

Identification of a Polymeric Ig Receptor Binding Phage-displayed Peptide That Exploits Epithelial Transcytosis without Dimeric IgA Competition*

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The polymeric Ig receptor (pIgR), also called membrane secretory component (SC), mediates epithelial transcytosis of polymeric immunoglobulins (pIgs). J Chain-containing polymeric IgA (pIgA) and pentameric IgM bind pIgR at the basolateral epithelial surface. After transcytosis, the extracellular portion of the pIgR is cleaved at the apical side, either complexed with pIgs as bound SC or unoccupied as free SC. This transport pathway may be exploited to target bioactive molecules to the mucosal surface. To identify small peptide motifs with specific affinity to human pIgR, we used purified free SC and selection from randomized, cysteine-flanked 6- and 9-mer phage-display libraries. One of the selected phages, called C9A, displaying the peptide CVVWMGFQQVC, showed binding both to human free SC and SC complexed with pIgs. However, the pneumococcal surface protein SpsA (*Streptococcus pneumoniae* secretory IgA-binding protein), which binds human SC at a site distinct from the pIg binding site, competed with the C9A phage for binding to SC. The C9A phage showed greatly increased transport through polarized Madin-Darby canine kidney cells transfected with human pIgR. This transport was not affected by pIgA nor did it inhibit pIgR-mediated pIgA transcytosis. A free peptide of identical amino acid sequence as that displayed by the C9A phage inhibited phage interaction with SC. This implied that the C9A peptide sequence may be exploited for pIgR-mediated epithelial transport without interfering with secretory immunity.

Mucosal epithelia form a barrier between the interior of the body and the external environment. At least 80% of all antibody-producing plasma cells of the body are located in the gastrointestinal and respiratory mucosae, and most of them are committed to production of dimers and larger polymers of IgA, collectively called pIgA⁵ (1, 2). These poly-

mers contain, in addition to α -heavy chains and light Ig chains, a short polypeptide named the J chain that is required for binding to the polymeric immunoglobulin receptor (pIgR) (3, 4). This receptor, also called membrane secretory component (SC), is synthesized by secretory epithelial cells and delivered to the basolateral plasma membrane where it can bind pIgA and pentameric IgM. The receptor-ligand complexes are endocytosed and transcytosed to the apical cell surface by vesicular transport. At the apical surface, proteolytic cleavage of pIgR results in the release of secretory IgA (SIgA), a complex of pIgA and bound SC. Pentameric IgM is exported in the same manner, forming secretory IgM. Transcytosis and cleavage of unoccupied pIgR give rise to free SC in the secretions (2, 5).

The pIgR is a type I transmembrane glycoprotein, consisting of an ~560-residue extracellular region, a 23-residue transmembrane region, and a 103-residue cytoplasmic tail. The extracellular region of pIgR contains five domains (D1–D5) that share sequence similarity with Ig variable regions (6). Recently, the crystal structure of the first domain (D1) of human pIgR was resolved (7).

SIgA performs several types of non-inflammatory defense functions. In addition, SC protects SIgA by delaying cleavage of the hinge/Fc region of the α -chain (8) and anchors SIgA to the mucus lining of the epithelial surface (9). Some pathogens, however, have developed strategies to exploit pIgR in order to invade the epithelium. IgA antibodies specific for herpes simplex virus and Epstein-Barr virus may facilitate internalization of the virus into pIgR-expressing epithelial cells (10–12). *Streptococcus pneumoniae* may use direct interaction with pIgR as an invasive mechanism (13–15). The binding to pIgR is mediated through *S. pneumoniae* SIgA-binding protein (SpsA), also known as choline-binding protein A (CbpA) or pneumococcal surface protein C (PspC). SpsA is able to bind human pIgR, free SC, or SIgA (bound SC) via a hexapeptide motif (YRNYPT) in its α -helical domain, which is conserved among many pneumococcal strains. This interaction is specific for human SC/pIgR and may mediate translocation of the bacteria across the epithelial barrier (15). We and others have recently shown that SpsA binds specifically to human pIgR via motifs in D3 and D4 (16, 17).

Ferkol *et al.* have exploited pIgR for the delivery of therapeutics to mucosal surfaces or epithelial cells (18–22). A fusion protein composed of a pIgR-specific antibody and human α_1 -antitrypsin showed basolateral-to-apical transcytosis similar to the transport of pIgA (19–21). Furthermore, a DNA-polylysine complex covalently coupled to pIgR-specific antibodies mediated functional transgene expression in epithelial cells when the protein DNA complex was administered intravenously (18, 22).

In this study, we used phage-display selection to identify short pep-

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⁵ The abbreviations used are: pIgA, polymeric IgA; pIgR, polymeric Ig receptor; SC, secretory component; SIg, secretory Ig; SpsA, *S. pneumoniae* SIgA-binding protein; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; NIP, 5-iodo-4-hydroxy-3-nitrophenylacetyl; TU, transducing unit.

A Phage-displayed Peptide Transported by Human pIgR

tides that could bind human SC/pIgR. Such pIgR binding peptides may be ideal to carry bioactive molecules to the lumen by pIgR-mediated transcytosis. We identified several phages from two different phage-display libraries that bound human SC. One of these phages had the capacity to be transported by pIgR *in vitro* through a polarized epithelial monolayer. This transport was not affected by the presence of pIgA, and the pIgA transport was not hampered by the phage. A free peptide with identical amino acid sequence to that displayed by the phage was able to inhibit binding of the phage to SC, suggesting that the peptide alone was sufficient for binding. The binding of the phage to SC was inhibited by SpsA, implying that the phage binds elements in D3 and/or D4 of human pIgR.

