriers (3,4). Small molecular carriers are able to accomplish increased transepithelial transport of drug by virtue of either their hydrophobic nature, e.g., fatty acid carriers (9), or their specific transporters, e.g., bile acids (10). In this paper we describe the use of poly(D-lysine) (PDL) as a non-specific, non-degradable carrier for controlling the transepithelial transport of tyramine (tyn), a stable tyrosine decarboxylation product serving as a model for small molecule drug delivery, linked to PDL via a reducible disulfide bond.

The primary goal of adsorptive drug delivery system design is to achieve targeted-controlled delivery of certain drugs by enhancing their adsorption to cell membranes followed by efficient transport through the epithelial or endothelial tissue and deposition of either the free or the conjugated drug into either the bloodstream or target organ. Several groups have investigated the feasibility of delivering drugs systemically via the oral route using naturally occurring mucoadhesive polymers (1,11) or synthetic adsorptive polymer systems (12) as carriers. Other groups have concentrated on achieving carrier-mediated transcytosis across the blood-brain barrier using specific and nonspecific adsorptive ligands as carriers (2,6). There are several important characteristics crucial to the design of an effective drug-carrier system, including minimizing the interaction of the carrier with non-targeted surfaces such as food in the gastrointestinal lumen or blood-borne proteins. Additionally, the linkage between the drug and carrier must be stable enough to resist degradation in general circulation or luminal viscera; yet, it must render the drug and carrier dissociable once delivered to the targeted area.

Soluble macromolecules, such as the polylysines, are excellent candidates for use as drug carriers (13). These macromolecules carriers have three basic advantages: (a) prolongation of residence time; (b) protection of the active moiety from inactivation while possibly reducing its immunogenicity; and (c) control of biodistribution (13). We have reported previously that polylysine based drug-carrier conjugate systems can enhance the vectorial transcytosis of a marker protein, horseradish peroxidase (3,4), across epithelial cells grown in culture. In these reports we have demonstrated that both the type of polylysine, e.g., the biodegradable poly(L-lysine) vs. the non-biodegradable poly(D-lysine), as well as the type of linkage between drug and carrier, e.g., the easily reducible disulfide linkage vs. the stable thioether linkage, are integrally important for determining the transcytotic nature of the drug-carrier conjugate. It has also been shown previously that the disulfide-linked conjugate of PDL to methotrexate (MTX-SS-PDL) is efficiently endocytosed and elicits inhibition of growth in MTX-

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0724-8741/94/0900-1250$07.00/0 

transport deficient CHO mutant cells (14). In this paper, we describe the transepithelial transport properties of tyn as PDL conjugates and depict the mechanism of transepithelial transport of this adsorptive drug-carrier system. Madin-Darby canine kidney (MDCK) cells were chosen for these studies due to the fact that, when grown to confluence on Transwell filter membranes, they develop the appropriate morphological characteristics required for studying drug transport across epithelial tissue (15–17).

## MATERIALS AND METHODS

Eagle's minimal essential medium (MEM) and fetal bovine serum (FBS) were obtained from GIBCO Laboratories (Grand Island, NY). Transwells with a 24.5-mm diameter and 0.4 μm pore size were obtained from Costar (Cambridge, MA). Poly(D-lysine, PDL) (MW = 65,000), tyramine (tyn), ammonium chloride ($NH_4Cl$), and bis-dithionitrobenzoic acid (DTNB) were obtained from Sigma Chemical Co. (St. Louis, MO). Heterobifunctional crosslinking agents N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and sulfo-succinimidyl 4-(*p*-maleimidophenyl)butyrate (sulfo-SMPB) were obtained from Pierce (Rockford, IL). Radioactive sodium iodide ($Na^{125}I$) was obtained from ICN Radiochemicals (Irvine, CA).

### Cell Culture

MDCK strain I epithelial cells were seeded in Transwells at a density of $2.2 \times 10^4$ cells/cm$^2$. Cells were maintained in a 5% $CO_2$ atmosphere at 37°C, and were supplemented with MEM spiked with varying concentrations of FBS as has been described previously (4). Briefly, cells were fed either 10%, 2.5%, or 1% FBS/MEM, with a reduction in FBS concentration as the cell monolayers approached confluence. Confluent cell monolayers were obtained 5–7 days post-inoculation, with each well demonstrating a transepithelial electrical resistance (TEER) of at least 2000 ohms · cm$^2$ as measured by an epithelial voltohmmeter (EVOM, World Precision Instruments, Sarasota, FL). Cell monolayers could be maintained at a constant resistance for up to 5 days without suffering leakage between apical and basal compartments or a loss of TEER.

