Brief Definitive Report

Targeting Mucosal Sites by Polymeric Immunoglobulin Receptor-directed Peptides

Kendra D. White and J. Donald Capra

Molecular Immunogenetics Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104

Abstract

Polymeric immunoglobulins provide first line humoral defense at mucosal surfaces to which they are specifically transported by the polymeric immunoglobulin receptor (pIgR) on mucosal and glandular epithelial cells. Previous studies from our laboratory suggested that amino acids 402–410 of the Cα3 domain of dimeric IgA (dIgA) represented a potential binding site for the pIgR. Here by binding human secretory component to overlapping decapeptides of Cα3, we confirm these residues and also uncover an additional site. Furthermore, we show that the Cα3 motif appears to be sufficient to direct transport of green fluorescent protein through the pIgR-specific cellular transcytosis system. An alternative approach identified phage peptides, selected from a library by the in vitro Madin Darby Canine Kidney transcytosis assay, for pIgR-mediated transport through epithelial cells. Some transcytosis-selected peptides map to the same 402–410 pIgR-binding Cα3 site. Further in vivo studies document that at least one of these peptides is transported in a rat model measuring hepatic bile transport. In addition to identifying small peptides that are both bound and transported by the pIgR, this study provides evidence that the pIgR-mediated mucosal secretion system may represent a means of targeting small molecule therapeutics and genes to mucosal epithelial cells.

Key words: mucosal immunity • IgA • transcytosis • secretory IgA • phage display library

Introduction

The humoral mucosal immune system is well adapted to deliver protective antibody to the mucosal surface where it prevents entry of a variety of pathogens to the body (1). A unique pathway exists within these tissues to specifically transport and secrete polymeric immunoglobulins, including polymeric IgA, at mucosal sites where they can act against microbial infection (2). Dimeric IgA (dIgA) binds specifically to the polymeric Ig receptor (pIgR), which is present on the basolateral surface of mucosal epithelial cells. The dIgA is subsequently endocytosed and transported across the cell to be secreted at the opposing apical cell surface as secretory IgA (sIgA). Through this process, sIgA retains most of the extracellular region of the pIgR, termed secretory component (SC; references 3–5). The pIgR-mediated transcytosis pathway for dIgA therefore provides an ideal mechanism to exploit for the specific delivery of drugs, antibodies, viruses, and genes to mucosal or glandular epithelial cells. The goal of the current study was to identify small peptide motifs that would both bind to the pIgR and be specifically transcytosed by the receptor and thus offer the possibility of targeting mucosal sites.

Address correspondence to Dr. J. Donald Capra, Oklahoma Medical Research Foundation, 825 N.E. 13th St., Oklahoma City, OK 73104. Phone: 405-271-7210; Fax: 405-271-8237; E-mail: Jdonald-Capra@omrf.ouhsc.edu

Materials and Methods

Solid-Phase Decapeptide Assay. The amino acid sequence of human IgA1 was used to construct decapeptides overlapping by eight amino acids from the Cα3 domain of human IgA as described previously (6). The overlapping decapeptides were simultaneously synthesized at the rounded ends of radiation derivatized polyethylene pins, arranged to fit into the wells of 96-well microtiter plates (Chiron Mimotopes). Positive control pins were synthesized from a known reactive sequence of the Sm B' protein (6). The identification of Cα3 reactive decapeptides was performed using a modified ELISA-based assay. Briefly, pins were blocked with 3% low-fat milk in PBS, pH 7.4 for 1 h at room temperature. After each step of the assay, pins were washed four times for 8 min in PBS with 0.05% Tween. Pins were first incubated with 100 μl/well of purified SC. Next, the pins were incubated with 100 μl/well of 1:500 sheep anti–rabbit SC (provided by Dr. Keith Mostov, University of California at San Francisco, San Francisco, CA) overnight at 4°C, followed by incubation with 100 μl/well 1:1,000 donkey anti–sheep IgG conjugated to alkaline phosphatase (Sigma-Aldrich) for 2–3 h at room temperature. The negative control assay to rule out reagent reactivity included all steps except incubation with SC. The positive control pins were tested with a known concentration of a standard control patient sera (provided by Dr. Judith James, Oklahoma Medical Research Foundation, Oklahoma City, OK). Paranitrophenyl phosphate was used as substrate and microtiter plates were read at 405 nm with a SpectraMax Plus Reader (Molecular Devices) until the positive control wells reached an OD of ~2.0. Results for each

plate were normalized by comparison with the positive control pins. After completion of each assay, the pins were regenerated as described previously (6).