EXPERIMENTAL PROCEDURES

Reagents—Native free SC was purified from human colostrum as described (23). Recombinant human pIgA and pentameric IgM were produced and purified as previously described (24, 25). Purified native human IgG was obtained from Kabi Pharmacia AB (Uppsala, Sweden). Recombinant expression of human and murine SC in 293E cells (CRL-10852; ATCC, LGC Promochem) has been described previously (16, 26). Madin-Darby canine kidney (MDCK) cells, untransfected or stably transfected with human or mouse pIgR, were produced and maintained as reported elsewhere (27, 28). Two phage-display libraries that present peptides of 6 or 9 random amino acids constrained by flanking cysteines, the Cys6 and Cys9 libraries, inserted in the gene 3 protein of the vector fUSE5 system have been detailed previously (29). The complexity of the libraries is $\sim 2.2 \times 10^7$ and 5.5×10^7 for the Cys6 and Cys9 libraries, respectively. One random phage clone from each library, C6K (CAGAWSRC) and C9K (CVLGLHWSLAC), together with a 7-mer, 7K (LHVSQRG) were used as negative controls. The free C9A peptide, KCVVWVGFEQVCK, and an amino acid scrambled version, KCVQFQVWGMVCK, were synthesized on a robotic system (Syro MultiSynTech, Bochum, Germany) with Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)/*O*-*tert*-butyl chemistry and 2-chlorotrityl resin (Senn Chemicals AG, Dielsdorf, Switzerland). The identity of the peptides was confirmed by electrospray mass spectrometry, and purity was analyzed by reverse-phase high performance liquid chromatography. Two lysines (K) flanking the cysteines were included to increase the solubility of the otherwise highly hydrophobic peptide. The lyophilized peptides were stored at -20°C and dissolved before use in distilled H_2O . The concentrations were determined by absorbance at 280 nm (A_{280} of 1 mg/ml solution, 3.66) for the free peptides. Recombinant fragments of SpsA designated SpsA SH2 (amino acids 37–283), SpsA SH3 (amino acids 37–158), and SpsA SM1 (amino acids 159–324) were produced as described (16).

Affinity Selection, Amplification, and Phage Titration—For affinity selection, MaxiSorp tubes (Nunc) were coated overnight at 4°C with 500 μl of native human free SC at 30 $\mu\text{g}/\text{ml}$ in isotonic phosphate-buffered saline (PBS), pH 7.4. The tubes were blocked with 1% (w/v) bovine serum albumin (BSA) or 1% (w/v) powdered skimmed milk in PBS for 1 h at room temperature and washed three times with PBS/0.05% Tween 20 (PBS/T). Approximately 10^{10} *Escherichia coli* K91K transducing units (TUs) from each library were preincubated for 30 min mixed 1:1 in PBS with 1% (w/v) BSA or skimmed milk, added to the tubes, and incubated for 1.5 h at room temperature. The tubes were then washed six times with PBS/T. Bound phage particles were eluted with 500 μl of 0.1 M HCl-glycine (pH 2.2) for 10 min and neutralized with 75 μl of 1.0 M Tris, pH 9.1. The recovered phages were amplified by infection of *E. coli* K91K, and phage supernatants of pools of amplified phages or individual isolates were prepared as described (30). Two more

rounds of selection were performed with $\sim 10^9$ and 10^8 TUs of amplified phage as input. Binding assays (elution-titration assays) were performed essentially as the affinity selection in wells of microtiter plates coated overnight with 200 μl of 20 $\mu\text{g}/\text{ml}$ of SC at 4°C . PCR products of individual phage clones were produced and sequenced as described (29).

ELISA—For SC binding ELISA, microtiter plates (Nunc) were coated with 30 $\mu\text{g}/\text{ml}$ of native free SC (or 5 $\mu\text{g}/\text{ml}$ for the analysis of the C9A variants) in PBS at 4°C overnight and blocked and washed as in the selection. 50 μl of the phage eluates after each round of panning or 50 μl (or 100 μl for the analysis of the C9A variants) of supernatant from individual clones (containing $\sim 1 \times 10^{10}$ TU/ml) were preincubated with an equal volume blocking solution for 30 min, added to the wells, and incubated for 1.5 h at room temperature. Bound phages were detected directly by incubation with a horseradish peroxidase anti-M13 IgG conjugate (Amersham Biosciences) diluted 1/4000 in PBS with 1% (w/v) BSA or skimmed milk for 1 h at room temperature and revealed by ABTS substrate solution (ABTS tablets from Roche Applied Science in citrate buffer, pH 4.0). Indirect detection was alternatively performed with anti-M13 mAb 1/1000 (Amersham Biosciences) and alkaline phosphatase-conjugated rabbit-anti-mouse antibody 1/1000 (DAKO, Glostrup, Denmark) incubated for 90 min and developed with *p*-nitrophenyl phosphate (Sigma) in diethanolamine buffer at room temperature. Absorbance was read after 10–60 min in a microtiter plate reader set at 405 nm (Tecan Sunrise Microplate Reader; Tecan Austria Gesellschaft, Salzburg, Austria) with Magellan 3.0 software.

