Polarized Transport of the Polymeric Immunoglobulin Receptor in Transfected Rabbit Mammary Epithelial Cells

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Abstract. A cDNA for the rabbit low M_r polymeric immunoglobulin (poly-Ig) receptor was expressed in an immortalized rabbit mammary cell line. The intracellular routing of the receptor and its cell surface expression was analyzed in stably transfected cells grown on permeable supports. Initially the cells formed a monolayer with no transmural electrical resistance. All monolayer cells expressed the poly-Ig receptor and cytokeratin 7 filaments characteristic of luminal mammary cells but absent in myoepithelial cells. Within 7 d in culture, the cells underwent cytodifferentiation and formed a bilayer with a transepithelial electrical resistance of ~500 $\Omega \times cm^2$. Upper layer cells formed tight junctions with adjacent cells and gap junctions with basal cells. Expression of the poly-Ig receptor and cytokeratin 7 was restricted to the cells from the upper layer. The kinetics of receptor biosynthesis and processing was similar to that reported for rabbit mammary gland and rat liver. The receptor was cleaved at the apical cell surface and release of secretory component into the apical medium occurred with a half-time of ~2 h. Selective cell surface trypsinization combined with pulse–chase experiments served to determine at which cell surface domain newly synthesized receptor appeared first. The receptor was digested with a half-time of ~60 min with trypsin present in the basolateral medium and 90 min with apical trypsin. These data are consistent with selective targeting of newly synthesized receptor to the basolateral surface. The results indicate that transcytosis of the receptor from basolateral to apical membrane in the presence or the absence of its ligand requires ~30 min. Cleavage of the receptor by endogenous protease is not concomitant with its appearance at the apical surface, but requires additional time, thus explaining the presence of intact receptor on the apical membrane.

THE (poly-Ig)[1] polymeric immunoglobulin receptor, which mediates transcytosis of polymeric-IgA antibodies across glandular or mucosal epithelia, plays a crucial role in mucosal immunity. The receptor allows the antibodies produced in the interstitial space by local plasma cells to reach the environment where they interact with and eventually cross-link or neutralize pathogens (12). It also stabilizes the antibodies. After cleavage of the receptor (54), the extracellular domain, called secretory component (SC), remains tightly bound to the antibodies rendering them resistant to proteolytic degradation (29).

The rabbit poly-Ig receptor has been cloned and sequenced (35), and its biosynthesis has been extensively studied in rat liver (57, 58, 63, 64), cultivated rat hepatocytes (38), rabbit mammary gland (53, 55), and in a human tumor cell line (32). The receptor is synthesized as a transmembrane glycoprotein (26, 34) which is terminally glycosylated in the Golgi complex in ~30 min (53, 63, 64). During its intracellular journey, the receptor is phosphorylated on its cytoplasmic tail (28, 55). The receptor binds ligand (polymeric IgA) at the basolateral cell surface (22). After endocytosis in coated pits, ligand-bound and unbound receptors are sorted and directed to the apical plasma membrane (8, 15, 22). During transcytosis or at the apical cell surface, the receptor is cleaved and SC released as secretory product whether or not it is bound to ligand (6).

A cDNA for the high M_r rabbit poly-Ig receptor has been expressed in the Madin-Darby canine kidney (MDCK) cell line in order to analyze the molecular mechanisms involved in the polarized delivery of the receptor (33). In MDCK cells the transfected receptor mediates vectorial transcytosis of IgA antibodies as well as antireceptor antibodies. No intact receptor is associated with the apical plasma membrane probably because the receptor is cleaved and selectively released in the apical medium (33). The cleavage location in the cell was not determined (33). Based on these data, it has been proposed that the receptor is sorted intracellularly and selectively targeted to the basolateral cell surface. This, however, was not supported by the biochemical detection of

1. *Abbreviations used in this paper*: MDCK, dog kidney cell line; poly-Ig, polymeric immunoglobulin; SC, secretory component.

newly synthesized poly-Ig receptor on the basolateral plasma membrane, as documented for the polarized transport of viral protein (17, 43, 46–48) and Na,K-ATPase in MDCK cells (7) and for the delivery of plasma membrane proteins in rat hepatocytes (2).

