Biochemical and Biophysical Research Communications **276,** 251–257 (2000)
doi:10.1006/bbrc.2000.3358, available online at http://www.idealibrary.com on IDEAL®

Peptide-Mediated Transcytosis of Phage Display Vectors in MDCK Cells

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Received July 12, 2000

Delivery of therapeutic macromolecules and gene vectors to certain tissues is hampered by endothelial or epithelial barriers. We show here that the transport of phage particles across epithelial cells can be facilitated by peptide ligands selected from a phage library of random peptides. Using MDCK cells, we identified a polycationic peptide sequence, RYRGDLGRR, containing a putative integrin-binding (RGD) motif that enhanced basal-to-apical transcytosis of peptide-bearing phage 1000- to 10,000-fold compared with phage with no peptide insert. Both the synthetic peptide RYRGDLGRR and the integrin-binding peptide GRGDSP inhibited phage transcytosis suggesting the involvement of integrins. Confocal immunofluorescence microscopy showed that following internalization at the basal cell surface, phage particles were delivered to the apical cytoplasm and released at the apical cell surface. These data suggest the feasibility of using short peptides for targeting transcytotic pathways and facilitating delivery of macromolecules across cellular barriers. © 2000 Academic Press

Key Words: **receptor-mediated transcytosis; phage display; integrins; MDCK cells.**

The barrier function of epithelial and endothelial cells hinders the access of macromolecular drugs and gene therapy vectors to target tissues. For instance, the intestinal epithelium prevents the passage of most orally administered macromolecular therapeutics into the bloodstream (1). Similarly, the "blood–brain barrier" (BBB) presents a major obstacle in targeting drugs to the central nervous system (2, 3). These cellular barriers are formed by tight junctions between neighboring cells that prevent the passage of macromolecules along a paracellular pathway (4, 5). The continuity and integrity of the endothelial lining strongly affects the delivery efficiency of viral gene therapy vectors from the blood to target tissues. While a high level of gene transfer was reported for liver tissue possessing sinusoidal vasculature and discontinuous endothelium, little or no transgene expression was detected in tissues with continuous endothelium such as skeletal muscles or brain (6, 7). Temporal osmotic disruption of the BBB, however, has been shown to facilitate the delivery of viral vectors to brain tissue (7, 8).

Receptor-mediated transcytosis has been used as a physiological means for transporting therapeutic agents across endothelial and epithelial cells. In this approach, a carrier, which is either a natural ligand to, or an antibody against a transcytosing receptor, is conjugated to the therapeutic agent. This carrier-drug conjugate can bind the cognate receptor and undergo vesicular trafficking across the barrier, delivering the therapeutic agent to the target organ (1, 3, 9, 10).

Previous efforts to develop transcytosing carriers have been limited to the analysis of known receptor-ligand pairs. We investigated here an alternative approach to identify novel and potentially more efficient transcytosing ligands by screening phage display combinatorial libraries. Using a phage library of random peptides and a model system of epithelial Madin–Darby canine kidney (MDCK) cells, we selected and characterized a polycationic peptide sequence RYRGDLGRR that greatly enhances the transcytosis of peptide-bearing phage across the cells. This novel approach of functional selection for transcytosis may be useful for generating peptide carriers capable of transporting macromolecules and viral vectors across cellular barriers.

MATERIALS AND METHODS

Cell culture. MDCK strain II (MDCKII) cells transfected with a rabbit receptor for polymeric immunoglobulins (pIgR) were kindly provided by Dr. K. E. Mostov (University of California) (11). MDCK strain I (MDCKI) cells (12) were kindly provided by Dr. K. Holmes (University of Colorado). Cells were maintained in Minimal Essential Medium (MEM; Life Technologies) supplemented with 10% heat-

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0006-291X/00 $35.00

inactivated fetal bovine serum (FBS) and antibiotic–antimycotic cocktail (Life Technologies) in 5% CO_2/95% air at 37°C. For transcytosis experiments, cells were plated at a density of 2×10^5 cells/cm^2 into 3-μm pore size Transwell-Clear chambers (Corning Costar). Cells formed confluent polarized monolayers 2 days after plating and were used for transcytosis experiments 3 days after plating. The integrity of monolayers was controlled by three techniques: visual inspection with a phase contrast microscope, the "leak test" (13), and measuring the transepithelial electrical resistance with a Millicell-ERS meter (Millipore). A fresh stock of cells was used after two to three passages.