For ELISA with *in vitro* reconstituted SigA, microtiter plates were coated with 100 μl of 5 $\mu\text{g}/\text{ml}$ of recombinant pIgA in PBS overnight at 4°C or with 5-iodo-4-hydroxy-3-nitrophenylacetyl (NIP)-BSA (2.5 $\mu\text{g}/\text{ml}$) in 0.05 M NaHCO_3 (pH 9.6) overnight at room temperature and NIP-specific pIgA in a second step to bind the NIP-BSA. The plates were then blocked with 1% (w/v) BSA for 1 h before recombinant human or mouse free SC was allowed to bind for 90 min. $\sim 10^9$ TU phages/well were added, and bound phage was detected by a horseradish peroxidase-conjugated anti-M13 IgG. Successful binding of human or mouse free SC to recombinant pIgA was determined by incubation with a rabbit antiserum to human SC diluted 1/3000 (DAKO) or to murine SC 1/10000 (gift from Dr. Blaise Corthesy, Laboratoire de Recherche et Développement, du Service d'Immunologie et d'Allergie, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland), followed by horseradish peroxidase-conjugated donkey antiserum to rabbit IgG diluted 1/2000 (Amersham Biosciences). In this ELISA, the TMB Microwell peroxidase substrate system (KPL) was used and the reaction stopped with an equal volume of 2 M H_2SO_4 . Absorbance was measured at 450 nm with a Tecan Sunrise Microplate Reader.

Inhibition of Phage Binding to SC—The C9A phage was co-incubated with a 2-fold dilution series of free peptides (starting concentration at 50 μM) or with recombinant pIgA, pentameric IgM, or SpsA fragments (starting concentration at 100 nM) in PBS/1% (w/v) BSA. The mix was incubated in SC-coated microtiter plates (30 $\mu\text{g}/\text{ml}$) for 1.5 h at room temperature. Bound phages were determined with anti-M13 antibody diluted 1/1000 (Amersham Biosciences) and alkaline phosphatase-conjugated rabbit anti-mouse antibody diluted 1/1000 (DAKO). A 2-fold dilution series of the C9A phage was used to determine the percentage phage binding compared with no competitor.

Amino Acid Substitutions of the C9A Phage—Variants of the C9A phage, each with 1 amino acid substituted with alanine (for the 9 internal amino acids) or serine (for either or both of the cysteines), were constructed with synthetic oligo nucleotides and PCR essentially as described for library construction (29). Single clone supernatants were prepared by infecting *E. coli* K91K cells as detailed elsewhere (30). The

sequence of each variant was confirmed by DNA sequencing, either in-house or at GATC Biotech AB.

In Vitro Transcytosis— $\sim 5.0 \times 10^5$ MDCK cells were seeded on 1-cm², 3.0- μ m collagen-coated PTFE filters (Transwell-COL 3494; Costar). The cells were grown to confluency for 3 days, providing a transepithelial resistance of ~ 150 – $200 \Omega/\text{cm}^2$. The filters were washed in fresh medium and transferred to new wells with 200 μ l of Hepes-buffered medium (20 mM Hepes; Invitrogen) containing 10 μ l phage of $\sim 1 \times 10^{10}$ TU/ml, and 200 μ l of medium was added to the apical chamber. To control for paracellular leakage, 100 nM (15 μ g/ml) native human IgG was included in the basolateral chamber; in some experiments 100 nM (37 μ g/ml) recombinant pIgA was added. The filters were incubated for 8 h at 37 °C after which the apical and the basolateral media were collected. The number of phages on either side was determined as *E. coli* K91K TUs. IgG and IgA were quantified by ELISA as described before (25).

RESULTS

Affinity Selection of Peptides That Bind to SC—To obtain peptides with affinity to human SC/pIgR, we screened two different phage-display libraries for binders to native free SC. We found that a higher percentage of phage input bound to SC for each round of selection. From the Cys6 library the recovery of acid-eluted phages was $3.0 \times 10^{-4}\%$ and $5.0 \times 10^{-1}\%$ of the input in the first and third rounds of selection, respectively. For the Cys9 library the recovery increased from $3.0 \times 10^{-4}\%$ to $2.0 \times 10^{-2}\%$ of the input from the first to the third round of selection. Furthermore, the SC binding ELISA (free SC as coat and

anti-M13 phage for detection) performed with phage eluate revealed increased signals for each round, which supported an enrichment of positive binders during selection (Fig. 1). 20 individual phages recovered from the second round of selection and 20 recovered from the third round (from each library) were picked at random and screened by the SC binding ELISA. Of these 80 phages, 41 bound to SC. The amino acid sequence of the displayed peptide was deduced from 29 of the 80 selected phages by DNA sequencing. The results summarized in Table 1, showed that of 14 SC binding isolates from the Cys6 library only two unique sequences were found. Of 7 SC binding phages sequenced from the Cys9 library, 6 were identical. An independent selection of SC binding phages was performed with the Cys9 library. Screening of 88 individual isolates after the first round of selection and a further 88 after the second round resulted in identification of 81 SC binding phages. The sequences of 31 of these were determined and revealed three different Cys9-derived sequences, the most frequent isolate being identical to the frequently isolated phage from the first screen. The SC binding ELISA revealed that three phages (C6A, C9A, and C9D) gave much stronger signals than the other sequenced phages, suggesting a higher affinity to SC for these phages (Table 1).

We used either skimmed milk or BSA as blocking reagents during the selection. Although skimmed milk may contain bovine SIgA and free SC, the recovery of SC binding phages was not affected by blocking agent (data not shown). Furthermore, all SC binding phages were negative for binding to BSA or skimmed milk when recovered from a library screen that used the opposite blocking agent.

