Antibodies to the Polymeric Immunoglobulin Receptor with Different Binding and Trafficking Patterns

Sanhita Gupta, Michael Heacock, Aura Perez, and Pamela B. Davis

Department of Pediatrics, Case Western Reserve University School of Medicine, Cleveland, Ohio

The polymeric immunoglobulin receptor (pIgR) has been proposed as a therapeutic target, but its potential depends on the efficiency of uptake and trafficking of the receptor ligand. Mouse monoclonal antibodies (Mabs) directed against pIgR, selected for strong binding to secretory component (SC) and secretory IgA (sIgA), were tested in a transcytosis assay in 16HBEo– cells (human bronchial epithelial cell line) transfected with human pIgR. Intracellular trafficking was followed by confocal microscopy. Mabs fell into two classes. For two Mabs, transcytosis from basolateral to apical surface is rapid, unidirectional, and little Mab is retained in the cell. For three Mabs, basolateral to apical transcytosis occurs to a significantly lesser extent, reverse transcytosis is permitted, and some of the Mab is retained in the perinuclear region even after 24 h. When tested for their ability to recognize and immunoprecipitate pIgR with systematic truncations and deletions of the five immunoglobulin (Ig)-like domains, all Mabs bound to the fifth Ig-like domain, but three of them also bound to the C-terminal region of pIgR near the plasma membrane. Different binding sites probably account for the different trafficking of these Mabs and may predict differential therapeutic utility.

Keywords: epithelial cells, basolateral and apical; pIgR; transcytosis

The polymeric immunoglobulin receptor (pIgR) is expressed in the airways from the sixteenth gestational week and is abundant in surface epithelium and the serous cells of the submucosal glands (1). This receptor takes up large quantities of polymeric immunoglobulins (Igs) at the basolateral surface and deposits them at the apical surface of the epithelium, beneath the mucous blanket. Its bulk flow characteristics, together with its distribution, have made it an attractive therapeutic target for pulmonary delivery of drugs that would be most effective at the apical surface (2, 3). The restriction of its expression to epithelium also offers a means of targeting gene transfer reagents specifically to epithelial cells, thereby increasing efficiency of gene transfer and limiting off-target expression and toxicity (4, 5). Moreover, this receptor facilitates invasion of *S. pneumoniae* by transporting this bacterium in retrograde fashion, so the pIgR offers a therapeutic approach to the lung interstitium via the airway (6). However, the efficacy of treatments targeting this receptor will depend on how the therapeutic complexes are trafficked. For delivery from the blood stream to the lumen, efficient and complete transcytosis is most desirable. However, for gene transfer, rapid and efficient cell entry at the basolateral surface, but pro-

tracted cellular retention would be better. This would allow a longer time for escape of the gene transfer vector from the endosome into the cytoplasm, and limit the amount of therapeutic reagent that is nonproductively expelled at the opposite surface.

The trafficking of pIgR, and its regulation, has been studied intensively in cell models. The pIgR is synthesized in the endoplasmic reticulum and traffics initially to the basolateral surface of polarized epithelial cells. Occupied or not, it can cluster into coated pits and undergo internalization. Many studies have elucidated the trafficking controls for the rabbit pIgR (7–15), but whether all these features are applicable to the human receptor is not yet clear (16). Moreover, although there has been extensive work defining the factors that regulate transcytosis, there is much less information on how the receptor is routed into the recycling pool, even though recycling occurs for as much as 45% of the receptors that are internalized.

To target the pIgR for therapeutic purposes, we prepared monoclonal antibodies (Mabs) directed against human secretory component (SC). Our initial studies targeting the pIgR for gene transfer were performed with Fab fragments prepared from polyclonal antisera, which proved to be immunogenic upon repeat administration (4, 17, 18). From monoclonal antibodies, single-chain Fvs (scFvs) can be cloned, which eliminate much of the constant region thought to be particularly immunogenic. Moreover, if they prove successful, these scFvs can be mutated to “humanize” their framework regions, further reducing immunogenicity. Five of these Mabs were selected for their ability to bind to both SC and secretory IgA (sIgA). Such Mabs do not bind at the same site as the natural ligand, dIgA, and so should not compete with dIgA for receptor access (2). In this study we investigate the properties of these Mabs. Two Mabs (4121 and 4214), like dIgA, undergo rapid and extensive basolateral to apical transcytosis and are not retained in the cell. Three of them (Mabs 7214, 7125, and 7221), on the other hand, are transported in the basolateral to apical direction to a much lesser extent than 4121 or 4214, can be transported retrograde, and are retained within the cell in compartments about the nucleus, even after 24 h. These different patterns of interaction with receptor bearing cells may presage differential therapeutic utilities.