Screening a phage library for peptides that mediate phage transcytosis. MDCKII cells transfected with pIgR were grown as confluent monolayers in 24-mm Transwells. The phagemid pC89 and the nonapeptide library of random peptides constructed using this vector in the N-terminal region of the major coat protein pVIII (Fig. 1) were kindly provided by Drs. F. Felici and A. Luzzago (Istituto di Ricerche di Biologia Molecolare, Rome, Italy) (14, 15). An aliquot of the library containing 7×10^{10} ampicillin-resistance transducing units (TU) was added into the basal chamber of two Transwell units and incubated with cells for 24 h at 37°C. Phage recovered in the apical medium were concentrated using MICROSEP microconcentrators with 300 kDa molecular weight cut off (Pall Filtron) and propagated by infection of *Escherichia coli* XLI-Blue bacteria (Stratagene) as described (16, 17). After four rounds of selection and propagation, peptide-encoding DNA inserts in individual clones were sequenced as described (17).

Phage transcytosis assay. Confluent monolayers of MDCKI cells were grown in Transwells. Phage were added into the medium of either the basal or apical chamber. Following incubation at 37°C for the indicated periods of time, aliquots were taken from the opposite chamber, and the number of phage was determined by infection of bacteria as described (18). To normalize the results obtained with Transwells of different size (6.5-, 12-, and 24-mm diameter), the total number of phage is divided by the area of the epithelial sheet and reported as TU/cm^2 throughout the presentation of data.

Immunofluorescence confocal microscopy. MDCKI cells were grown as confluent monolayers in 24-mm Transwell units. Phage (10^{11} TU) were added into the basal chamber and incubated with cells for 4 h at 37°C. Following incubation, nonattached phage were removed from the apical cell surface and the basal surface of the filter by extensive washing with 60 ml of Hanks' balanced salt solution containing Ca^{2+} and Mg^{2+}. Cells were fixed in 3.7% formaldehyde (Fisher Scientific) in PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ for 10 min, and filters with cells were cut in half. Each half of the filter was processed for immunostaining of either extracellular and intracellular phage (Protocol A), or extracellular bound phage only (Protocol B). In Protocol A, fixed cells were permeabilized with 0.1% saponin (Sigma) in PBS for 15 min, incubated with the blocking solution (1% BSA and 0.025% NaN_3 in PBS-saponin) for 15 min, and then incubated with rabbit anti-fd bacteriophage IgG (Accurate Chemical and Scientific Corp.) diluted 1:200 in PBS-saponin-BSA for 1 h. In Protocol B, cells were processed in the same manner as in Protocol A, except saponin was excluded from solutions. Consequently, cells remained impermeable to the primary anti-phage antibody, and only extracellular bound phage could react with the antibody. The following steps were the same in both Protocol A and B. Cells were washed 3×5 min with PBS, 10 min with PBS-saponin, blocked for 10 min with PBS–saponin–BSA containing 100 μg/ml RNAse A (Gentra Systems), and incubated for 30 min with rat anti-ZO-1 monoclonal antibody (Chemicon) diluted 1:100 in PBS–saponin–BSA–RNAse. Subsequently, cells were washed 4×10 min with PBS–saponin and incubated for 1 h with secondary antibodies and propidium iodide (1 μg/ml) (Molecular Probes) diluted in PBS–saponin-BSA. The following secondary antibodies were used: FITC-conjugated swine anti-rabbit immunoglobulins (Dako) diluted 1:30, and TRITC-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories) diluted 1:50. Cells were washed 5×10 min with PBS and mounted in ProLong Antifade (Molecular Probes). The samples were examined with a Zeiss LSM 510 confocal microscope using a C-Apochromat 63×, 1.2 water immersion objective.