Phage Binding to Human SC Compared with Mouse SC Complexed with pIgA—To further characterize the selected phages, we used ELISA to analyze binding to recombinant human or mouse SC that had been complexed with pIgA, i.e. reconstituted SIgA. Only the C9A phage was able to bind recombinant human SC in this assay (Fig. 2). None of the other phages showed binding to human SC in SIgA, and no binding activity was detected against mouse SC present in reconstituted SIgA. The C9C phage was not tested in this assay due to its low affinity for SC as compared with C9A and C9D. These results suggested that for C9B, C9D, C6A, and C6B the binding site on human SC was either masked in the reconstituted SIgA or these phages bound to epitopes exposed only on free SC when coated directly onto the microtiter wells. Importantly, however, the C9A phage showed binding specific for human SC, both when it was coated in free form directly onto plastic and when present as bound SC in reconstituted SIgA.

Inhibition of the C9A Phage by Other SC Ligands—An abundance of pIgA-producing plasma cells in mucosal tissues ensures a high local concentration of pIgA available for pIgR-mediated epithelial export. Thus, an important aspect of pIgR-mediated targeting of mucosal therapeutics would be whether the phage would compete with pIgA for

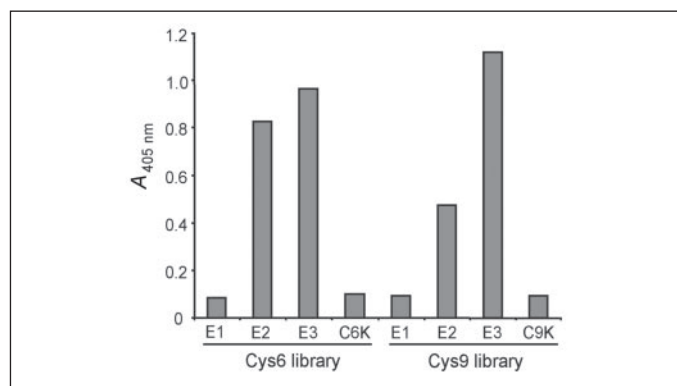


FIGURE 1. Phage eluates from selection against immobilized SC are enriched in SC binding phages. Two different phage-display libraries were selected for binders to purified native human free SC as described under “Experimental Procedures.” The eluates ($\sim 1 \times 10^9$ TUs) from three rounds of successive selection (E1–3) with the Cys6 and Cys9 libraries were measured for SC binding in an ELISA ($A_{405 \text{ nm}}$) and compared with one negative phage from each of the libraries (C6K and C9K).

TABLE 1
Sequences of the selected phage-displayed peptides that bound immobilized human free secretory component

16 and 13 individual clones from the Cys6 and Cys9 selection, respectively, and 35 clones from the second screen of Cys9 selection were picked randomly from the second and third rounds and DNA sequenced. Six different sequences were positive SC binders in phage ELISA. The deduced amino acid sequences of the insert of these six phages and their frequency of isolation (remaining clones tested negative in the phage ELISA) are shown. The $A_{405 \text{ nm}}$ (mean \pm 1 S.D.) in phage ELISA ($\sim 7 \times 10^8$ TU/well) for the first screen and $A_{405 \text{ nm}}$ (mean \pm 1 S.E.) of several phage clones of the second screen of Cys9 selection are shown, except 7K that represents mean of duplicates. The amino acids of the sequences are given in the one-letter code, and cysteines are written in bold.

Screen	Name	Sequence	Frequency	A _{405 nm}
1.Cys6	C6A	–CQDPICFCGADGACYCTSRNC–	12/16	1.22 \pm 0.04
	C6B	–CAWHYRFCGAAHSADGACREVLVC–	2/16	0.32 \pm 0.02
1.Cys9	C9A	–CVVWMGFQQVC–	6/13	1.89 \pm 0.07
	C9B	–CWTSGARWRLC–	1/13	0.33 \pm 0.01
	C6K	–CAGAWSRC–	-	0.16 \pm 0.003
	C9A	–CVVWMGFQQVC–	13/35	1.26 \pm 0.03
2.Cys9	C9C	–CIIVPHAYAWC–	6/35	0.31 \pm 0.03
	C9D	–CALVSEAGCLVWAA–	12/35	0.70 \pm 0.06
	7K	–LHVSQRG–	-	0.06