### Synthesis of [$^{125}$I]tyn-SS-PDL and [$^{125}$I]tyn-S-PDL Conjugates

To prepare the disulfide-linked conjugate, 10 mg of PDL was dissolved in 1 ml PBS, pH 7, and 2 mg of SPDP was dissolved in 0.2 ml of *N,N*-dimethylformamide, 0.15 ml of the SPDP solution was added, dropwise, to the PDL solution, while vortexing at room temperature, to obtain a 30:1 molar ratio of SPDP:PDL. After 2 hr at room temperature, 0.1 ml of dithiothreitol (DTT, 1M) was added to the reaction. The reaction was then allowed to sit at room temperature for 30 min, after which the entire reaction volume was eluted through a 10 ml Sephadex G-50 column. Eluted fractions were subjected to a standard Ellman's Reagent test to determine those high MW fractions having free thiol groups, which were then added to SPDP-modified tyn (5 mg tyn/0.5 ml PBS, pH 7) and reacted for 24 hr at room temperature. The conjugation reaction was then concentrated to 1 ml using a Centricon-10 filter concentrator (Amicon Corp), loaded onto a Sephadex G-50 column, and high MW eluted fractions were analyzed for tyn modification spectrophotometrically at 280 nm. The final ratio of tyn:PDL in the tyn-SS-PDL conjugate was determined to be 36:1 by calculating the [tyn] using its molar extinction coefficient and the [PDL] using the Trypan Blue Precipitation assay (18). Tyn-S-PDL was prepared similarly, the only difference being that tyn was modified using sulfo-SMPB as opposed to SPDP, thus creating the thioether linkage. Both conjugates were iodinated using the Chloramine-T method, and were then purified on a 10 ml Sephadex G-50 column to >99% pure [$^{125}$I]tyn-SS-PDL or [$^{125}$I]tyn-S-PDL having specific radioactivities of 0.736 or 4.27 μCi/μg tyn, respectively. Conjugates were determined to remain stable (>99% pure) prior to their use in experiments using filter-grown MDCK cells.

### Transport of Tyn-PDL Conjugates Across MDCK Cells

MDCK monolayers grown to confluence on Transwells (as described above) were used for both transport and pulse-chase experiments. In all experiments, 1% FBS/MEM was used as the incubation media. For transport experiments, 3 μg/ml PDL of tyn-PDL conjugate was added to either the apical or basal side (donor compartment) of the Transwells and hourly samples amounting to 10% of the total recipient compartment volume were taken. Following sample removal from the recipient compartment, fresh media was added as replenishment to insure constant volume in both Transwell compartments for the duration of the experiment. Collected samples were measured for $^{125}$I-radioactivity in a Packard gamma counter. At the end of the experiments, the monolayers were washed three times with cold PBS and the entire filter membrane/cell monolayer was analyzed for $^{125}$I-radioactivity. For pulse-chase experiments, the Transwells were cooled to 4°C for 30 min prior to conjugate administration, conjugate was pulsed from the donor compartment for 1 hr at 4°C, and then all media was removed and the cells were washed three times with cold PBS. Transwells were then incubated for 2 hr at 37°C, and the entire apical and basal compartment volumes were counted; in addition, the filter membrane/cell monolayers were washed three times with cold PBS and counted. In certain cases, recipient compartment media were analyzed using a Sephadex G-50 column to determine the molecular size of the transported $^{125}$I-radioactivity.

### Treatment of MDCK Cells with Biochemicals Affecting Tyn-PDL Transport

In certain experiments, biochemicals were added to either the donor or recipient compartment of the Transwells in order to dissect the mechanisms involved in the transport process. The following biochemicals were used: $NH_4Cl$ (20 mM), DTNB (100 μM), DTT (5 mM), and heparin (30 μg/ml). The treatment of cell monolayers with these biochemicals did not result in any significant decrease in TEER throughout the course of the transport experiments.

## RESULTS

Both [$^{125}$I]tyn-SS-PDL and [$^{125}$I]tyn-S-PDL were prepared as has been described in the Materials and Methods section and in (19). Conjugates were tested for their stability

for a period of 8 weeks; both preparations were found to be >99% pure, unfragmented conjugate as determined using Sephadex G-50 column chromatography. Upon evaluation of the polarity of conjugate transport, [$^{125}$I]tyn-SS-PDL was found to transport [$^{125}$I]tyn in the apical-to-basal direction predominantly in filter-grown MDCK monolayers (Fig. 1). Over the course of a 6 hr experiment, [$^{125}$I]tyn transport in the apical-to-basal direction was approximately 20-fold greater than that amount transported in the basal-to-apical direction. Throughout the course of the experiment, the TEER of the MDCK monolayers was monitored and was determined to be >3000 ohms · cm$^2$. The basal media containing transported $^{125}$I-radioactivity was collected and run through a 10 ml Sephadex G-50 column to determine the molecular size of the transported ligand; it was found that ~95% of the recovered $^{125}$I-radioactivity was indeed released [$^{125}$I]tyn as opposed to intact conjugate. In order to further demonstrate the polarity inherent to this transport process, pulse-chase experiments were done using MDCK cells; these results are shown as Table I. It is notable that a comparable polarity was established in these experiments, i.e., the apical-to-basal transport was 15-fold greater than basal-to-apical transport.