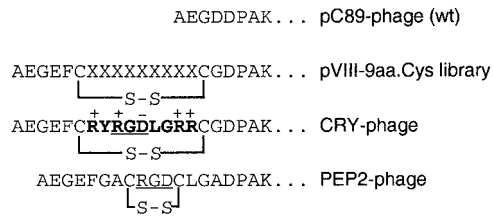


FIG. 1. Amino acid sequence of the N-terminal portion of the wild-type and recombinant phage coat protein pVIII. pC89-phage (14) represents the original vector and wild-type pVIII. In the pVIII-9aa.Cys library (15), the random peptide sequences are flanked by two cysteines to favor the presentation of peptides in a conformationally constrained cyclic form. The randomized residues are marked with (X). CRY-phage was selected from the pVIII-9aa.Cys library by transport across MDCKII cells. The identified peptide motif is shown in bold, and the charge of amino acid residues is indicated. In PEP2-phage, an integrin-binding peptide (26) is fused with pVIII as described (18). The integrin-binding tripeptide motif is underlined, and the putative disulfide bonds are indicated.

RESULTS

Selection of phage that undergo transcytosis in MDCK cells. Initial selection experiments were performed using MDCKII cells transfected with pIgR. Basolateral-to-apical transcytosis of pIgR in these cells has been extensively characterized (11, 19). Therefore, we hypothesized that at least one transcytosing receptor, pIgR, may be targeted in these cells by phage display vectors. After four rounds of selection of a phage library representing approximately 2×10^7 different peptides, selected phage were delivered across the monolayer of MDCKII cells approximately 1000-fold as well as the original library (data not shown). Sequencing of DNA from eight individual clones of isolated phage inferred the identical RYRGDLGRR amino acid sequence. Phage that express this peptide are designated as "CRY-phage," named for the first three amino acid residues of the peptide insert including the flanking cysteine (Fig. 1). Surprisingly, the identified peptide contained a putative integrin-binding (RGD) motif suggesting the involvement of integrins, but not pIgR, in phage transcytosis.

To characterize transcytosis of CRY-phage in more detail, and to lessen the possibility of paracellular diffusion of phage particles, we performed further experiments using MDCKI cells. This cell line forms epithelial sheets with high transepithelial electrical resistance (TER) indicating the presence of a highly impermeable tight junctions (12). The TER of MDCKI cells was in the range of 1000–1200 ohm · cm^2, approximately ten times higher than that for MDCKII cells (TER of 100–200 ohm · cm^2).

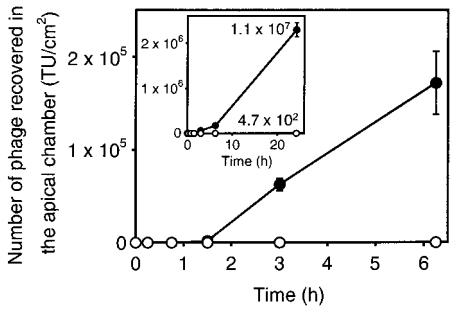


FIG. 2. Time dependence of phage transcytosis in MDCKI cells. Cells were grown on 24-mm Transwells. CRY-phage (●) or pC89-phage without peptide insert (○) (5×10^{10} TU) were applied into the basal chamber of each Transwell unit. Following incubations at 37°C for indicated periods of time, aliquots were taken from the apical chamber, and phage titers were determined. The inset shows the same data, but with expanded *x* and *y* axes. The numbers in the inset indicate total numbers of phage recovered in the apical chambers after 24 h incubation. Data represent means ± SD from four Transwell units and are representative of four experiments.

Time and temperature dependence of CRY-phage transcytosis in MDCKI cells. CRY-phage were transported across MDCKI cells in the basal-to-apical direction approximately 10,000 times more efficiently than pC89-phage without a peptide insert as shown at the 24-h time point in Fig. 2. Incubation of cells with phage for 24 h caused no noticeable decrease of TER, thus indicating the integrity of tight junction was not influenced by the presence of phage. These results suggest that the transport of CRY-phage was mediated by peptides displayed on the surface of phage particles. We observed a 1-h lag time in phage transportation (Figs. 2 and 3) similar to that described in other transcytosis systems (20–22).