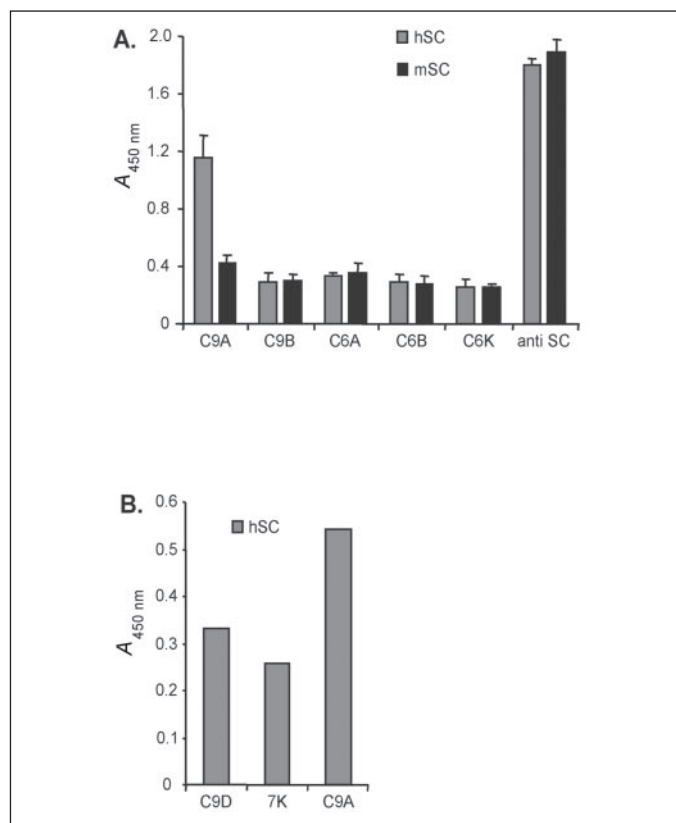


FIGURE 2. The C9A phage binds to human SC but not to mouse SC present in reconstituted secretory IgA. *A*, purified pIgA was coated onto microtiter plates, and either recombinant human SC (hSC) or mouse SC (mSC) was added to make reconstituted SIgA before the different isolated phages were added ($\sim 1 \times 10^9$ TUs). After washing, bound phages were detected with specific antibodies and measured by ELISA ($A_{450\text{ nm}}$). Human or mouse SC bound to pIgA was measured with polyclonal anti-human or anti-mouse SC antibodies, respectively. Mean (± 1 S.D.) of one representative experiment of three is shown. *B*, the C9D phage from the second screening was also tested for binding to reconstituted human SIgA. In this ELISA, SIgA was reconstituted in three steps: first, NIP-BSA was coated onto the wells; second, NIP-specific pIgA was added; and third, human recombinant SC was added. Remaining steps were performed as described above. The columns show mean of duplicates. The amino acid sequences of the phages tested in panels *A* and *B* are given in Table 1.

binding to the receptor. We therefore performed an SC binding ELISA with pIgA, pentameric IgM, or different fragments of SpsA as competitors of the phage C9A. Neither pIgA nor pentameric IgM competed with the phage for binding to SC, even at 100 nM concentration of these natural ligands (Fig. 3). Thus, the C9A phage utilizes a different binding site on SC from that of pIgA and pentameric IgM. However, the *S. pneumoniae* SpsA SH2 and SM1 fragments competed effectively with the C9A phage for binding to SC (Fig. 3). These fragments included the hexapeptide motif known to interact with SC/pIgR. A related fragment from SpsA, SpsA SH3, which lacks the SC/pIgR-binding hexapeptide, did not compete with the phage for binding to SC (Fig. 3).

The SC binding ELISA performed with the C6A, C6B, C9B, and C9D phages showed that pIgA or pentameric IgM did not inhibit the binding of any of these phages to SC. However, the C6A phage was inhibited by the SpsA fragments SH2 and SM1 but not by SH3. The C6B, C9B, and C9D phages were not competed by SpsA (data not shown).

Alanine/Serine Substitution Analysis of the C9A Phage—The Cys9 library contained $\sim 5 \times 10^7$ distinct phages. However, screening of this library identified only one phage that interacted with both human free SC and bound SC in reconstituted SIgA. To further dissect the C9A sequence, a series of recombinant phages, each with 1 amino acid substitution, were constructed. The 9 internal amino acids were each sep-

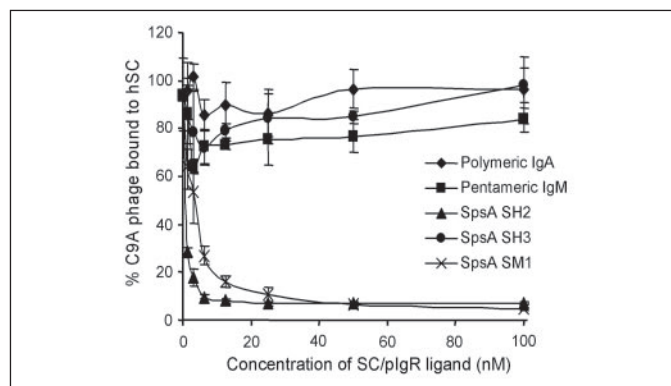


FIGURE 3. Binding of C9A phage to human SC is blocked by SC binding SpsA fragments but not by pIgA or pentameric IgM. Increasing amounts of pIgA, pentameric IgM, or different fragments of recombinant SpsA were mixed with the C9A phage ($\sim 1 \times 10^{10}$ TU) before incubation in SC-coated ELISA plates. Bound phages were detected as described under "Experimental Procedures" and expressed as percentage of binding observed in the absence of competitor. SpsA SH2 and SpsA SM1 contain the SC binding hexapeptide motif found in full-length SpsA, whereas it is lacking in SpsA SH3. Mean (± 1 S.D.) of one representative experiment of three is shown.