MATERIALS AND METHODS

Anti-pIgR Mabs

The hybridoma clones making the anti-pIgR Mabs were originally made by the Cystic Fibrosis Hybridoma core at Case Western Reserve University. Human SC was purified from human milk by incubation with a mouse anti-human SC antibody (Sigma, St. Louis, MO) immobilized on sepharose beads. Free SC was separated from secretory immunoglobulins by size-exclusion gel chromatography (19). Mice were injected intraperitoneally with 50 µg purified hSC in 100 µl sterile PBS emulsified in an equal volume of complete Freund's adjuvant. The mice were boosted with 25 µg hSC in incomplete Freund's adjuvant three times at 3- to 4-wk intervals.

Sera from these mice were screened against hSC by ELISA, and mice with high antibody titer were killed and spleen cells fused with Sp2/0 murine myeloma cells. The hybridoma cells were selected in HAT medium and cloned by limiting dilution. Positive clones were identified

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Correspondence and requests for reprints should be addressed to Pamela B. Davis, Department of Pediatrics, Case Western Reserve University at Rainbow Babies and Childrens Hospital, Biomedical Research Bldg, Rm 831, 2109 Adelbert Road, Cleveland, OH 44106-6006. E-mail: pbd@po.cwru.edu

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by screening medium by ELISA against purified hSC. Six antibodies had high titer binding to both hSC and human sIgA, but one hybridoma was lost. Antibodies were named with the first digit (in this case, 4 or 7) designating the mouse from which the spleen cell was derived, and the next three digits numbering the clones. Thus, 4121 is clone #121 from mouse #4. The cells were sent to Harlan BioProducts for Science (Madison, WI) for production of ascites. The IgG was precipitated from the ascites using EZSTEP one spin antibody partitioning kit (Middlesex Sciences, Norwood, MA) following the manufacturer's protocol. IgG was purified from the precipitated pellet using Affi-Gel Protein A MAPS II Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's recommendation.

Cell Culture: Creation of MDCK-pIgR Line and 16HBE-pIgR Line

Madin-Darby canine kidney (MDCK) type II cells were maintained on uncoated tissue culture plasticware (Falcon, Bedford, MA) in 90% Eagles' Minimal Essential Media with Earle's salts (EMEM; Biowhitaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS), 15 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid (HEPES) (Sigma) 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA) in a humidified 37°C incubator with 5% CO₂. The human bronchial cell line 16HBE140⁻ (a gift of Dr. Dieter Gruenert, University Pacific Medical Center, San Francisco, CA) was maintained similarly on the plasticware coated with a mixture of 1 mg/100 ml human fibronectin (Invitrogen), 1 ml/100 ml Vitrogen-100 (Collagen Biomaterials, Palo Alto, CA), and 1 mg/ml bovine serum albumin (Sigma) in LHC Basal Media (Biofluids, Rockville, MD). Cell culture media consisted of 90% Minimum Essential Media with Earle's salts (MEM; Invitrogen), 10% FBS, and 2 mM L-glutamine. MDCK II and 16HBE-140⁻ cells were transfected with human pIgR cDNA (a gift of Dr. Charlotte Kaetzel, University of Kentucky) with a neomycin resistance gene using Lipofectin reagent (Invitrogen) according to the manufacturer's directions. Stably transfected MDCK-pIgR cells or 16HBE-pIgR cells were selected, sorted, and maintained in appropriate media containing 400 µg/ml G-418 sulfate (Calbiochem, San Diego, CA). These cells form tight junctions and transport the natural ligand, dIgA, from the basolateral to the apical surface (see Figure E1 in the online supplement).

Fluorescence-Activated Cell Sorting

Stably transfected cells were labeled using a goat polyclonal antibody to human secretory component (Sigma) followed by a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Molecular Probes, Eugene, OR). Positive cells were sorted with the Coulter Elite ESP (Fullerton, CA), 525-band path using a 488-nanometer laser line from a 15-milliwatt argon ion-cooled laser, grown to confluence, and repeatedly resorted until the population was > 90% positive. Subsequent passages of MDCK-pIgR and 16HBE-pIgR were monitored for continued positive expression.