When the capacity for apical-to-basal transport of [$^{125}$I]tyn using [$^{125}$I]tyn-SS-PDL was compared with that of [$^{125}$I]tyn-S-PDL, it was found that [$^{125}$I]tyn-SS-PDL resulted in an amount of transported [$^{125}$I]tyn approximately 10-fold greater than [$^{125}$I]tyn-S-PDL (Fig. 2A), thus demonstrating the importance of the type of linkage on this transport process. Fig. 2B shows that the disulfide-linked conjugate's predominance over the thioether-linked conjugate with respect to apical-to-basal transepithelial [$^{125}$I]tyn transport does not simply reflect the amount of conjugate entering the cell monolayers; in fact, the thioether-linked conjugate demonstrates a 2-fold greater cell-associated amount of [$^{125}$I]tyn after 6 hr.

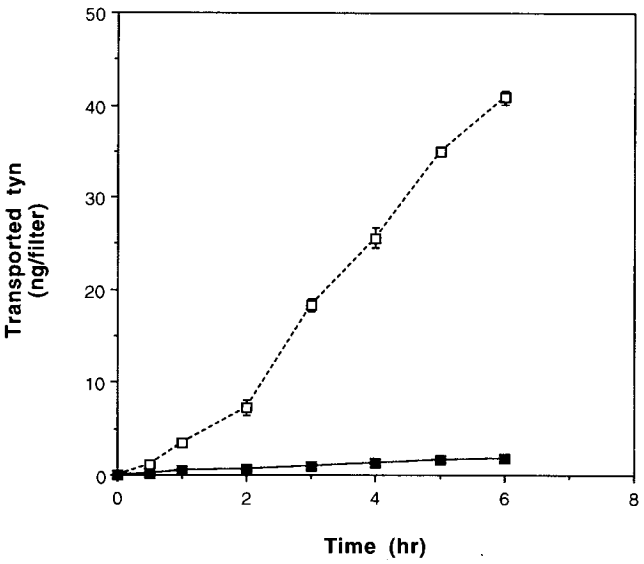


Fig. 1. Basal-to-apical vs. apical-to-basal transport of [$^{125}$I]tyn across MDCK-grown Transwells. [$^{125}$I]tyn-SS-PDL conjugate was added at a concentration of 3 μg/ml PDL to either the apical or the basal chamber of Transwell monolayers and then incubated at 37°C for 6 hr. Samples (0.1 ml) were taken each hour from the chamber opposite to that of conjugate administration, and fresh media (1% FBS-spiked MEM) was added as replenishment to insure a constant volume in the recipient chamber. Samples were analyzed for $^{125}$I-radioactivity in a Packard gamma counter, and the amount of [$^{125}$I]tyn transported in the apical-to-basal direction (□) was plotted against the amount transported in the basal-to-apical direction (■). Each point represents the mean of measurements from N = 3 monolayers, and the S.E.M. is either represented as a bar or is smaller than the size of the symbol.

Table I. Recovery of [$^{125}$I]tyn following a 2 hr pulse (4°C)/1 hr chase (37°C) of [$^{125}$I]tyn-SS-PDL in filter-grown MDCK cells.

| | Percent of Total Radioactivity | | |
|---|---|---|---|
| Donor Compartment[a] | Transcytosed[b] (%) | Recycled[c] (%) | Membrane-Associated[d] (%) |
| apical | 15 | 57 | 28 |
| basal | <1 | 35 | 64 |

[a] [$^{125}$I]tyn-SS-PDL conjugate applied to either the apical or basal chamber of Transwell-grown MDCK monolayers after cooling to 4°C for 30 min.

[b] Percentage of total [$^{125}$I]tyn recovered in acceptor compartment post-chase. Sephadex G-50 analysis showed >95% of transcytosed $^{125}$I-radioactivity to be [$^{125}$I]tyn fragments.

[c] Percentage of total [$^{125}$I]tyn recovered in donor compartment post-chase. Sephadex G-50 analysios showed 34% of recycled $^{125}$I-radioactivity to be [$^{125}$I]tyn fragments.

[d] Percentage of total [$^{125}$I]tyn recovered in cell/filter membrane post-2 hr chase.

Certain experiments were done to determine the nature of the charge interaction between the PDL carrier and the cell membrane. Specifically, an excess of heparin (30 μg/ml), a highly negatively charged polyanion, was added to the apical media concomitant to [$^{125}$I]tyn-SS-PDL conjugate administration in order to neutralize the charge on the PDL and prevent the adsorption of polycations to the cell membrane. As shown in Fig. 3, heparin addition to the apical media resulted in nearly total inhibition of [$^{125}$I]tyn transport to the basal media, demonstrating that the positively charged PDL is necessary for binding to the cell membrane and subsequent transport of [$^{125}$I]tyn.