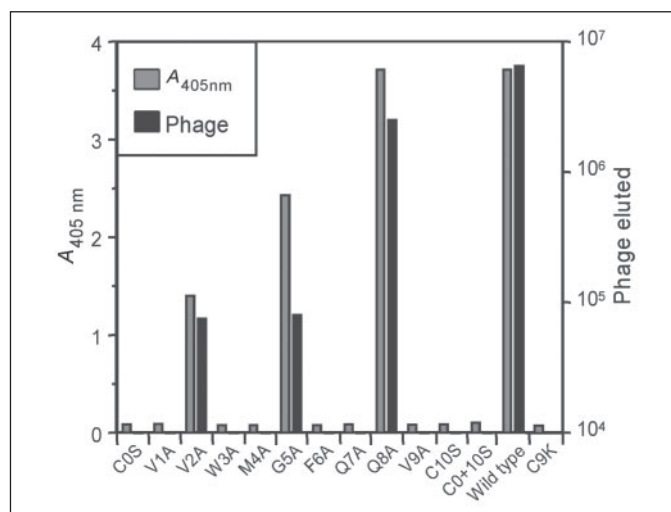


FIGURE 4. Mutational analysis of the requirements for SC binding by the C9A phage. The 11 amino acids of the C9A phage-displayed peptide were mutated individually into alanine (for the 9 internal amino acids) or serine (for the 2 flanking cysteines). In addition, both cysteines were mutated together into serine. All C9A mutants were expressed as phages and analyzed for binding to SC by ELISA and compared with the wild-type C9A phage and a negative control phage (C9K). $1\text{--}3.5 \times 10^9$ TU phages was added to the ELISA well s, and bound phages were analyzed by ELISA ($A_{405\text{ nm}}$), subsequently eluted with 0.1 M HCl/glycine, pH 2.2, and counted as described under "Experimental Procedures." The columns show mean of duplicates of one representative assay of three.

arately changed into alanine, while the 2 cysteines were altered to serine. In addition, both cysteines were mutated together into serine. These 12 new phage clones were analyzed by the SC binding ELISA as well as by acid elution of bound phage and enumeration by phage titration. Of the 12 substitutions made in the C9A sequence, only 3 retained some affinity to SC (Fig. 4). The same trend was observed with both methods of analysis. Exchange of the glutamine in position 8 for alanine had the least effect on binding to SC. In addition, substitution of glycine in position 5 or valine in position 2 into alanine was tolerated but reduced the level of phage binding almost 100-fold. Both flanking cysteines and the remaining internal amino acids of the C9A sequence were essential for the binding of the phage to immobilized SC (Fig. 4).

Inhibition of the C9A Phage Binding to SC by Free Peptide—To test whether the identified amino acid sequence encoded by phage C9A was able to bind to SC independently of the phage particle, we performed

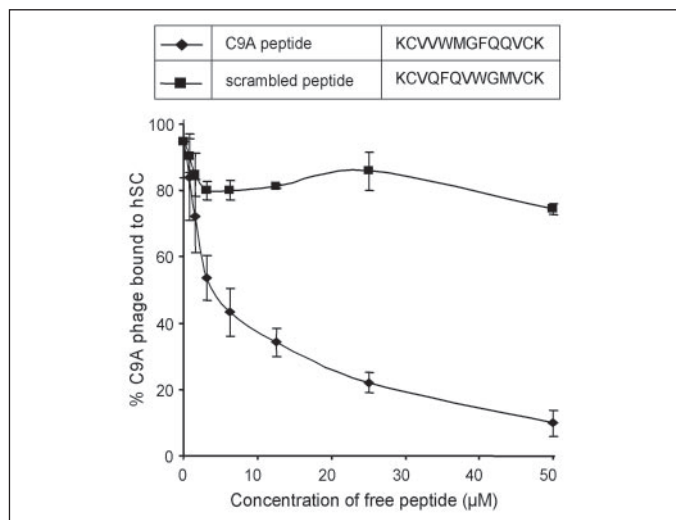


FIGURE 5. Free peptide competes with the C9A phage for binding to SC. Increasing amounts of a free peptide with an amino acid sequence identical to the insert of the C9A phage or a scrambled control peptide were mixed with the C9A phage ($\sim 1 \times 10^9$ TU) before incubation in SC-coated ELISA plates. Bound phages were detected as described under "Experimental Procedures" and expressed as percentage of binding without addition of peptide. Mean (± 1 S.D.) of one representative experiment of three is shown.

competition assays with a synthetic peptide of identical sequence. Because the amino acid sequence displayed by the C9A phage was very hydrophobic, we flanked each cysteines in the free peptide with a lysine (KCVVWMGFQQVCK). A peptide with the same amino acid composition but with a scrambled sequence (KCVQFQVWGMVCK) was used as a control. The free C9A peptide clearly inhibited the binding of the C9A phage in SC binding ELISA, whereas the scrambled peptide did not (Fig. 5). This suggested that the binding of C9A phage to SC was sequence-specific and not dependent on peptide fusion to the phage.

Transcytosis of the C9A Phage through pIgR-transfected MDCK Cells—The C9A phage specifically bound free SC as well as bound SC within reconstituted SIgA. To determine whether the C9A phage could be transcytosed by pIgR-expressing epithelial cells, we used human pIgR-transfected MDCK cells as well as mouse pIgR-transfected and untransfected MDCK cells. MDCK cells were seeded onto collagen-coated filters and allowed to form tight polarized monolayers. $\sim 1 \times 10^8$ TUs of either the C9A phage or a control phage were added to the basolateral chamber and incubated for 8 h at 37 °C before the number of phages in the apical chamber was determined. The C9A phage was translocated 30–250-fold more efficiently by human pIgR-transfected MDCK cells than by untransfected MDCK cells or MDCK cells expressing mouse pIgR (Fig. 6A). Translocation of the control phage across MDCK cells was not significantly affected by pIgR expression being similar to the level of translocation of the C9A phage by MDCK cells lacking human pIgR. The C6A, C6B, C9B, and C9D phages were also tested by *in vitro* transcytosis but showed no pIgR-mediated transport compared with the negative control phage (data not shown). IgG was added to the basolateral chamber as a control for monolayer integrity. The amount of IgG in the apical medium was below 30 ng/ml for all filters analyzed (data not shown).