Baculovirus Expression of Human Secretory Component. The hexahistidine-tagged extracellular region of human pIgR (reference 7; provided by Dr. Jean-Pierre Kraehenbuhl, University of Lausanne, Lausanne, Switzerland) was cloned into the pH-360EX baculovirus transfer vector using standard molecular biology techniques (8). Transfer vector containing pIgR was cotransfected with Bsu36I-digested BacPAK6 viral DNA using Bacfectin (CLONTECH Laboratories, Inc.) into *Spodoptera frugiperda* cells (Sf9) to produce recombinant virus. Growth, plaque purification, and amplification of recombinant virus were standard. For protein production, High Five cells (Invitrogen) infected at an MOI of 5 were maintained in serum free medium in shaking flasks at 2×10^6 cells/ml. Human SC was then purified from cell supernatants on Ni-NTA agarose columns (QIAGEN) and dialyzed to PBS. Quantification was performed using Ni-NTA HisSorb plates (QIAGEN).

Cell Culture. Type II Madin Darby Canine Kidney (MDCK) cells and Type II MDCK cells stably transfected with rabbit pIgR (provided by Dr. Keith Mostov, University of California at San Francisco) were maintained in DMEM containing 5–10% fetal bovine serum (HyClone Laboratories) and antibiotics in 5% CO_2 at 37°C (9). For transcytosis assays, transfected or nontransfected MDCK cells were grown to confluence in 6-well plates on 0.45 or 1.0 μm pore size Falcon cell culture inserts (BD Biosciences; reference 9). The cells were fed every other day and used for the various transcytosis assays 4 to 5 d after plating.

Construction of Green Fluorescent Protein Fusion Peptides. Monomeric (TWASRQEPSQGTTTFAVTSGP $(G)_6$PG-GFP) and dimeric (TWASRQEPSQGTTTFAVTSGP$(G)_6$PGPGTWASRQEPSQGTTTFAVTSGP$(G)_6$PG-GFP) IgA pIgR-binding peptides and the corresponding monomeric IgG peptide (TTPPVLDSDGPFFLYSGP$(G)_6$PG-GFP) were cloned into the high level bacterial green fluorescent protein (GFP) expression vector pQBI T7-GFP (Quantum Biotechnologies) with a small linker sequence, resulting in NH_2-terminal peptide fusions. Bacterial expression was induced with IPTG and crude protein extracts generated by standard freeze-thaw methods. The peptide fusion proteins were analyzed by SDS-PAGE and by Western blot with detection using GFP monoclonal antibody (Quantum Biotechnologies). The amounts of GFP fusion proteins were normalized by measuring Absorbance at 590 nm on an fmax fluorescence plate reader (Molecular Devices).

pIgR-mediated Transport of GFP Fusion Peptides. GFP fusion proteins were added to the basolateral chamber of polarized MDCK cells or pIgR-transfected MDCK cells grown on 0.45 μm cell culture inserts. After 4 h of incubation at 37°C, the apical media was collected and GFP fluorescence determined.

Selection of Random Phage Peptide by Transcytosis. The random 40-mer peptide library RAPID 40 (DGI Biotechnologies) with a total diversity of 1.55×10^{10} has been described previously (10). A similar 20-mer random peptide library was also used (provided by Dr. Stephen Johnston, The University of Texas Southwestern Medical Center, Dallas, TX; reference 11). For phage selection, 100 library equivalents were added to the basolateral chamber of polarized pIgR-transfected MDCK cells grown on 1.0 μm cell culture inserts. After 4 h of incubation at 37°C, the apical media was harvested. Phage rescue and amplification were performed in *Escherichia coli* strain TG1 according to standard procedures (12). After eight rounds of selection, random phage clones were selected and propagated for sequencing.

DNA Sequencing and Analysis. DNA sequencing was performed on double-stranded plasmid or phagemid DNA using an ABI 377 Prism automated sequencer. Alignments of deduced peptide sequences with immunoglobulin constant regions were performed using the program LALIGN (13).

pIgR-Mediated Transport of Selected Phage Peptides. 5×10^{10} phage particles were added to the basolateral chamber of polarized MDCK cells or pIgR-transfected MDCK cells grown on 1.0-μm cell culture inserts. After 4 h of incubation at 37°C, the apical media was collected and the titers of transported phage were determined.