In another set of experiments, DTNB (100 μM) was added to either the apical, basal, or both apical and basal media concomitant to apical administration of [$^{125}$I]tyn-SS-PDL conjugate and the transport of [$^{125}$I]tyn to the basal media was monitored for 6 hr (Fig. 4). DTNB, an effective inhibitor of disulfide- reducing reactions (20), is impermeable to the cell membrane; therefore, this experiment allowed us to determine if either apical- or basal-membrane associated reducing mechanisms were responsible for reduction of the disulfide bond in the [$^{125}$I]tyn-SS-PDL conjugate. From the graph shown as Fig. 4, it is apparent that approximately 70% of the disulfide reduction involved in [$^{125}$I]tyn apical-to-basal transport is accomplished by reducing mechanisms located on the apical surface of the cell monolayers, and the basolateral surface of the monolayers is not significantly responsible for [$^{125}$I]tyn-SS-PDL reduction. Thus, it is safe to assume that the other 30% of [$^{125}$I]tyn-SS-PDL reduction occurs intracellularly (see Discussion section).

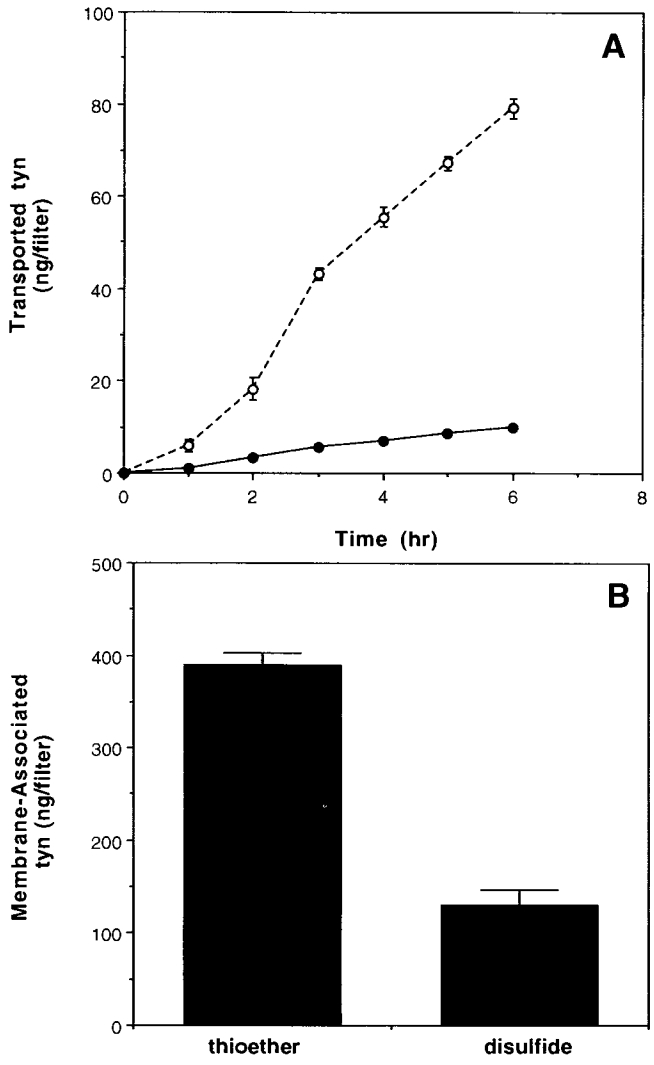


Fig. 2. Apical-to-basal transport of [$^{125}$I]tyn across MDCK-grown Transwells using either a disulfide-linked or a thioether-linked conjugate of [$^{125}$I]tyn to PDL. *Panel A:* Either [$^{125}$I]tyn-SS-PDL (○) or [$^{125}$I]tyn-S-PDL (●) conjugate was added to the apical chamber of Transwell monolayers at a concentration of 3 μg/ml PDL and the apical-to-basal transport of [$^{125}$I]tyn was monitored hourly for 6 hr at 37°C by sampling 0.1 ml basal media and analyzing the sample in a gamma counter. The basal chamber volume was kept constant by replenishing with an equivalent amount of 1% FBS-MEM each hour. *Panel B:* At the conclusion of the experiment, all remaining media were removed, the monolayers were washed extensively with cold PBS, and the Transwell filters were analyzed for remaining cell/filter membrane-associated [$^{125}$I]tyn. Each point or bar represents the mean of measurements from N = 3 monolayers, and the S.E.M. is either represented as a bar or is smaller than the size of the symbol.