Simultaneous Transcytosis of the C9A Phage and pIgA through MDCK Cells Transfected with Human pIgR—Inhibition experiments in the SC binding ELISA demonstrated that pIgA did not compete with the C9A phage for binding to human SC (see Fig. 3). Thus, the C9A phage and pIgA probably interacted with different sites on SC/pIgR. We therefore questioned whether pIgA normally present at the basolateral side of pIgR-expressing epithelium might inhibit the transcytosis of the C9A

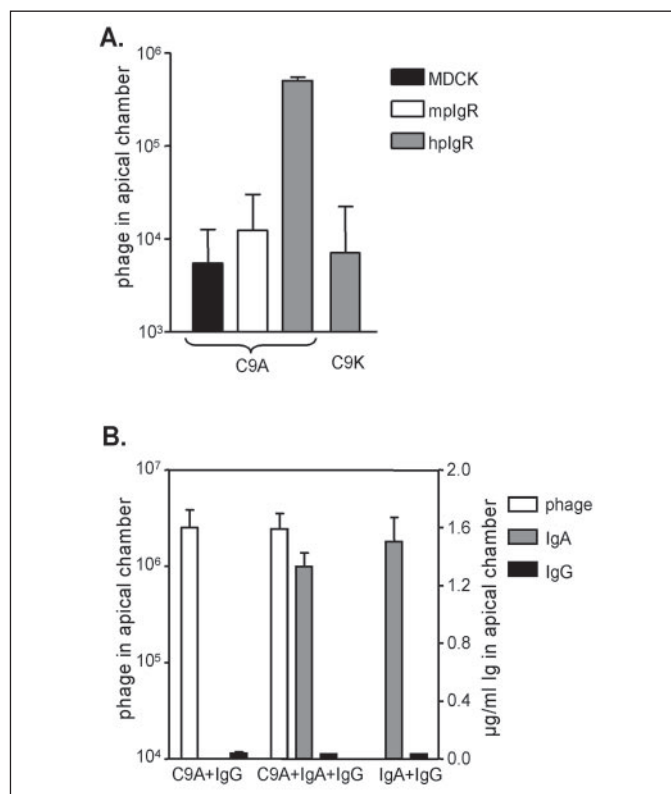


FIGURE 6. The C9A phage is transcytosed by epithelial cells expressing the human pIgR. A, Madin-Darby canine kidney (MDCK) cells, untransfected or transfected with either human (hplgR) or mouse (mplgR) pIgR, were seeded onto collagen-coated filter supports and allowed to form a tight monolayer. The C9A phage or a negative control phage, C9K, (both $\sim 1 \times 10^8$ TU) was added to the basolateral chamber and the cells incubated at 37 °C. After 8 h the medium in the apical chambers was harvested and analyzed for the amount of phage as described under "Experimental Procedures." Mean (± 1 S.D.) of one representative experiment of three is shown. B, the C9A phage ($\sim 1 \times 10^{10}$ TU) or pIgA was added alone or in combination to the basolateral chamber for transcytosis by MDCK cells transfected with human pIgR. IgG was added as a control for paracellular leakage. After 8 h the medium in the apical chambers was harvested and analyzed for phage, IgA, and IgG as described under "Experimental Procedures." Mean (± 1 S.D.) of one representative experiment of three is shown.

phage or vice versa. MDCK cells transfected with human pIgR were allowed to form monolayer as described above, and the C9A phage or pIgA was added alone or in combination to the basolateral chamber. IgG was added as a control for monolayer integrity. After 8 h the amounts of phage particles, IgA, and IgG in the apical chamber were determined (Fig. 6B). No significant difference in the C9A phage transcytosis was found when pIgA was included in the basolateral chamber. Similarly, the presence or absence of the C9A phage in the basolateral chamber did not affect pIgA transcytosis. As before, the monolayers were relatively impermeable to IgG.

DISCUSSION

~ 3 g of pIgA is transported by pIgR per day in an adult human. This receptor is therefore interesting as a potential vehicle for targeting of bioactive molecules to the vast mucosal surfaces. We used phage display to identify a peptide with affinity to human pIgR. The phage carrying this peptide, called C9A, did not interfere with the natural ligands (pIgA or pentameric IgM) for binding to pIgR. The C9A phage was preferentially transported by MDCK cells expressing human pIgR in an *in vitro* transcytosis assay. Importantly, this transport was not inhibited by concurrent pIgA transcytosis nor did the phage inhibit the transport of pIgA.

Affinity Selection of Peptides That Bind to Secretory Component—For each successive round of selection, both the titer of recovered phages and

A Phage-displayed Peptide Transported by Human pIgR

the $A_{405\text{ nm}}$ measurements in the SC binding ELISA were increased. However, a few binders dominated the selected population even after only one or two rounds of selection. Analyses of single isolates by sequencing and SC binding ELISA identified six different SC binding phage-displayed peptides. In contrast to what we have seen previously when selecting for binders to a number of different mAbs (29), there were no obvious sequence similarities between the six binders. Furthermore, both SC binding phages identified from the Cys6 library contained double oligonucleotide inserts that encoded cysteine-rich peptides longer than expected. Similarly, one of the four SC binders from the Cys9 library had an unexpected insert size, which might have arisen from a mutation or incorrectly ligated oligonucleotide during library construction. Such rare deviant phages have not been observed during library characterization or panning against other targets (29, 31) suggesting that a 6-amino acid epitope could be insufficient for binding SC/pIgR.

Characterization of Phages That Bind to Secretory Component—Only the C9A phage was able to bind *in vitro* reconstituted SIgA. We can only speculate whether this could be due to blocking by pIgA, lower affinity for SC/pIgR, or that free SC immobilized to plastic might expose “artificial” binding sites. None of the phages was competed by pIgA for binding to SC (Fig. 3 and data not shown), and the phages C9B, C9D, C6A, and C6B failed to be transported *in vitro* by human pIgR-expressing MDCK cells. However, as we did not directly assay the different phages for affinity to free SC or its membrane variant, pIgR, we cannot formally exclude that these phages bind native SC.