Rat Transport Experiments. Male Wistar rats were anesthetized with ketamine hydrochloride, an I.V. saline line established, and the bile duct cannulated with the outlet of the cannula situated below the rat. Bile collection began after I.V. injection of 10^6 test phage and 10^6 negative control phage particles (in 0.2 ml PBS) and was continued for 3.5 h, collecting bile at 30 min time points in separate tubes. After bile collection was complete, the animals were killed. Phage titers were determined and 20 random phage clones were sequenced from each time point. Experiments were performed in accordance with institutional guidelines for animal care and use.

Results and Discussion

We previously reported the localization of an important pIgR-binding site on dimeric human IgA to a predicted loop structure in the Cα3 domain (10). To further localize this interaction, the binding of pIgR to overlapping decapeptides comprising Cα3 was evaluated. When the Cα3 decapeptides bound to pins were screened for reactivity with the polyclonal anti-SC serum and anti–sheep IgG conjugate, no significant background was demonstrated (Fig. 1 a). However, screening the peptides with SC identified two pIgR-reactive regions within the sequence of Cα3, peptide numbers 35–36 and 48–50 (Fig. 1 b). Decapeptide numbers 35–36 correspond to amino acids 404–415 of Cα3 and decapeptides 48–50 correspond to amino acids 430–443. Previous phage display selection of random peptides with cell-expressed pIgR coupled with subsequent alanine substitution provided evidence for amino acids 402–410 of Cα3 in dIgA being involved in the interaction with pIgR (10). The current results for decapeptides 35–36 correspond to the same region in Cα3 and thus provide further evidence for the importance of this specific interaction. When the region of Cα3 identified by decapeptides 48–50 (Cα3 amino acids 430–443) are compared with the corresponding region of Cγ3 (amino acids 424–437), part of the region would be predicted to form a loop structure and thus be accessible for ligand binding based on the crystal structure of IgG Fc (14). Therefore, amino acids 430–443 of Cα3 may represent another part of dIgA that cooperates with the 402–410 amino acid region for the initial high affinity interaction of dIgA with pIgR.

To identify a motif on IgA dimer molecules or unique peptide motifs that lead to secretion onto mucosal surfaces via the pIgR-mediated transcytosis pathway, experiments were designed to localize the smallest possible peptide(s) required for the pIgR–dIgA interaction. The native region from IgA (amino acids 402–410) that we have shown pre-

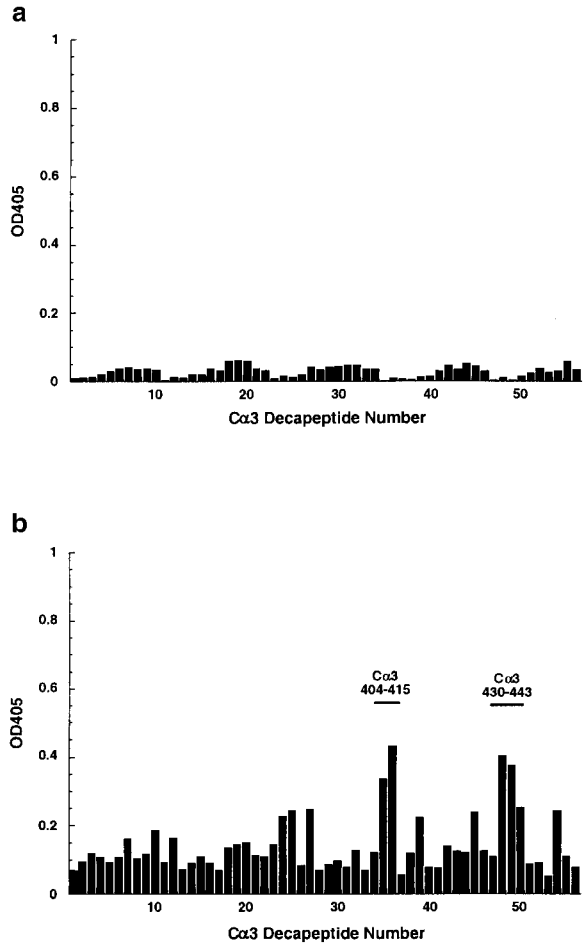

Figure 1. Reactivity of human pIgR with Cα3 decapeptides overlapping by eight amino acids. (a) Background reactivity of the decapeptides with pIgR detection reagents sheep anti-pIgR and donkey anti–sheep IgG alone. (b) Interaction of the decapeptides with human SC detected with sheep anti-pIgR and donkey anti–sheep IgG alkaline phosphatase. Results are means from three assays.