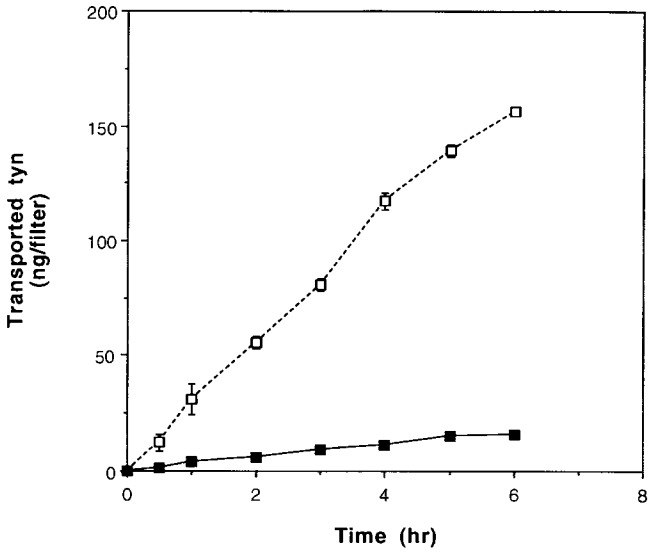


Fig. 3. Apical-to-basal transport of [$^{125}$I]tyn across MDCK-grown Transwells in the presence of heparin. [$^{125}$I]tyn-SS-PDL conjugate was added at a concentration of 3 μg/ml PDL to the apical chamber of Transwell monolayers and then incubated at 37°C for 6 hr. In certain monolayers, heparin (30 μg/ml) was added to the apical chamber immediately prior to conjugate administration. Samples (0.1 ml) were taken each hour from the basal chamber, and fresh media (1% FBS-MEM) was added as replenishment to insure a constant volume in the recipient chamber. Samples were analyzed for [$^{125}$I]tyn-radioactivity in a gamma counter, and the amount of [$^{125}$I]tyn transported in the control monolayers (□) was plotted against the amount of [$^{125}$I]tyn transported in the presence of heparin (■). Each point represents the mean of measurements from N = 3 monolayers, and the S.E.M. is either represented as a bar or is smaller than the size of the symbol.

In transport experiments designed to determine whether or not reduction of the disulfide linkage during [$^{125}$I]tyn transport was proceeding via a lysosomal pathway, 20 mM $NH_4Cl$ was added to the apical media concomitant to either [$^{125}$I]tyn-SS-PDL or [$^{125}$I]tyn-S-PDL conjugate and transport of [$^{125}$I]tyn was monitored for 6 hr. In both cases, no significant inhibition of [$^{125}$I]tyn transport was noted. Other experiments were designed to demonstrate the existence of an active transporter of tyn across MDCK cell monolayers; the results presented as Table II show that a concentration gradient between the apical and basal chambers of MDCK-grown Transwell's appeared when [$^{125}$I]tyn-SS-PDL conjugate was reduced using DTT prior to its addition to the apical media. [$^{125}$I]tyn-SS-PDL was treated with 0.5 mM DTT for 20 min at 37°C, resulting in >90% reduction of the disulfide linkage within the conjugate as measured using Sephadex G-50 analysis. Transport experiments were continued for 6 hr, at which point the entire apical and basal media was collected and analyzed for $^{125}$I-radioactivity. As shown in Table II, a concentration gradient favoring the basal media was noted after 6 hr whether the DTT-reduced conjugate was added to the apical or the basal media at the start of the experiment. For experiments involving DTNB inhibition, DTT reduction, and heparin charge neutralization, Fig. 5 depicts the effects of these treatments on the cell/filter-membrane associated [$^{125}$I]tyn or [$^{125}$I]tyn conjugate activity.

## DISCUSSION

Presently, a generic, clinically applicable method to achieve efficient systemic absorption of the many protein, peptide, and hydrophilic drug entities which are presently only biologically available when delivered invasively does not exist (21). Thus, the apical-to-basal polarity noted for PDL-mediated [$^{125}$I]tyn transepithelial transport across fil-