Transcytosis Experiments

Cells were plated on 24-well size 1.0 µm pore size Falcon tissue culture inserts (filters), uncoated for MDCK-pIgR (0.3 × 10⁶ cells) or the fibronectin mixture-coated for 16HBE-pIgR (0.5 × 10⁶ cells), grown for 48 h with only apical media (0.3 ml), then subsequently with both apical and basolateral (0.5 ml) media, for 9–16 d. Confluent, polarized monolayers were washed extensively, checked for leakage, and dIgA (15 µg) (purified from human ascites, gift of Michael Lamm, M.D., Case Western Reserve University, Cleveland, Ohio) or anti-human secretory component Mabs (15 µg) were applied to either apical or basolateral sides of the filters. Fresh media was added to the opposite side. At the indicated times, the entire media on the opposite side of the filter was collected and assayed for dIgA, IgG, or SC as indicated. Each well was committed to a single time point, so each value is accumulated over that period of time. For some experiments, the filter was washed six times on the apical and basolateral sides, then the cells remaining on the filter were extracted with RIPA buffer, and the extracts assayed as noted below.

ELISA for dIgA and Anti-HSC Antibodies

For dIgA, media samples were incubated in microtiter plates coated with rabbit anti-human IgA as the capture antibody, and bound protein was detected with horseradish peroxidase-conjugated goat anti-human IgA, using dimeric IgA for standardization. For IgG, a similar procedure was followed except capture antibody was rabbit anti-mouse IgG, with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Pharmacia, Piscataway, NJ) as secondary antibody, and IgG1κ from mouse myeloma as the standard. All other antibodies were purchased from Sigma Chemical Co.

Stain to Visualize Antibodies

After transcytosis of anti-HSC Mabs and dIgA from basolateral media through the cells to apical media, cells on filters were fixed with Periodate-Lysine-Paraformaldehyde (McLean's fix) at each of the time points and stored at 4°C overnight. Filters/cells were sequentially: washed with PBS (without Calcium or Magnesium, pH 7.4), washed with PBS containing 0.1% ovalbumin and 0.05% sodium azide, and incubated with 20 µg/ml of goat anti-mouse Alexa Fluor-488 (Molecular Probes) in the same buffer for 1 h. DAPI was used to stain nucleic acids in nuclei. After washing in PBS-ovalbumin-sodium azide, filters were excised from supports using #11 scalpel blade, mounted on slides with cells up, covered with Fluoromount (Fisher Scientific, Suwanee, GA) and #10r #1.5 thickness coverslips. Each experiment was repeated at least three times.

Confocal Scanning Laser Microscopy

Confocal images were acquired using a Zeiss LSM 510 (Thornwood, NY) inverted laser-scanning confocal fluorescence microscope in the Case Western Reserve University Ireland Comprehensive Cancer Center confocal microscope facility with a Plan-Neofluor 40×/1.3 oil immersion DIC objective. Confocal images of Alexa 488 were collected using an excitation wavelength of 488 nm from an argon laser and emission wavelength of 505-nm-long pass filter. Images of Hoechst fluorescence were collected using a tunable pulsed Ti:sapphire laser, a 700-nm dichroic mirror, and a 390- to 469-nm band pass filter. Control images of anti-HSC antibodies alone, Alexa-fluor 488 alone, or cells alone, even with increased laser power, had no detectable immunofluorescence.

Construction of Mutants of pIgR Lacking One or More Ig-Like Domain

c-DNA encoding pIgR was amplified from wild type pIgR c-DNA (gift of Dr. Charlotte Kaetzel) by PCR and cloned into pCDNA 3.1 vector between the *EcoRI* and *NotI* sites. The oligonucleotides used in construction of pIgR and its mutants are as shown in Table 1. For the progressive deletions, domains 1, 1 and 2, 1–3, 1–4, 1–5, and 1–6 (leaving aa 607–754) and the domain beginning at aa 588 to the end of the sequence (called Δ6a) were deleted. The mutants of pIgR lacking any one of the domains 2, 3, 4, or 5 were made by megaprimer directed PCR (19) (Table 1). The megaprimers are then used in a second round of PCR using the forward or reverse primer (as needed) for pIgR to generate the mutants. Mutations were verified by standard sequencing analysis.

In Vitro Translation of pIgR and Mutants of pIgR

The following proteins were translated using TNT system (Promega, Madison, WI) in the presence of ³⁵S methionine/³⁵S cysteine and according to manufacturer's instructions: wt pIgR, pIgRΔ1, pIgRΔ1,2, pIgRΔ1–3, pIgRΔ1–4, pIgRΔ1–5, and pIgR Δ1–6 through aa 606, pIgRΔ2, pIgRΔ3, pIgRΔ4 and pIgRΔ5, and pIgR 588–674 (Δ6a).