viously to be involved in pIgR-binding was synthesized in both monomeric (mApep) and dimeric (dApep) form with a small linker as GFP fusion proteins. As a negative control, the corresponding region from Cγ3 (amino acids 398–403) was also synthesized as a fusion protein in monomeric form (mGpep). The ability of mApep, dApep, and mGpep to be specifically transported by the pIgR was assessed in the MDCK transcytosis assay by using both pIgR-transfected and nontransfected cells (Fig. 2). The data indicate that a peptide derived from the native region from the CH3 region of IgA in dimeric form, and to a lesser extent in monomeric form, can indeed direct transport of a fusion protein through pIgR-transfected MDCK cells but not through nontransfected MDCK cells. mGpep, the negative control peptide derived from the sequence of IgG, was not transported by the pIgR.

It is conceivable that other structures, including smaller peptides, could substitute for polymeric immunoglobulins in the pIgR-mediated transcytosis process. To identify such molecules, the MDCK transcytosis system was used to se-

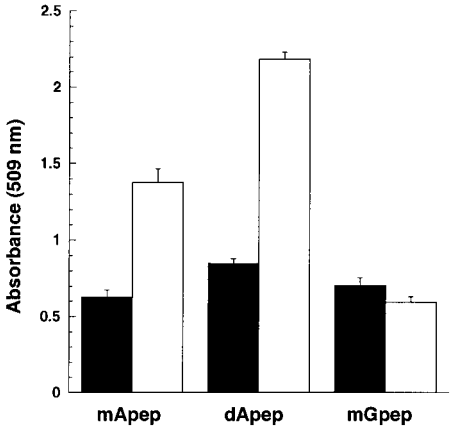

Figure 2. Transcytosis of GFP fusion proteins through nontransfected and pIgR-transfected MDCK cells. The native region from dIgA (amino acids 402–410) that was previously identified to be involved in pIgR-binding has been synthesized in both monomeric (mApep) and dimeric (dApep) form as GFP fusion proteins. The corresponding region from IgG that does not bind to the pIgR was also synthesized in monomeric form as a negative control (mGpep). The ability of the GFP fusion proteins to be specifically transported by the pIgR in the MDCK transcytosis system was assessed by measuring fluorescence from the apical medium after the MDCK transcytosis assay was performed using both nontransfected (shown as solid fill) and pIgR-transfected (shown as no fill) cells. Results represent means of three assays.

lect random phage-displayed peptides transcytosed in vitro. Three independent MDCK-pIgR transcytosis system phage display selection experiments were performed. Two experiments used a 40-mer random peptide library (experiments no. 1 and no. 2) and one experiment used a 20-mer random peptide library (experiment no. 3). Both phage libraries were constructed as pIII peptide fusions as this results in low number of peptides per phage being expressed and thus should allow selection of high affinity interactions. After eight rounds of selection, 20 clones from experiment no. 1 and 18 clones from experiments no. 2 and no. 3 were chosen at random and sequenced. Shown in Fig. 3 are the eight phage peptides selected from the three experiments and the frequency of selection of each phage clone.

EXP1
SAMFVPFDIAVGVRDGQQGLGGSRRKGARLREAISSYAE 9/20
IPSVTRMTVGGTLRKEFQDVVLGVIFGLVLVINRCSFL 3/20
LVLRGNQVFAFCRSDNNRQQAPAGCCYVGFSLFVTRGGYE 1/20

EXP 2
WQAYPVQYLFVVATGYGGKVINHLRGKVRRESADQVPGYF 4/18
MFVCVDAKQCLLGAAGGLRLIFA 3/18
VDDLTLQSRSPPSQLNSQHLLLSQLCGYWMFRVRSRSCCG 1/18
RSRMFVLGVLEVDSGLLNCLCWVGVSVDGRKSSCRWTAY 1/18