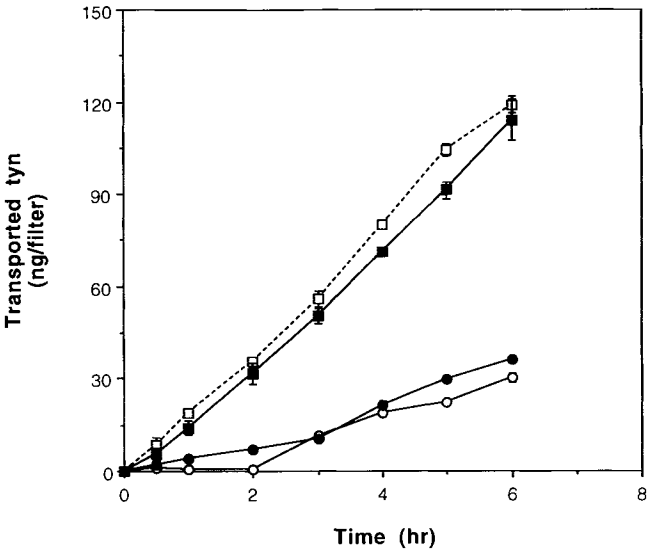


Fig. 4. Apical-to-basal transport of [$^{125}$I]tyn across MDCK-grown Transwells in the presence of DTNB. [$^{125}$I]tyn-SS-PDL conjugate was added at a concentration of 3 μg/ml PDL to the apical chamber of Transwell monolayers and then incubated at 37°C for 6 hr. In certain monolayers, DTNB (100 μM) was added to either the apical or basal chamber immediately prior to conjugate administration. Samples (0.1 ml) were taken each hour from the basal chamber, and fresh media (1% FBS-MEM) was added as replenishment to insure a constant volume in the recipient chamber; those monolayers exposed to DTNB in the basal chamber received DTNB-spiked media. Samples were analyzed for [$^{125}$I]-reactivity in a gamma counter, and the amount of [$^{125}$I]tyn transported in the control monolayers (□) was plotted against the amount of [$^{125}$I]tyn transported in the presence of DTNB administered to either the basal media (■), the apical media (○), or both apical and basal media (●). Each point represents the mean of measurements from N = 3 monolayers, and the S.E.M. is either represented as a bar or is smaller than the size of the symbol.

ter-grown MDCK cells is a pharmaceutically relevant phenomenon due to the fact that this transport directionality is analogous to the absorption of molecules across various types of epithelia.

The experiments describing the administration of heparin, a polyanionic species, to neutralize the polycationic charge inherent to the PDL carrier demonstrated that the electrostatic binding between the PDL carrier and the apical membrane of MDCK cells is an integral part of disulfide-linked, PDL-mediated transepithelial transport of [$^{125}$I]tyn. As shown in Fig. 3, when heparin is present in the apical media, there is negligible apical-to-basal transport of [$^{125}$I]tyn. Additionally, these data show that the serum present in the apical chamber, which is at a concentration of 1% in the cell culture media, is not responsible for reduction of the disulfide linkage within the conjugate. By using DTNB, a membrane-impermeable inhibitor of disulfide reduction (20), it is possible to determine if either apical or basal membrane-bound mechanisms are responsible for reduction of the disulfide bond within the conjugate (19). As depicted in Fig. 4, apical administration of DTNB inhibits [$^{125}$I]tyn-SS-PDL transport by ~70%, basal administration of DTNB elicits no significant inhibition of [$^{125}$I]tyn transport, and the combination of apical and basal administration of DTNB inhibits [$^{125}$I]tyn transports by 70%. These data imply that 70% of [$^{125}$I]tyn-SS-PDL reduction resulting in apical-to-basal [$^{125}$I]tyn transepithelial transport is accomplished on the apical membrane of the MDCK cells, possibly mediated by protein disulfide isomerase (22), while the remaining 30% occurs intracellularly. Interestingly, is appears as though enzymes located on the basolateral domain of the MDCK cells are not integral for processing apically-administered [$^{125}$I]tyn-SS-PDL conjugate. These data are consistent with previous studies using cultured Chinese hamster ovary cells with respect to inhibition of apical disulfide reduction and internalization of [$^{125}$I]tyn-SS-PDL conjugate via DTNB administration (19).

Table II. A concentration gradient is established between the apical and basal compartments of Transwell-grown MDCK monolayers for DTT-reduced [$^{125}$I]tyn-SS-PDL.

| Donor Compartment[a] | Apical [tyn] (ng/ml) | Basal [tyn] (ng/ml) | $[tyn]_{basal}$: $[tyn]_{apical}$[b] |
|---|---|---|---|
| apical | 25.8 ± 0.5 | 48.0 ± 0.1 | 2:1 |
| basal | 9.7 ± 0.3 | 88.8 ± 0.6 | 9:1 |

[a] [$^{125}$I]tyn-SS-PDL conjugate treated with 0.5 mM DTT for 20 min at 37°C prior to addition to the apical side of MDCK Transwells. Sephadex G-50 analysis showed that >95% of [$^{125}$I]tyn-SS-PDL was reduced to free [$^{125}$I]tyn.

[b] The entire apical and basal media was collected and analyzed after 6 hr to determine the ratio of basal [tyn]:apical [tyn].

The importance of having an easily reducible disulfide linkage within the PDL conjugate in order to achieve efficient transepithelial transport of the conjugated ligand has been demonstrated previously (4). Similarly, in this study, we have shown that having the proper linkage between the PDL carrier and [$^{125}$I]tyn is an integral consideration, and this fact is illustrated in Fig. 2A. Experiments in which the non-reducible thioether-linked [$^{125}$I]tyn-S-PDL conjugate was administered to the apical side of the MDCK cells and its transport properties were compared with those of the disulfide-linked [$^{125}$I]tyn-SS-PDL conjugate show quite clearly that the thioether linkage does not promote efficient release of [$^{125}$I]tyn from the PDL carrier. It has been demonstrated previously that a hydrophilic ligand conjugated to a polylysine polymer will be able to achieve an exceptionally high degree of non-specific uptake into cultured epithelial cells (3,4) or fibroblasts (5). As shown in Fig. 2B, transepithelial transport of [$^{125}$I]tyn is not dependent upon uptake of either conjugate into the cells; in fact, the thioether conjugate registers a 2-3-fold greater uptake of [$^{125}$I]tyn than does the disulfide conjugate. Thus, the type of linkage used to conjugate the PDL carrier to [$^{125}$I]tyn is of critical importance to the efficiency of this transepithelial transport system.