Immunoprecipitation

The pIgR and its mutants labeled by *in vitro* translation in the presence of ³⁵S methionine and cysteine (Amersham Pharmacia). Each monoclonal antibody was separately incubated for 90 min at 4°C with each of the pIgR mutants to allow binding followed by incubation with protein A agarose (Roche Molecular Biochemicals, Indianapolis, IN) for 30 min at 4°C. The precipitated proteins were analyzed on a 10% SDS-polyacrylamide followed by standard fluorography and autoradiography.

TABLE 1. OLIGONUCLEOTIDES USED IN CONSTRUCTION OF pIgR AND MUTANTS

Construct name	Forward Primer	Reverse Primer
pIgR	5'CCGGAATTCGAAATGCTGCTCTCTGCT CACCTGC 3'	5'ATTGCGGCCGCTAGGCTTCCTGGGGG CCGTCGTG 3'
pIgR Δ1	5'CCGGAATTCGAAATGCTGGGCTCTAAATGACACTAA 3'	5'ATTGCGGCCGCTAGGCTTCCTGGGGG CCGTCGTG 3'
pIgR Δ1,2	5'CCGGAATTCGAAATGCTAAAGCCGAGCCGAGCTGTTAT 3'	5'ATTGCGGCCGCTAGGCTTCCTGGGGG CCGTCGTG 3'
pIgR Δ1-3	5'CCGGAATTCGAAATGATCCCCGAGCCC CACTGTGG 3'	5'ATTGCGGCCGCTAGGCTTCCTGGGGG CCGTCGTG 3'
pIgR Δ1-4	5'CCGGAATTCGAAATGGAACCAACCTCAAG GTACCAGGAATGTC 3'	5'ATTGCGGCCGCTAGGCTTCCTGGGGG CCGTCGTG 3'
pIgR Δ1-5	5'CCGGAATTCGAAATGGGGTCCCGCATGTC AGCCTAGCGAAGGCA 3'	5'ATTGCGGCCGCTAGGCTTCCTGGGGG CCGTCGTG 3'
pIgR Δ1-6	5'CCGGAATTCGAAATGGAGGAAAGGCGGTGGCAGAT 3'	5'ATTGCGGCCGCTAGGCTTCCTGGGGG CCGTCGTG 3'
pIgRΔ6a	5'CCGGAATTCGAAATGCTGCTCTCTGCT CACCTGC 3'	5'ATTGCGGCCGCTAGCTAGCACCTCTCATCAG-3'
	For Mega Primer	Forward/Reverse Primer
pIgR Δ2	5'CAGCTCGGGCTCGGGCTTTAGAGGACCTGGCTGAC 3'	pIgR forward primer in cycle 1 PCR and pIgR reverse primer in cycle 2 PCR
pIgR Δ3	5'CACAGTGGGGTGGCGGAATCACTTGGAGGTGAGC 3'	pIgR forward primer in cycle 1 PCR and pIgR reverse primer in cycle 2 PCR
pIgR Δ4	5'CTTCGTCAATGAGGAGTCCACGCTCAAGGTACAGGG 3'	pIgR reverse primer in cycle 1 PCR and pIgR forward primer in cycle 2 PCR
pIgR Δ5	5'GATTATCGAAGGAGAACCAACGATGTCAGCCTAGCG 3'	pIgR reverse primer in cycle 1 PCR and pIgR forward primer in cycle 2 PCR

Definition of abbreviation: pIgR, polymeric immunoglobulin receptor.

Statistical Analysis

The *P* values were calculated using unpaired *t* test (Sigmaplot 8.0).

Binding Studies and Binding Constant Analysis

Binding studies with the antibodies and SC were performed in the BIAcore 1000 (BIAcore, Piscataway, NJ). Purified SC was bound to CM5 sensor chip surface by amine coupling, and different concentrations of purified Mabs were directed over the surface. The response curves were generated by subtracting the background obtained from control chips. The binding constant (K_D) values were calculated using the BIAevaluation 3.0 Software (BIAcore).