EXP 3
QRNPRLRLIRRHPTLRIPPI 11/18

Figure 3. Phage peptides selected by transcytosis and frequency of selection. The in vitro MDCK-pIgR transcytosis system was used to identify phage peptides that were transported from the basolateral medium to the apical medium. Two experiments used a 40-mer random peptide library (experiments no. 1 and no. 2) and one experiment used a 20-mer random peptide library (experiment no. 3). Phage peptides are referred to by the first three amino acids of their sequence.

```
       400        410
IGA  LTWASRQEPSQGTTTFAVTSIL
     ::  ::. ::: ..   . : :
VDD  LTLQSRSPPSQLNSQHLLLSQL
            10         20

         380        390
IGA  FSPKDVLVRWLQGSQEL
     : : :. :   .:.: :
SAM  FVPFDIAVGVRDGQQGL
          10         20

    410        420        430
IGA  TTFAVTSILRVAAEDWKKGDTF
     : ..: . ::   .:   :  :
IPS  TRMTVGGTLRKEFQDVVLGVIF
            10         20
```

Figure 4. Three phage peptides selected by transcytosis map to Cα3 of human IgA. The transcytosis-selected phage peptides were aligned to the sequence of IgA using the computer program LALIGN. The symbol : = amino acid identity and the symbol . = amino acid similarity.

When the eight selected phage peptides were aligned to the sequence of IgA, three peptides (VDD, SAM, and IPS) show significant identity in and around the 402–410 region which we have previously shown to be important for pIgR binding (Fig. 4). The SAM peptide shares 41.2% identity in a 17 amino acid overlap with IgA, the VDD peptide has 40.9% identity in a 22 amino acid overlap, and the IPS peptide has 31.8% identity with IgA in a 22 amino acid overlap. In addition, several of the peptides show high homology to sequences within J chain (unpublished data), are currently under further investigation, and will subsequently be submitted for publication. The use of the RAPID 40 phage library has been recently reported for the identification of protein partners (15). As a negative control, the peptide sequences were aligned with irrelevant proteins such as human serum albumin and do not show significant homology (unpublished data). Identity values for the peptides compared with albumin do not exceed 20.0% in an average of 18 amino acid overlap. When the peptide sequences were compared with each other using peptide alignment computer programs, no common sequence motifs were found.

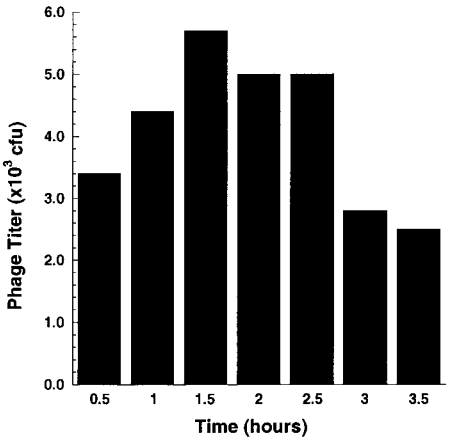

Figure 6. Kinetics of IPS phage peptide secretion into rat bile. The bile ducts of male Wistar rats were cannulated. Bile collection began after I.V. injection of 10^6 specific phage (IPS) and 10^6 negative control phage particles and was continued for 30 min time points in separate tubes. Phage titers from each time point were determined by standard procedures.

Eight phage peptides were selected for their positive transport through epithelial cells expressing pIgR. However, the definitive mechanism of transport was not determined. Therefore, the movement of the phage peptides through both pIgR-transfected and nontransfected MDCK cells was measured. The results indicate that the RSR, SAM, IPS, VDD, QRN, and MFV peptides displayed on the surface of the phage allow the phage to be selectively transported by the pIgR through MDCK cells (Fig. 5, a–c). In contrast, the WQA and LVL phage peptides likely direct apical transport of phage by an alternate transcytosis path-