In this paper, we have expressed the low M_r rabbit poly-Ig receptor in an immortalized rabbit mammary cell line, using a pSV-2 expression vector. The mammary cells undergo cytodifferentiation in culture and form within 7–10 d a bilayer when cultivated on porous supports. All cells of early cultures express the poly-Ig receptor as well as cytokeratin filaments specific to mammary luminal cells. With time, expression of poly-Ig receptor and cytokeratin becomes restricted to the superficial cells of the bilayer. We have analyzed the kinetics of cell surface expression of newly synthesized receptors by combining biosynthetic labeling and selective cell surface trypsinization. We show that the receptor is selectively targeted to the basolateral cell surface. The receptor undergoes transcytosis in the absence of ligand. Receptor cleavage which occurs at the apical plasma membrane and not intracellularly is a rate-limiting step in receptor processing.

Materials and Methods

Reagents

L-^{35}S-Cysteine, α^{32}P-ATP, antihuman actin antibodies, and biotinylated antibodies were obtained from Amersham (UK). Leupeptin, antipain, pepstatin A, chloroquine, soybean trypsin inhibitor, human placental collagen (type IV), glucose-oxidase type II, and lactoperoxidase (bovine milk) were obtained from Sigma Chemical Co. (St. Louis, MO). Hygromycin B was purchased from Calbiochem AG (Luzern, Switzerland). TPCK-treated trypsin was from Worthington Biochemical Corp. (Freehold, NJ) and PMSF from Serva Fine Biochemicals Inc. (Heidelberg, FRG). The enzymes for cDNA cloning, mouse anticytokeratin 7 (67), and antivimentin mAbs were from Boehringer Mannheim Biochemicals (FRG). RNase H was from Enzo Biochem, Inc. (NY), and RNasin from Promega Biotec (Madison, WI). dTP and the large fragment of polymerase 1 (Klenow fragment) were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). Sequenase was purchased from Biochem. Corp. (Cleveland OH). Transwell plates (0.22 μm^2) were from Costar Data Packaging Corp. (Cambridge, MA). Urea was obtained from Schwarzmann Biotech (Cleveland, OH). Dimeric monoclonal IgA antibodies were prepared according to Weltzin et al. (71).

Cloning of the Poly-Ig-Receptor and Insertion of Its cDNA into an Expression Vector

Cytoplasmic RNA was prepared from the mammary gland of lactating New Zealand rabbits according to Suard et al. (61) and the poly $(A)^+$ RNA was purified according to Schibler et al. (51). The poly$(A)^+$ RNA, enriched in receptor-translatable activity by size fractionation on a 5–20% linear sucrose gradient, served as a template for the synthesis of cDNA. The first cDNA strand was synthesized according to Wahli et al. (70) and the second strand according to the DNA polymerase I-RNase H method (19). The double-stranded and dC-tailed cDNA was fractionated on a 1% low melting agarose gel and cDNAs with a size ranging between 1.5 and 7 kb were eluted and annealed to dG-tailed pBR322 (42). DH1 cells were transformed with the annealed DNA (21). Transformed bacteria were lysed and the DNA was transferred onto nitrocellulose filters (66) and probed with two synthetic oligonucleotides (corresponding to nucleotides 186–206 and 2421–2437 of the published sequence (35) labeled with T4 polynucleotide kinase and α^{32}P-ATP (18). The cDNA clones hybridizing to both probes were further characterized by hybrid-selected translation (44). pSC-1 with a 2.95-kb insert and pSC-11 with a 3.5-kb insert were fragmented by restriction enzymes and sequenced by the method of Sanger et al. (49). Based on the sequence data, pSC-1 corresponds to the low M_r from of the poly-Ig receptor with deletion of domains 2 and 3 (10), while pSC-11 encodes the high M_r form. The cDNA for pSC-1 was introduced into the expression vector pSV-2 (59). The construct is referred to as pSV2-SC1.

Northern Blot Analysis

RNA from untransfected rabbit mammary cells grown on plastic was extracted by the citric acid method (69). The glyoxylated RNA, separated by electrophoresis and transferred to nitrocellulose filters, was hybridized to nick-translated cDNA probes from pSC-1, pCasein β (50), and pWAP-1 (11) with specific activities of 0.2–1.0 $\times$ 10^8 cpm/μg (30).