The interaction of C9A phage with bound SC in reconstituted SIgA was specific for human SC compared with mouse SC. This species difference was also confirmed in the *in vitro* transcytosis assay, which showed no increased transport of the C9A phage by the mouse pIgR compared with untransfected MDCK cells. SpsA, like the C9A phage, is also specific for human SC. We and others have recently mapped its binding site on SC/pIgR to D3 and D4 (16, 17). The ability of SpsA to compete with the C9A phage for binding to SC suggested that the C9A phage also binds D3 and/or D4. In contrast to the species specificity of the C9A phage, the phages identified by White *et al.* (32) bound to decapeptides from human SC and were transported by MDCK cells transfected with rabbit pIgR as well as by rat hepatocytes expressing pIgR *in vivo*.

Binding of C9A phage to SC was competed with free peptide with the same amino acid sequence as that encoded by the insert in the C9A phage (KCVVWMGFQQVCK). A peptide with the same amino acid composition but with a scrambled sequence (KCVQFQVWGMVCK) did not compete. Thus, binding of the C9A phage to SC depended on the amino acid sequence of the displayed peptide but not on fusion of the peptide to the phage gene 3 protein. Both the Cys6 and Cys9 libraries have random amino acids flanked by cysteines to constrain the peptides, which leads to increased affinity of the peptides to the selected target (33, 34). The gene 3 protein is presented as 5 copies on the tip of the phage (30), allowing the five copies to form a structural motif. However, a synthetic peptide with an intramolecular disulfide bridge inhibited binding of the phage to SC in ELISA (data not shown), indicating that single free peptides also facilitated binding to SC/pIgR.

To further characterize the nature of the SC binding C9A phage, we introduced alanine substitutions at each position of the 9-mer insert and exchanged each of the cysteines with serine. As expected, most of the amino acids were important for the binding to SC. One exception was the glutamine in position 8, as the phage with this amino acid altered to alanine showed similar binding capacity as the wild-type C9A phage. This polar amino acid is frequently involved in hydrogen bonding networks at the interaction site of two molecules, but it appeared to be

dispensable here. Phage clones with the small amino acids valine and glycine in position 2 and 5, respectively, still bound to SC when mutated into alanine. This suggested that the role of these amino acids is primarily structural. Surprisingly, substituting the valine with alanine in position 1 or 9 completely abolished binding to SC. These amino acids are located next to the flanking cysteines and may be important for the disulfide formation. Thus, the amino acids most likely involved in the binding interaction are tryptophan, methionine, phenylalanine, and glutamine in position 3, 4, 6, and 7, respectively. Recently, Luo *et al.* (35) suggested that hydrophobic interactions are involved in the binding of SpsA to SC. Although the sequences of the C9A peptide and the binding motif in SpsA (YRNYPT) show no apparent amino acid homology, competition of phage C9A binding by SpsA suggests proximity of the two binding sites. Thus, both the C9A phage and SpsA may interact with SC/pIgR via a hydrophobic docking site involving D3 and D4.

Epithelial Transport of the C9A Phage—A short peptide sequence that, via binding to the pIgR, could facilitate epithelial transport of coupled therapeutic molecules should not interfere with the export of endogenous pIgA to provide SIgA antibodies at the mucosal surfaces. *In vitro* transcytosis assays with the C9A phage showed greatly increased transport by MDCK cells transfected with human pIgR compared with mouse pIgR or untransfected cells. Polarized MDCK cells with pIgR expression might increase unspecific uptake of basolateral macromolecules because of increased endocytosis. However, the lack of transport over MDCK cells transfected with mouse pIgR showed that this was not the case and confirmed that the human pIgR-driven transport of the C9A phage was specific. Significantly, cocubation of the phage and pIgA did not influence the transport of either ligand. Thus, the C9A phage and pIgA did not compete for pIgR-mediated epithelial transport, which is an important prerequisite of any future clinical application of the C9A peptide.

Ferkol *et al.* (19–21) developed a therapeutic strategy for cystic fibrosis by exploiting properties of cellular pIgR routing. They linked agents for targeted delivery across respiratory epithelia to a mouse mAb directed at the extracellular domain of human pIgR. The anti-SC provided greater specificity for the target cells than the natural ligand pIgA, which can bind to alternative receptors (*e.g.* Fc α Rs) on other cell types. Similarly to antibody-mediated targeting, the peptide sequence of the C9A phage can be exploited for pIgR-mediated transport across respiratory epithelia, acting as targeting molecule fused with a therapeutic agent, such as human α_1 -antitrypsin (19–21). A peptide may include fewer potentially immunogenic epitopes than whole mAb or scFv, thereby preventing undesired immune responses when used in humans (36).

Preliminary studies indicate that construction of functional fusion proteins with the C9A sequence will need a systematic and accurate approach. Important features for usefulness in a clinical setting of such a targeting peptide are high affinity to its ligand, low immunogenicity, and absence of unwanted side effects. We believe that the ability of the short C9A peptide sequence to exploit epithelial pIgR-mediated transport without inhibiting SIgA and secretory IgM generation makes this ligand an interesting candidate for further studies.

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Identification of a Polymeric Ig Receptor Binding Phage-displayed Peptide That Exploits Epithelial Transcytosis without Dimeric IgA Competition

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