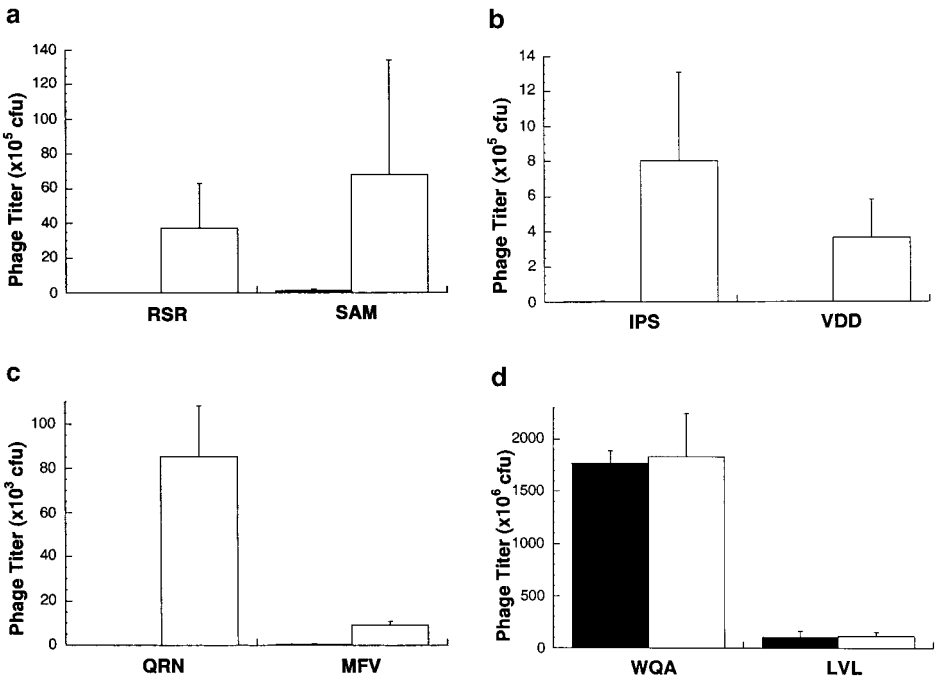

Figure 5. Basolateral to apical transport of phage peptides measured in the MDCK transcytosis system. Phage were added to the basolateral medium of wells containing 1.0 μm pore inserts confluent with either polarized nontransfected (negative control shown as solid fill) or pIgR-transfected (shown as no fill) MDCK cells. Transcytosis was allowed to occur for 4 h. The apical supernatant fluids were collected and phage titers determined. (a) Transport of RSR and SAM peptides; (b) transport of IPS and VDD peptides; (c) transport of QRN and MFV peptides; and (d) transport of WQA and LVL peptides. Results represent means from three independent experiments.

way as there is no difference in transport by MDCK cells whether they are transfected with pIgR or not (Fig. 5 d).

To determine if the phage-displayed peptides are transported in vivo, a mixture of pIgR transcytosis-selected IPS phage and nonspecific phage were injected intravenously into rats. Bile was collected at 30 min time intervals to measure hepatic transport. The kinetics of IPS phage transport were similar to that reported previously for dIgA (16; Fig. 6). 100% of the phage recovered from all time points displayed the IPS peptide (unpublished data). No nonspecific phage were recovered at any time point (unpublished data). Therefore, at least one of the phage peptides identified by in vitro transcytosis is transported specifically by the pIgR in vivo in a rat model for pIgR transport.

This study aimed to identify structural determinants, either derived from the sequence of dIgA Fc or dIgA-independent, required for interaction with the highly specific pIgR-mediated transcytosis pathway for the secretion of polymeric immunoglobulins onto mucosal surfaces. With this information, it may be possible to exploit the IgA-specific pIgR mucosal secretion system as a means of targeting therapeutic recombinant molecules and/or genes to mucosal epithelial cells or their surfaces. We previously identified a nine amino acid structural motif in the Cα3 domain of IgA that is important for binding of dIgA to the pIgR. This motif, when expressed as a monomer, but especially when expressed as a dimer appears to be sufficient to direct transport of the 23-kD GFP fusion protein through the pIgR-specific cellular transcytosis system. In addition, we have selected a number of phage peptides that are transcytosed by pIgR using an in vitro transcytosis assay with pIgR-transfected MDCK cells and phage display techniques. Three of the peptides show considerable homology with the native pIgR-binding region of dIgA, four have significant homology with human J chain, and some bear no resemblance to the IgA constant region sequence. Interestingly, six of the eight selected peptides appear to be specifically transported by the pIgR while two appear to be transported through MDCK cells via an alternate pathway. In vivo studies indicate that at least one of the peptides, IPS, is transported by the pIgR in the rat. Therefore, the results of this study validate the feasibility of using a small pIgR-binding motif to deliver therapeutics to mucosal sites. Furthermore, tissue-specific mucosal epithelial cells may display unique receptor molecules, which can be identified by random peptide selection upon live cells. Linking structures specific for the pIgR with additional tissue antigens may yield molecules of high avidity capable of the selective or site-specific delivery of a variety of therapeutic molecules.