RESULTS

Basolateral to Apical Transport of the Mabs

We performed transcytosis studies with antibodies using monolayers grown on filters of MDCK cells and 16HBEo⁻ cells that were transfected to express pIgR. MDCK and 16HBEo⁻ cells that were not transfected with human pIgR failed to support transcytosis of any of the antibodies in either direction (data not shown). Figure 1A shows that apical delivery of antibodies 7221 and 7125 is significantly less than apical delivery of antibodies 4124 and 4121 in 16HBE-pIgR cells from the outset of the experiment. To assure that this phenomenon was not an accident of the 16 HBEo⁻ cell line or of airway cells, MDCK-pIgR cells

were studied. Substantially similar results were obtained, though in MDCK-pIgR cells, the differences between the two groups of antibodies become significant later in the course of the experiment (Figure 1B). To determine whether these differences could result from better receptor binding of 4121, K_D was determined for antibodies of both classes by Biacore (Table 2). The affinity for SC was somewhat less for 4121, so better binding of 4121 is unlikely to account for these results. The amount of SC released into the apical media was the same in the presence of either 4121 or 7221 antibodies (Figure 2), so the difference in transport is not accounted for by changes in production or apical delivery of the receptor *per se*, nor does either ligand accelerate or retard receptor delivery to the apical surface. Two other possibilities arise. First, 7221, 7125, and 7214 antibodies could be recycled from the apical side and transported back into the cell. Alternatively, they could be diverted within the cell into an intracellular compartment.

7221, but Not 4121 Antibodies Are Transported in an Apical to Basolateral Direction

Apical to basolateral transport of the antibodies ("reverse transcytosis") was studied in 16 HBE-pIgR cells. 7221 antibody was efficiently transported apical to basolateral, but 4121 antibodies are very poorly transported in this direction (Figure 3). Signifi-

Figure 1. Transcytosis of anti-pIgR Mabs. (A) Mabs were applied to the basolateral side of 16HBEo⁻ cells expressing pIgR plated on tissue culture inserts, grown to confluence. At each time point, indicated on the x-axis, the entire apical media was saved for ELISA. This graph shows the mean of five separate experiments, with each point done in triplicate (three wells per point in each of five experiments). The amount transported was compared between 4121 and 4214 as a class and 7221, 7125, and 7214 antibodies at 7 h and 24 h time points by unpaired *t* test. *Different from 4121 and 4214, *P* < 0.002. Values for irrelevant IgG were less than 10 ng/ml at 24 h. (B) A similar experiment was performed in MDCK cells expressing pIgR. Graph shows the mean of five experiments, each point in triplicate. *Different from 4121 and 4214, *P* < 0.002. Values for irrelevant IgG were less than 30 ng/ml at 24 h.

TABLE 2. BINDING CONSTANTS DETERMINED IN BIACORE FOR Mabs FOR SC

Sample	K_D (10^{-6} M)
Mab 7221	0.637
Mab 7214	0.207
Mab 7125	0.198
Mab 4121	2.46

Definition of abbreviations: K_D , binding constant; Mab, monoclonal antibody; SC, secretory component.

cant differences are seen at 6 and 24 h. Consistent with these observations, immunofluorescence tracking of antibodies added to the apical surface show that 4121 antibodies are seen only at the apical surface, whereas at 24 h, the 7221 antibodies are visible within the cytoplasm of the 16HBE-pIgR cells in punctate compartments (data not shown).

Different Trafficking Patterns Observed in the Two Classes of Antibodies

16HBE-pIgR cells grown on filters, when treated with 4121 antibody in the basolateral medium, display the presence of the antibody at or near the apical membrane by 2 h (Figure 4A). At this time, some of the immunofluorescent staining is also observed in intracellular compartments. By 6 h, 4121 is mostly in the apical membrane of the cell as seen in orthogonal sections from confocal images (Figure 4C). At 21 h, the stain is still concentrated at the apical membrane of the cell (data not shown). This corroborates the transcytosis results (Figure 1). For 7221, the pattern is different. At 2 h the label is visible mostly within the cells, and appears to be punctate and compartmentalized (Figure 4B). This pattern becomes more pronounced at 6 h (Figure 4D) and 24 h (data not shown) with significant retention of antibody within the cell, as shown by orthogonal sections from confocal images (Figure 4). The 7221 antibody is retained within the cell longer than 4121. Results for 4214 are

Figure 2. Assay of SC in the apical media. 16HBE⁺ cells expressing pIgR were plated on tissue culture inserts. Mab (10 μ g) or plain media (control) was added to the basolateral surface. Fresh media was applied on the apical side. At each time point, indicated on the x-axis, the entire apical media was saved for ELISA for SC. This graph shows the mean of three separate experiments, with each point done in triplicate. No comparisons showed significant difference (including 4121 versus 7221 at any time point) except the comparison of no antibody versus 4121 at 7 h ($P < 0.003$ before correction for multiple comparisons).