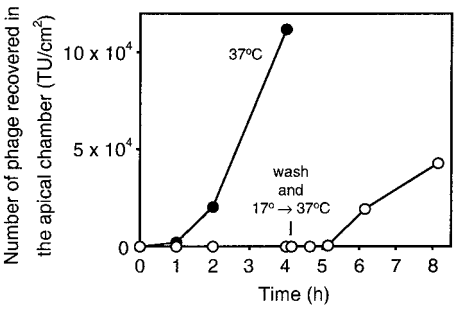


FIG. 3. Temperature dependence of CRY-phage transcytosis across MDCKI cells. Cells were cultured on 24-mm Transwells. Phage (3×10^{10} TU) were applied into the basal chamber of each Transwell unit followed by incubation at either 37°C (●), or 17°C (○). In this experiment, tissue culture medium was buffered with 20 mM Hepes, pH 7.5. After 4 h incubation at 17°C, the bottom surface of apical chambers was extensively washed with tissue culture medium. The apical chambers were transferred into new wells containing fresh medium without phage, and incubation was continued at 37°C. Aliquots were taken from the apical chambers at indicated time points, and the phage titer was determined. Data are mean values from two Transwell units.

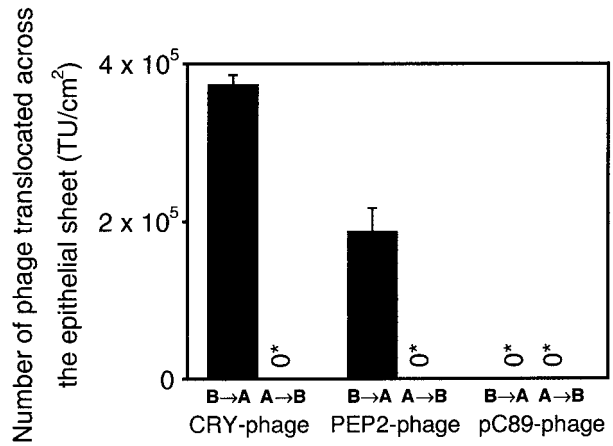


FIG. 4. Unidirectional basal-to-apical phage transport in MDCKI cells. Cells were cultured on 12-mm Transwells. Phage were applied either into the basal chamber (1.8×10^{10} TU), or apical chamber (0.9×10^{10} TU) of Transwell units. Following incubation at 37°C for 5 h, aliquots were taken from the opposite chambers, and the phage titer was determined. Data are mean values ± SD from four Transwell units. 0* indicates that the number of recovered phage was less than the minimum detection limit which was 300 TU/cm². B → A, basal-to-apical phage transport. A → B, apical-to-basal phage transport.

We next determined whether the temperature dependence of phage transport shares common features with other models of transcytosis. It has been shown that temperatures between 15 and 20°C inhibit transcytosis of IgG (23) and IgA (24), although endocytosis of these proteins does occur at these temperatures. In agreement with these observations, we found that transcytosis of CRY-phage was inhibited at 17°C (Fig. 3). However, at this temperature, cells were able to bind and, possibly, internalize phage. Warming the cells to 37°C resulted in the delivery of these loaded phage into the apical medium as evident in Fig. 3 (6- and 8-h time points). The similarity in temperature dependence of transcytosis of phage particles and proteins, such as IgG and IgA, suggests the likeness of transcytotic transport mechanisms.

Involvement of integrins in phage transcytosis. Several pieces of evidence suggest that transcytosis of CRY-phage is mediated by a direct interaction with integrins on the basal surface of MDCKI cells. First, the RGD motif present in the phage-displayed RYRGDLGRR peptide is a known recognition sequence for integrins (25). We further examined whether the RGD motif is sufficient for triggering phage transcytosis by using PEP2-phage (18) that display an RGD tripeptide (26) in a context different from that presented by CRY-phage (Fig. 1). PEP2-phage were specifically transported in the basal-to-apical direction across MDCKI cells although approximately twofold less efficiently than CRY-phage (Fig. 4), suggesting that the RGD motif is sufficient for mediating phage transcytosis.