The authors would like to thank Dr. Judith James (Oklahoma Medical Research Foundation) for the synthesis and production of the Cα3 solid phase decapeptides and technical guidance with the assays. We also wish to acknowledge Sherry Hart and Andy Duty for technical assistance, Danny Moore for help with the rat transport experiments, and Sheryl Christofferson for DNA sequencing.

This work is supported by a grant from the National Institutes of Health (AI 44206) and Epicyte Pharmaceuticals, Inc.

Submitted: 12 April 2002
Revised: 21 June 2002
Accepted: 16 July 2002

References

1. Underdown, B.J., and J.M. Schiff. 1986. Immunoglobulin A: strategic defense initiative at the mucosal surface. *Annu. Rev. Immunol.* 4:389–417.
2. Mostov, K.E., and G. Blobel. 1982. A transmembrane precursor of secretory component. The receptor for transcellular transport of polymeric immunoglobulins. *J. Biol. Chem.* 257: 11816–11821.
3. Lindh, E., and I. Bjork. 1974. Binding of secretory component to dimers of immunoglobulin A *in vitro*. *Eur. J. Biochem.* 45:261–268.
4. Fallgreen-Gebauer, E., W. Gebauer, A. Bastian, H.D. Kratzin, H. Eiffert, B. Zimmermann, M. Karas, and N. Hilschman. 1993. The covalent linkage of secretory component to IgA. *Biol. Chem. Hoppe Seyler.* 374:1023–1028.
5. Underdown, B.J., J. De Rose, and A. Plaut. 1977. Disulfide bonding of secretory component to a single monomer subunit in human secretory IgA. *J. Immunol.* 118:1816–1821.
6. James, J.A., and J.B. Harley. 1992. Linear epitope mapping of an Sm B/B' polypeptide. *J. Immunol.* 148:2074–2079.
7. Rindisbacher, L., S. Cottet, R. Witteek, and J.-P. Kraehenbuhl. 1995. Production of human secretory component with dimeric IgA binding capacity using viral expression systems. *J. Biol. Chem.* 270:14220–14228.
8. Hasemann, C., and J.D. Capra. 1990. High-level production of a functional immunoglobulin heterodimer in a baculovirus expression system. *Proc. Natl. Acad. Sci. USA.* 87: 3942–3946.
9. Mostov, K.E., and D.L. Deitcher. 1986. Polymeric immunoglobulin receptor expressed in MDCK cells transcytoses IgA. *Cell.* 46:613–621.
10. Hexham, J.M., K.D. White, L.N. Carayannopoulos, W. Mandecki, R. Brisette, Y.S. Yang, and J.D. Capra. 1999. A human immunoglobulin (Ig) Cα3 domain motif directs polymeric Ig receptor-mediated secretion. *J. Exp. Med.* 189:747–751.
11. Barry, M.A., W.J. Dower, and S.A. Johnston. 1996. Toward cell-targeting gene therapy vectors: selection of cell-binding peptides from random peptide-presenting phage libraries. *Nat. Med.* 2:299–305.
12. Hexham, J.M. 1998. Production of human Fab antibody fragments from phage display libraries. *Methods Mol. Biol.* 80: 461–474.
13. Huang, X., and W. Miller. 1991. A time-efficient, linear-space local similarity algorithm. *Adv. Appl. Math.* 12:337–357.
14. Deisenhofer, J. 1981. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9 and 2.8 Angstroms resolution. *Biochemistry.* 20:2361–2370.
15. Pillutla, R.C., K. Hsiao, R. Brissette, P.S. Eder, T. Giordano, P.W. Fletcher, M. Lennick, A.J. Blume, and N.I. Goldstein. 2001. A surrogate-based approach for post-genomic partner identification. *BMC Biotechnol.* 1:6.
16. Vaerman, J.P., A. Langendries, D. Giffroy, P. Brandtzaeg, and K. Kobayashi. 1998. Lack of SC/pIgR-mediated epithelial transport of a human polymeric IgA devoid of J chain: *in vitro* and *in vivo* studies. *Immunology.* 95:90–96.