Figure 3. Apical to basolateral transcytosis of anti-pIgR Mabs. Mabs were applied to the apical surface of 16HBE⁺ cells expressing pIgR. At each time point, indicated on the x-axis, the entire basolateral media was saved for ELISA. This graph shows the mean of three separate experiments with each point done in triplicate. *Different from 4121, $P < 0.02$ and **different from 4121, $P < 0.001$.

similar to those shown for 4121 and antibodies 7214 and 7125 are similar to 7221. When dIgA was added to the basolateral surface, and at intervals cells were fixed, permeabilized, and treated with fluorescent antibodies against dIgA, its trafficking resembles that of 4121 (Figure E1).

Cells treated with antibody but no secondary FITC-anti-mouse IgG showed no immunofluorescence, nor did cells treated with no primary antibody but stained with FITC-anti-mouse IgG (data not shown).

Neither 4121 nor 7221 Antibodies Increase Delivery of dIgA to the Apical Medium

To test whether anti-SC antibodies affect the trafficking of dIgA, as would be expected if they entrain intracellular signaling that affects all pIgR-ligand traffic, we measured the transcytosis of dIgA in the presence of 4121, 7221, or an anonymous isotype matched mouse IgG antibody, and compared it with the transcytosis of dIgA alone. At 2 h, transport of dIgA was greater in the presence of 4121 compared with 7221, but at later time points, transport was comparable. Thus, 4121 did not accelerate, nor did 7221 retard, delivery of dIgA to the apical surface (Figure 5). Surprisingly, the anonymous isotype-matched IgG appeared to inhibit dIgA transcytosis compared with no additions, but neither of the active antibodies had any significant effect.

All Antibodies Bind to the Fifth Ig-Like Domain of pIgR, but 7221, 7214, and 7125 Antibodies also Bind to the C-Terminal Portion of pIgR

Since 7221, 7125, and 7214 antibodies traffic in distinct fashion from 4121 and 4214 antibodies, we tested the hypothesis that the two groups of antibodies bind to the pIgR at different sites. Mutants of pIgR lacking one or more of Ig like domains were constructed (Figure 6). These progressive truncations were cloned into the *EcoRI* and *NotI* sites of pCDNA3.1. The T7 promoter upstream of the cDNA in pCDNA3.1 allowed *in vitro* translation of the wt and mutant pIgR with ³⁵S-labeled amino acids. Translation products were immunoprecipitated with each antibody. Each experiment was repeated three times. Results shown in Figure 7 for 4121 are similar to those obtained for 4214, and those obtained for 7221 are similar to those obtained

Figure 4. Confocal images of 4121 and 7221 Mabs at different times during transcytosis. Mabs, 4121 and 7221 (15 μ g) were added to 16HBEo[−] cells expressing pIgR on the basolateral side. At the time points indicated filters/cells were washed, fixed, stained, and excised from supports. Confocal images are shown including orthogonal sections for each image. The scale bar shows 20 μ m. Green fluorescence represents antibodies, and blue fluorescence DAPI-stained nuclei. (A) 4121, 2 h. (B) 7221, 2 h. (C) 4121, 6 h. (D) 7221, 6 h. Photomicrographs are representative of three separate experiments.

for 7214 and 7125. All antibodies interact with domain 5, because they do immunoprecipitate the mutants lacking domains 1–4, but they do not immunoprecipitate those lacking domains 1–5. However, when a further deletion was tested, and in addition to domains 1–5, a portion of the extracellular stem to aa 606 was deleted, 7221, 7125, and 7214 also interact with the remaining portion of the extracellular stem (aa 607–754). Thus, a second site of interaction for these antibodies is observed when the primary binding site, the fifth Ig-like domain, is missing. This site is not readily accessible to the antibody unless a portion of the extracellular stem is missing. In addition, other mutants of the pIgR were tested, each of which was missing only one of the five domains. All of these deletion mutants interacted with 4121 antibodies except those missing Ig-like domain 5. However, all of the individual Ig-like domain mutants of pIgR interacted with 7221, though the interaction of the mutant missing domain 5 was faint (Figure 7F). These results confirm that 7221 interacts with the C-terminal region of the pIgR as well as domain 5.

DISCUSSION

All of the anti-pIgR antibodies are transported across polarized epithelial layers in a receptor specific fashion, that is, cells that do not express the pIgR do not effectively transport these antibodies. Antibodies 4121 and 4214 are transported efficiently from the basolateral to apical surface in polarized epithelial cells engineered to express human pIgR. However, antibodies 7221, 7214,

and 7125 have comparable initial transport, but, after 6–24 h, accumulate in apical medium to a much lesser extent than 4121 or 4214. Since the binding constants for these antibodies are all in the same range (and even favor 7221, 7214, and 7125), it is likely that these differences result from differential trafficking rather than more avid binding of 4121. Several possibilities present themselves. The binding of 7221, 7214, and 7125 antibodies to pIgR may result in conformational changes in the receptor that favor routing to a basolateral recycling pool, or that inhibit progression of the antibody–SC complex into the apical recycling endosome pool. Alternatively, these antibodies, once released at the apical surface, might dissociate from SC and be available to bind either to intact pIgR at the apical surface, or to the stem of the pIgR which is left after receptor cleavage, which may be subject to reuptake. These possibilities were tested in a series of experiments.