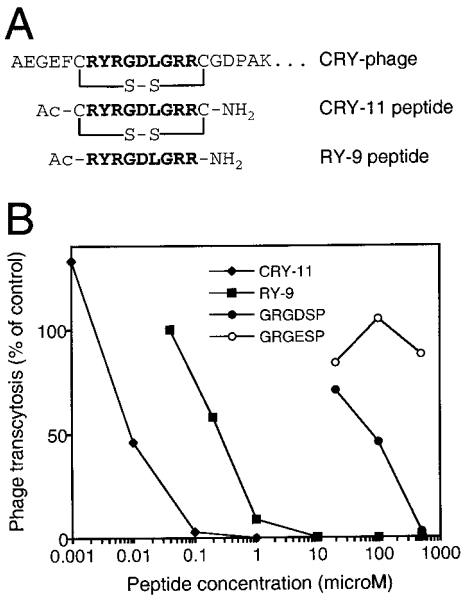


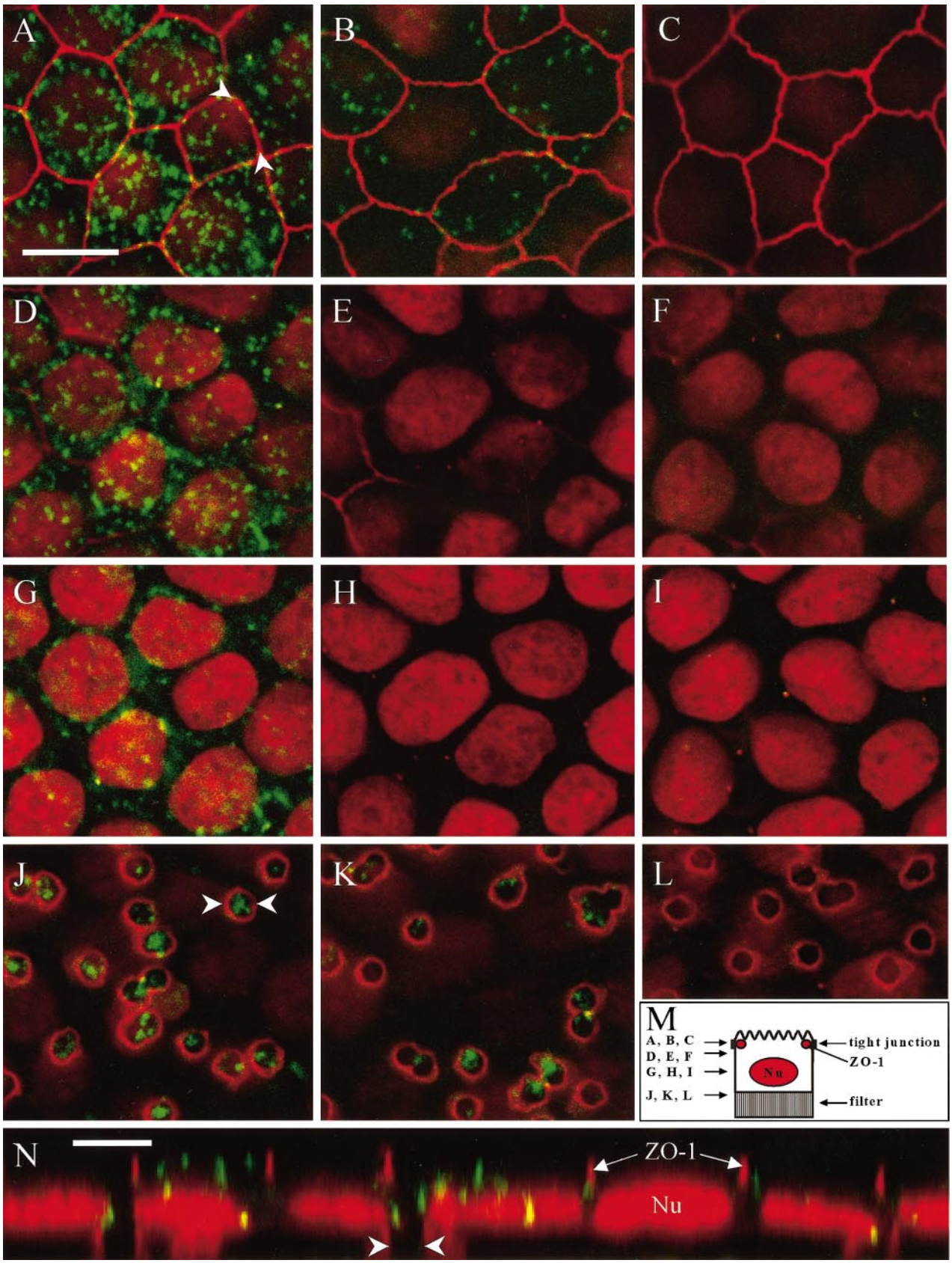
FIG. 5. Synthetic peptides derived from CRY-phage are potent inhibitors of phage transcytosis. (A) Amino acid sequence of the N-terminus of the coat protein pVIII in the isolated CRY-phage, and two derived peptides (CRY-11 and RY-9). The peptides were custom synthesized (Chiron Technologies) in N-terminus-acetylated and C-terminus-amidated form in order to eliminate any possible effects of electrical charge of the terminal amino and carboxyl groups. The identified peptide motif is shown in bold. (B) Synthetic peptides competitively inhibit basal-to-apical transcytosis of CRY-phage. GRGDSP and GRGESP peptides were obtained from Life Technologies. MDCKI cells were grown on 6.5-mm Transwells. Phage (0.875×10^{10} TU) with or without synthetic peptide were added into the basal chamber of each Transwell unit, and the number of phage transported into the apical chamber after 5 h incubation at 37°C was determined. The effect of synthetic peptides was assessed as percentage of phage transcytosis in the control Transwell units containing no peptide. The graph combines the results from three experiments. Data are either mean values from two to seven Transwell units or values from single Transwell units.