Transfection of Rabbit Mammary Cells

Immortalized mammary cells (passage 17) were grown to confluency in petri dishes (10 cm^2). The medium was changed 3 h before transfection. The cells were transfected by the calcium phosphate coprecipitation method (72) with 9 μg pSV2-SC1, 150 ng pX343, a plasmid carrying the hygromycin resistance gene (3), and 12 μg of salmon sperm carrier DNA. The cells were then incubated for 3 h at 37°C and for another 4 h in the presence of 0.5 mg chloroquine (16). The cells were treated for 3 min at room temperature with 10% glycerol in PBS, washed several times in PBS, and cultivated for 2 d in serum-free medium. The cells were then grown for 3 wk in serum-free medium containing 120 μg/ml of Hygromycin B. The medium was changed twice a week. Cell clones resistant to hygromycin were isolated, amplified, and tested for poly-Ig receptor expression. The positive clones were subcloned twice by limiting dilution.

Cell Culture Conditions

Transfected mammary cells were cultivated in serum-free medium as described elsewhere (14). The cells were plated at 3 $\times$ 10^4 cells/cm^2 on plastic and at 3 $\times$ 10^5 cells/cm^2 on Transwell filters (0.45 μm, 4.7 cm^2), coated with collagen type I, III, or IV (60). Transepithelial potential difference and short circuit current were measured in modified Ussing chambers (41).

Biosynthetic Labeling

Transwell filters containing confluent transfected mammary cells were washed three times with cysteine-free medium and incubated 30 min in the same medium. The cells were pulsed with 200 μCi (1 mCi/ml) of ^{35}S-cysteine. At the end of the pulse, the cells were washed three times with medium containing 10 mM cold cysteine and then transferred into serum-free medium and chased for the indicated time. At the end of the chase, the cells were washed three times with ice-cold PBS. Lysis buffer (10 mM Tris-HCl, pH 7.4, 1% NP-40, 0.4% deoxycholate, 5 mM EDTA, 1 mM PMSF, and 5 mg/μl leupeptin, antipain, and pepstatin) (0.5 ml) was added and the cells were scraped off the filter using a rubber policeman. The cell lysate was vortexed for 1 min, centrifuged 3 min, and the supernatant was either processed or stored at −70°C. 10-μl aliquots from each sample were removed, TCA precipitated, and counted by liquid scintillation. Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Richmond, CA).

Cell Surface Radioiodination

Transfected mammary cells grown on collagen-coated filters for 7–10 d were washed three times with ice-cold PBS. 1 ml of ice-cold labeling mixture (1 mCi $Na^{125}I$ carrier free, 0.1 mg/ml of lactoperoxidase and glucose oxidase diluted 1:5,000 in PBS) were added to the apical or the basolateral side of the chamber and the filters were incubated for 30 min on ice. The reaction was started by adding 20 mM glucose. After 15 min, 20 μl of 20% NaN_3 was added to stop the reaction. The filters were extensively rinsed with cold PBS and processed as indicated under biosynthetic labeling (23).

Selective Cell Surface Trypsinization

Transfected mammary cells grown to confluency on filters (electrical resistance ~500 $\Omega \times cm^2$) were washed twice with PBS and incubated at 4°C or at 37°C with 2 ml of serum-free medium containing 100 μg/ml of L-(tosylamido 2-phenyl) ethyl chloromethylketone-trypsin added either to the apical, the basolateral, or both sides. When trypsin was added to one side only, soybean trypsin inhibitor at 200 μg/ml was added to the opposite side (31). Potential difference and short circuit current were monitored during trypsinization. In pulse-chase experiments, trypsin was added after the