Immunofluorescence studies indicate that 4121 and 4214 antibodies travel rapidly to the apical surface of the cell, so that even at 2 h, much of the antibody resides at or near the apical surface, and by 6 h most of the staining is at the apical membrane. The natural ligand, dIgA, follows this pattern. In contrast, 7221, 7214, and 7125 antibodies accumulate in the perinuclear region, where they remain for at least 24 h after transcytosis has begun. Thus, there is increased intracellular retention of these three antibodies. If the 4121 and 4214 antibodies traffic more rapidly because they stimulate transcytosis at the level of intracellular signaling (e.g., promoting tyrosine phosphorylation and intracel-

Figure 5. Transcytosis of dlG in the presence of Mabs. 16HBE⁺ cells expressing pIgR were plated on tissue culture inserts. Dimeric IgA (15 μ g) was added to the basolateral surface, either alone, or together with Mabs as indicated (10 μ g). Fresh media was applied on the apical side. At each time point, indicated on the x-axis, the entire apical media was saved for ELISA for dlG. Data shown are mean of three separate experiments, with each point done in triplicate. Comparison between 4121 and 7221 showed a significant difference only at 2 h ($P < 0.04$).

lular free calcium release), then transcytosis of the empty receptor and/or dIgA added at the same time should also increase. However, there was no increase in release of SC or of dIgA added at the same time in the presence of 4121 antibody compared with cells treated with 7221 antibody. Conversely, if intracellular signals are sent by 7221 antibody receptor binding that retard transcytosis of pIgR or route it into a basolateral recycling compartment, we might expect less free SC or dIgA to be released at the surface in the presence of 7221 IgG compared with controls treated with 4121 Mab. However, apical release of SC and dIgA was not reduced. Therefore, the differential response to the two classes of Mabs is not explained by intracellular signals that influence pIgR trafficking. An alternative hypothesis is that this

difference resides in the interaction of the specific IgG with the pIgR and the ensuing conformational changes. Therefore, the two classes of Mabs may interact differently with the receptor.

These Mabs were selected for their binding to sIgA, and dlG does not compete with these Mabs for transport (2). Therefore, the Mabs are predicted to bind to pIgR in the cleaved portion of the receptor (i.e., SC), but not where the natural ligand binds—primarily in the first Ig-like domain of the pIgR, with some participation of the third and fourth domains. Consistent with this expectation, immunoprecipitation studies show that 4121 antibodies bind to the fifth Ig-like domain, but the 7221 antibodies bind both to the fifth Ig-like domain and to the C-terminal portion of the receptor as well. The second binding site was unexpected, because the Mabs were selected for their binding to the cleaved portion of the receptor, SC. However, because the precise cleavage site for pIgR is not certain, it may be that SC purified from human milk (used as the immunogen to generate the Mabs) included sequences that are included in the C-terminal region of our test constructs. Nevertheless, there are different binding sites for the two classes of antibodies, which may entrain different conformational changes in the receptor when binding occurs, which in turn could stimulate different trafficking patterns. It is also possible that the binding of 7221, 7214, and 7125 antibodies, with one binding site in the fifth Ig-like domain and one closer to the membrane, inhibits cleavage of SC at the apical surface. If less of the pIgR bound to these antibodies is cleaved and released, more of it might undergo reuptake. If this were the case, one would expect less SC to be released in the presence of 7221, 7214, and 7125 antibodies than with 4121 or 4214, but no such reduction was detected. However, the total quantity of SC that is released is far in excess of the quantity of IgG that is transported, so changes in SC release caused by antibody binding may be too small to detect, relative to total SC release. We also considered the possibility that, given their capacity to bind to the region of the pIgR closest to the membrane, which remains at the apical surface after SC is cleaved, 7221, 7214, and 7125 antibodies may dissociate from SC after cleavage and bind to the pIgR stem, or to intact receptors at

Figure 6. Domain structure and deletion mutants of pIgR. Diagram (not to scale) represents the polymeric immunoglobulin receptor. The five Ig-like extracellular domains (I–V), cleavage site, stem, and membrane spanning regions are shown. Below, the line diagrams illustrate the truncation and deletion mutants tested in pull-down assays.