With respect to that amount of [$^{125}$I]tyn-SS-PDL conjugate which is processed intracellularly, we conducted certain experiments to determine the nature of the compartment accessed within the cells which is responsible for disulfide reduction, i.e., is the reduction accomplished within lysosomes, or does it occur at another intracellular location? In these experiments, a lysosomal inhibitor, $NH_4Cl$, was added to the apical media in apically-pulsed MDCK cells in order to

neutralize lysosomal pH (23). Both the [$^{125}$I]tyn-S-PDL and [$^{125}$I]tyn-SS-PDL conjugates demonstrated no significant inhibition of [$^{125}$I]tyn transport in the presence of 20 mM $NH_4Cl$; thus, lysosomes are not responsible for the reduction or degradation of that amount of conjugate processed intracellularly. Therefore, for apical-to-basal transport of [$^{125}$I]tyn-SS-PDL, 30% of the [$^{125}$I]tyn transported which has not been released from the conjugate at the apical surface results from non-lysosomal intracellular reduction of the disulfide linkage.

The amount of cell/filter membrane-associated [$^{125}$I]tyn was measured at the end of the various transport experiments, as is shown in Fig. 5. It is apparent that only [$^{125}$I]tyn-SS-PDL conjugate, as opposed to a reduced fragment (DTT treated) or electrostatically neutralized conjugate (heparin treated), results in a significant amount of uptake/adsorption of [$^{125}$I]tyn to the cell/filter membrane. This is an important observation when attempting to determine the mechanism(s) responsible for [$^{125}$I]tyn transport across the MDCK cells; specifically, the DTT treated conjugate transport data from Table II indicates that there exists an active transport mechanism which greatly contributes to the transepithelial transport of the [$^{125}$I]tyn fragment. Understandably, it is quite probable that the small [$^{125}$I]tyn fragment is able to transport paracellularly through the MDCK monolayer; yet, the concentration gradient established between the apical and basal media is indicative of an energy-dependent process (Table II). If solely a passive diffusion mechanism were involved in [$^{125}$I]tyn transport, an equivalent concentration of [$^{125}$I]tyn fragment would ultimately appear in both apical and basal media at the conclusion of the 6 hr experiment. Additionally, in other experiments, the apical-to-basal transport of tyn fragments is inibitable by the presence of the ATP poison $NaN_3$ (0.5%), reinforcing the active nature of [$^{125}$I]tyn transepithelial transport in MDCK cells (data not shown). It is important to note that, even though the [$^{125}$I]tyn fragment can be transported by an active process, its generation from [$^{125}$I]tyn-SS-PDL is dependent upon adsorption of the conjugate to the apical membrane of the epithelial cells (Fig. 3); therefore, its transport is an adsorptive-mediated process. Furthermore, we have reported previously that a non-membrane permeable drug, leu-enkephalin, is able to achieve apical-to-basal transepithelial transport across MDCK cell monolayers via disulfide linkage to PDL (24); yet, leu-enkephalin transport proceeds via a different mechanism than that of tyn transport, and thus is not elaborated upon in this paper.

To summarize the results of these experiments, we propose a model for the apical-to-basal transepithelial transport of [$^{125}$I]tyn via its disulfide linked PDL carrier (Fig. 6). Supported by the heparin treatment experiments, binding of the conjugate to the apical membrane is an integral first step. Next, as is supported by the DTNB treatment data, 70% of the [$^{125}$I]tyn fragment transported across the cells in the apical-to-basal direction is a result of disulfide reduction of the conjugate at the apical membrane followed by transepi-