Second, we compared the efficacy of phage transcytosis across the MDCKI cell layer in the basal-to-apical direction with phage transcytosis in the apical-to-basal direction. Both CRY-phage and PEP2-phage were transported across MDCKI cells in the basal-to-apical, but not in the apical-to-basal direction (Fig. 4). These results are consistent with observations that MDCK cells express integrins predominantly on the basolateral surface, with very little expression of integrins on the apical surface (27, 28).

Third, we demonstrated that the transcytosis of CRY-phage was competitively inhibited by a synthetic integrin-binding GRGDSP peptide added into the basal chamber ($IC_{50} \approx 10^{-4}$ M) (Fig. 5B). The control GRGESP peptide had no effect (Fig. 5B). Remarkably, synthetic peptides derived from the isolated CRY-phage (Fig. 5A) appeared to be far more potent inhibitors of phage transcytosis than the GRGDSP peptide (Fig. 5B). Cyclic CRY-11 peptide inhibited phage transcytosis at lower concentrations ($IC_{50} \approx 10^{-8}$ M) than the linear RY-9 peptide ($IC_{50} \approx 3 \times 10^{-7}$ M) indicating the importance of peptide conformation for receptor recognition. These data suggest that the basal-to-apical delivery of CRY-phage is mediated by the interaction of displayed peptides with integrins.

Together, these results suggest that the binding of CRY-phage to integrins on the basal surface of MDCK cells triggers internalization of phage particles similar to that described in other tissue culture systems (18, 29). The internalized phage are likely transported across the cells and are subsequently released into the apical medium. It is not yet known whether internalized integrins remain in association with phage particles and follow the same transcytotic pathway. Addition of the integrin-binding GRGDSP peptide (1 mM) into the apical chamber consistently increased the number of phage released into the apical medium (1.5–3.1 times, $P < 0.001$; five independent experiments). The control GRGESP peptide (1 mM) had no effect (data not shown). These findings may suggest that CRY-phage are transported from the basal to the apical cell surface in association with integrins. Consequently, the apically applied GRGDSP peptide may facilitate dissociation of transcytosed phage from integrins, and thus increase the number of phage in the