Figure 7. Immunoprecipitation of pIgR and its mutants with 4121 and 7221 antibodies. (A) The pIgR and mutants lacking one or several of the Ig-like domains were translated *in vitro* and labeled with ^{35}S methionine/cysteine mix. The weaker translation of intact pIgR is probably because of the larger size and inclusion of the transmembrane domain. Autoradiogram of a representative translation is shown. (B) 4121 monoclonal antibody was incubated for 90 min at 4°C with each of the ^{35}S labeled pIgR mutants to allow binding followed by immunoprecipitation. The immunoprecipitated proteins were analyzed on a 10% SDS-polyacrylamide gel, an autoradiogram of which is shown. (C) Same as B, except 7221 Mab was used instead of 4121. Autoradiogram is a representative

of three independent experiments. (D) The pIgR mutants lacking single Ig-like domains as indicated (see Figure 6) were translated *in vitro* and labeled with ^{35}S methionine/cysteine mix as in A. A representative autoradiogram of the translation is shown. (E) Immunoprecipitation by Mab 4121 of *in vitro* translated ^{35}S -labeled mutant pIgR proteins shown in D by Mab 4121. Autoradiogram is representative of three independent experiments. (F) Immunoprecipitation of *in vitro* translated proteins shown in D by Mab 7221. Gel is representative of three independent experiments.

the apical surface, and be taken up again in this manner. However, this seems unlikely, as there is an ever-increasing excess of free SC in the apical medium to which the small quantity of dissociated antibody could bind under the conditions of these experiments. Mabs 7221, 7214, and 7125, but not 4121 or 4214, do undergo reverse transcytosis, from the apical to the basolateral surface, when they are added to the apical medium. Our studies do not permit us to distinguish whether this occurs because 7125, 7221, and 7214 antibodies bind to the stem of pIgR that remains after cleavage, and are transported retrograde in this fashion, or whether they bind to intact pIgR at the surface to trigger a conformational change that permits the intact receptor to undergo retrograde transcytosis or, because they allow the intact receptor to resist cleavage and therefore increase its probability of being recycled.

The differences in trafficking patterns of the two classes of antibodies may have implications for their use in therapeutic complexes directed at the lung. An scFv cloned from 4121, incorporated into a fusion protein with α_1 -antitrypsin, is quite effective in delivering its payload to the apical surface of airway epithelial cells. In a culture model of well-differentiated human airway epithelial cells grown at the air-liquid interface, delivery of functional α_1 -antitrypsin is rapid, not blocked by a hundredfold molar excess of dIgA, and results in micromolar concentration of α_1 -antitrypsin in the apical medium (2). Moreover, when it is injected intravenously, the fusion protein is delivered preferentially (compared with free α_1 -antitrypsin) to the lumen of xenografts seeded with human airway epithelial cells and maintained subcutaneously in immunocompromised mice (3). Thus, pIgR binding reagents derived from 4121 antibody are quite effective in transcytotic delivery, *in vitro* and *in vivo*, as predicted from their trafficking pattern. However, the scFv derived from 4121,

when included in a gene transfer complex, guided gene transfer specifically to receptor bearing cells, but was inefficient (5). It is possible that much of the gene transfer complex was simply expelled from the cell, bound to SC, and was not retained in the cell long enough for the genetic cargo to escape. Because they linger longer inside the cell, we speculate that 7221, 7125, and 7214 antibodies will make more effective reagents for gene delivery, and because they can undergo transport in the retrograde direction, they may be appropriate carriers of therapeutic payloads from the apical to basolateral surface and into the interstitium.

Conflict of Interest Statement: S.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.B.D. is a coinventor of a U.S. Patent held by Case Western Reserve University (CWRU) that describes a method of delivery of therapeutic payload using fusion proteins directed at the polymeric immunoglobulin receptor. This patent has been sublicensed to Arizeke, Inc. P.B.D., as well as CWRU, has received royalty payments for this sublicense (\$13,500 to P.B.D.), and P.B.D. serves on the Scientific Advisory Board of Arizeke, for which she was compensated \$16,666 in 2004. P.B.D. is a coinventor on several other U.S. Patents held by CWRU aimed at gene transfer/gene therapy that have been licensed to Copernicus Therapeutics, Inc., a company of which she is a founding scientist. She has received no royalties from this license, though she does hold stock and options from this company, which are difficult to value since the company is not publicly traded. The best guess is that the holdings of P.B.D. and her immediate family are worth less than \$12,500. In 2002, she received sponsored research support for her laboratory from this company for \$100,000.

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