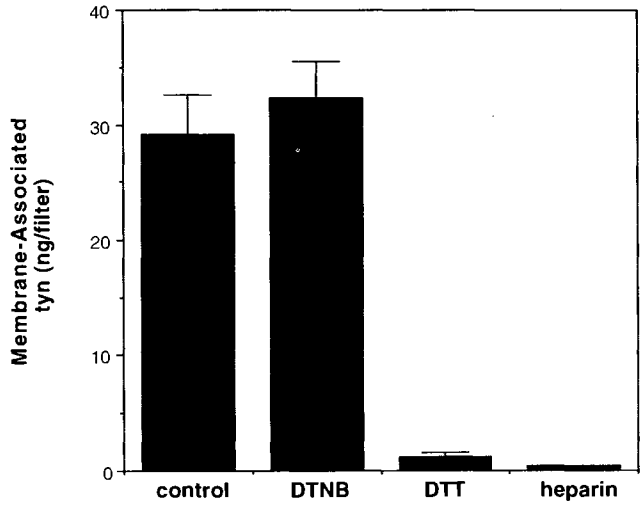


Fig. 5. Cell/filter membrane-associated [$^{125}$I]tyn in MDCK-grown Transwells following apical administration of [$^{125}$I]tyn-SS-PDL conjugate in the presence of either DTNB, DTT, or heparin. In all monolayers, [$^{125}$I]tyn-SS-PDL conjugate was added at a concentration of 3 μg/ml PDL to the apical chamber then incubated at 37°C for 6 hr. In the first set of monolayers, DTNB (100 μM) was added to the apical chamber immediately prior to conjugate administration; in the second set of monolayers, heparin (30 μg/ml) was added to the apical chamber immediately prior to conjugate administration. A third set of monolayers received conjugate only, thus serving as the untreated controls. In a fourth set of monolayers, the [$^{125}$I]tyn-SS-PDL conjugate was pre-treated with 5 mM DTT for 20 min at 37°C prior to adding it to the apical chamber. At the conclusion of the experiment, all media were removed, the monolayers were washed extensively with cold PBS, and the Transwell filters were analyzed for cell/filter membrane-associated [$^{125}$I]tyn. Each bar represents the mean of measurements from N = 3 monolayers, and the S.E.M. is represented as a bar.

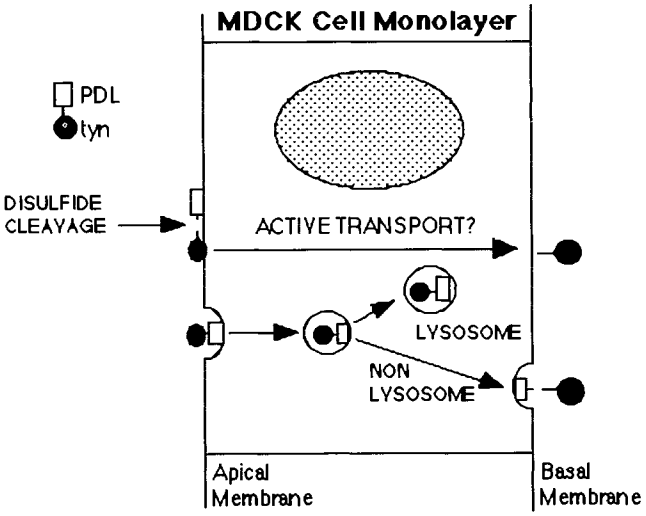


Fig. 6. A proposed model depicting the process of apical-to-basal transport of [$^{125}$I]tyn via its disulfide-linked PDL conjugate across Transwell-grown MDCK cells. Binding of conjugate proceeds non-specifically on the apical side of the monolayer, followed by either reduction of the disulfide linkage on the apical membrane itself or within the cell following endocytosis. The [$^{125}$I]tyn released as a result of apical disulfide reduction may undergo active transport across the cell, which contributes to about 70% of total transported [$^{125}$I]tyn. Endocytosed conjugate reaches a non-lysosomal intracellular compartment where disulfide reduction occurs; released [$^{125}$I]tyn is then exocytosed from the basolateral membrane of the cell into the basal media of the Transwell chamber.

thelial transport of the released [$^{125}$I]tyn fragment. The remaining 30% of that amount of [$^{125}$I]tyn fragment transported across the cells proceeds via internalization of the [$^{125}$I]tyn-SS-PDL conjugate, non-lysosomal disulfide reduction, and then transport of the [$^{125}$I]tyn fragment to the basal membrane of the cells and release of [$^{125}$I]tyn into the basal media. Most likely, the conjugate reduced on the apical surface is transported in the apical-to-basal direction via an as yet unidentified active transport mechanism. According to our data from the pulse/chase experiments (Table I), only 15% of bound and apically-internalized [$^{125}$I]tyn eventually is released from the basal side of the cells; the majority of internalized /[$^{125}$I]tyn is either recycled back to the apical media or remains trapped within the cells and/or filter membrane.

These studies describing the transepithelial transport of radiolabeled [$^{125}$I]tyn via a easily reversible PDL conjugate clearly demonstrate that it is possible to immobilize, via a reducible disulfide linkage, a significant number of small [$^{125}$I]tyn molecules to a large, non-degradable polymeric carrier. Specifically, in this paper, we have shown that the [$^{125}$I]tyn-SS-PDL conjugate is able to adhere to the apical membrane domain of filter-grown MDCK cells, followed by reduction of the disulfide linkage, transport of [$^{125}$I]tyn across the cell, and release of [$^{125}$I]tyn from the basal membrane. More generally, we have designed a novel system for controlling the delivery of a small drug molecule across epithelial cell monolayers using an adsorptive macromolecular carrier. Thus, this study represents an approach which is contributive to the improvement of drug targeting (13).

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