FIG. 6. Localization of CRY-phage in MDCKI cells by confocal microscopy. Cells were grown on 24-mm Transwells. CRY-phage (A, B, D, E, G, H, J, K, N) or pC89-phage (C, F, I, L) (10^{11} TU) were added into the basal chamber of Transwell units and incubated with cells for 4 h at 37°C. Phage particles were detected using FITC-conjugated secondary antibody (green), and the tight junction protein ZO-1 was detected using TRITC-conjugated secondary antibody (red). The nuclei were stained with propidium iodide (red). The position of horizontal optical sections (A–L) is shown in the diagram (M): (A, B, C) at the level of tight junctions; (D, E, F) below tight junctions; (G, H, I) at the level of nuclei; (J, K, L) at the level of the uppermost portion of the filter. The images in the left column (A, D, G, J) show localization of intracellular plus extracellular CRY-phage. To stain both intracellular and extracellular phage, cells were permeabilized with saponin prior to incubation with the primary anti-phage antibody as described under Materials and Methods. Arrowheads in A indicate ZO-1. Arrowheads in J indicate a pore of the filter. Bar in A, 10 μm. The images in the middle column (B, E, H, K) show localization of extracellular CRY-phage only. To stain solely extracellular phage, cells were permeabilized with saponin only after incubation with the primary anti-phage antibody. The images in the right column (C, F, I, L) show absence of the control pC89-phage bound or internalized by cells. Cells in C, F, I, and L were permeabilized before the incubation with the primary anti-phage antibody. (N) Confocal section in the vertical plane demonstrates distribution of phage particles (green) throughout the cell cytoplasm with predominant localization in the apical region of the cell. Cells in (N) were permeabilized prior to incubation with the primary anti-phage antibody. Tight junctions are seen as red dots between neighboring cells. Nu, nucleus. Arrowheads indicate a pore within the filter. Bar, 5 μm.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apical medium. However, alternative interpretations of these results can not be excluded. Additional studies are needed, therefore, to determine whether integrins undergo transcytosis in MDCK cells.

Immunolocalization of phage particles in MDCKI cells. Confocal fluorescence microscopy revealed CRY-phage particles bound to the basal cell surface of MDCKI cells (Figs. 6J and 6K). CRY-phage were also localized within cells (Figs. 6D and 6G) mainly in the apical region of the cell (Figs. 6A and 6N). Immunostaining of extracellular phage prior to cell permeabilization showed that some phage were released out of cells and stayed bound to the apical plasma membrane (Fig. 6B). pC89-phage demonstrated neither binding, nor internalization by cells (Figs. 6C, 6F, 6I, and 6L). Taken together, these data provide direct evidence of CRY-phage transcytosis in MDCKI cells.

DISCUSSION

We report here a novel selection strategy for the identification of peptides that enhance transcytosis of phage vectors through epithelial cells. A potential use for such selection strategy is in the development of peptide "carriers" capable of binding transcytosing receptors and mediating delivery of macromolecules, including viral vectors, across epithelial and endothelial barriers. This approach complements recent studies aimed at using phage-peptide libraries for targeting cell-specific receptors that undergo endocytosis (reviewed in 30). The advantage of using peptides as targeting ligands includes the ease of their synthesis and incorporation by chemical conjugation or cloning into a variety of delivery vehicles, such as drug conjugates, liposomes, and viral or artificial gene delivery vectors. In addition, small peptides are less likely to induce immune response compared to protein ligands. However, the structural simplicity of peptides may impose limitations on their use as targeting ligands. It is not yet clear whether peptide binders can be efficiently selected for any target receptor, or preferentially for those receptors whose normal function involves recognition of short amino acid sequences. Thus, in the model system of MDCKII cells transfected with pIgR, we were able to isolate peptide ligands to integrins, but not peptides that bind pIgR. This result is surprising considering the high transcytosing capacity of pIgR and the effective release of transcytosed ligands on the apical surface of these cells (11). It is possible that regardless of the high transcytotic capacity of pIgR, this receptor is not efficiently recognized by short peptides due to their small size and the lack of distinct conformation. Conversely, integrins may be efficiently targeted by peptide-presenting phage because of their remarkable affinity for short amino acid motifs (25).

In addition to peptide selection, the strategy described here is likely to be applicable for screening phage libraries of antibodies (31) and variants of natural protein ligands (32). Antibody libraries may be especially helpful for selecting ligands to those receptors that evade recognition by peptides, or for optimization of established transcytosing antibodies.

In conclusion, we demonstrated the ability of bacteriophage to undergo receptor-mediated transcytosis in epithelial cells. This finding offers a new way to discover targeting ligands to transcytosing receptors by functional screening of phage combinatorial libraries. This approach may be helpful for generating novel carriers capable of delivering macromolecules via transcytotic pathways.

ACKNOWLEDGMENTS

We thank Drs. F. Felici and A. Luzzago (Istituto di Ricerche di Biologia Molecolare, Rome, Italy) for their gifts of pC89 vector and pVIII-9aa.Cys library. This work was supported by the Program of Excellence in Molecular Biology of the Heart and Lung (POEMB) NIH-HL41496 and through the generous support of the Caroline Halfter Spahn Trust for cystic fibrosis research.

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