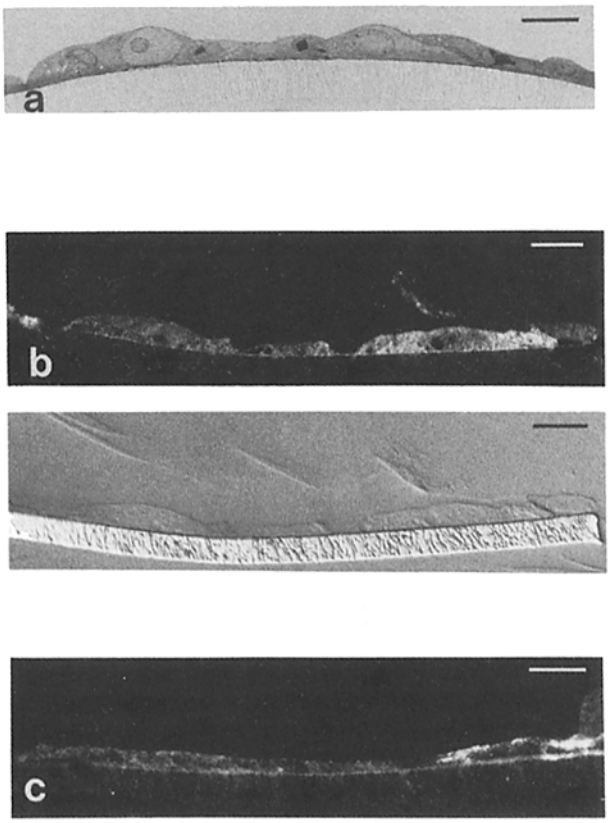

Figure 1. Light micrographs of the transfected mammary cells cultured for 2 d. Transfected mammary cells, cloned twice by limiting dilution, were plated at 1.5×10^5 cells/cm^2 on collagen type IV-coated polycarbonate filters and cultivated for 2 d in a chemically defined medium. The cells were fixed in 0.5% glutaraldehyde–3% paraformaldehyde, embedded in Epon (*a*) or processed for immunocytochemistry (*b* and *c*) as described in Materials and Methods. (*a*) Section stained with toluidine blue. The cells form a monolayer. (*b*) 0.5-μm frozen section incubated with an anticytokeratin 7 antibody. All cells in the monolayer express cytokeratin 7. (*c*) 0.5-μm frozen section incubated with mAb antitail. The poly-Ig receptor is found in all cells. A mouse IgG_1 mAb directed against the amphibian Na,K-ATPase β subunit served as a control. The cells remained unlabeled. Bars, 10 μm.

pulse and maintained during the chase. In surface radioiodination experiments, trypsin was added at 4°C and the filters were incubated for 30 min at 4°C. Before lysis of the cells, filters were washed three times with PBS containing soybean trypsin inhibitor.

Immunoprecipitation

Samples containing the same amount of protein were boiled 5 min in the presence of 3% SDS (final concentration) and diluted 10-fold with dilution buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100). Immunoprecipitation was performed using Sepharose-immobilized affinity-purified antirabbit SC antibodies or anti-poly-Ig receptor mAbs (56) after a protocol previously described (53). The immunoprecipitated labeled material was separated on 5–13% SDS-polyacrylamide gels. The autoradiograms or fluorograms were scanned by densitometry at 515 nm.

Immunocytochemistry and Electron Microscopy

Cells grown on filters were fixed in 3% glutaraldehyde, 0.1 M Na cacodylate, pH 7.4, for 2 h at room temperature, and embedded in Epon 812. The sections were examined in a Phillips EM 400 electron microscope. For immunocytochemistry the cells were fixed in 3% paraformaldehyde, 0.5% glutaraldehyde for 4 h at 4°C. 0.5-μm frozen sections were prepared according to Tokuyasu (68) and incubated with the primary antibody (anti-poly-Ig receptor antibodies) at 0.1 mg/ml, followed by a biotinylated second antibody and streptavidin-phycoerythrin.

Results

Expression of the Poly-Ig Receptor in Immortalized Mammary Cells

The mammary cell line, which was used in the transfection experiments, was established by microinjecting linearized SV-40 DNA into the nucleus of primary mammary secretory cells derived from the mammary glands of midpregnant rabbits (14). The primary cells, at the time of microinjection, retained their differentiated phenotype and were able to express milk protein genes in response to hormonal stimulation (20, 62). The immortalized cells lost their capacity to express the tissue-specific genes, as indicated by the absence of accumulation of β casein, whey acidic protein (14), and poly-Ig receptor mRNAs (data not shown). To express the poly-Ig receptor in these cells, mammary cells were cotransfected with pSV2 expression vectors (59) containing the cDNA coding for the low M_r rabbit poly-Ig receptor and the hygromycin resistance gene (3). The amount of poly-Ig receptor expressed in the hygromycin-resistant clones was determined by immunoprecipitation of ^{35}S-cysteine–labeled cells using polyclonal anti-SC antibody. In two clones, 2 and 5, the amount of biosynthetically labeled receptor recovered after a 4-h pulse corresponded to 0.1 and 0.025% of the total labeled proteins, respectively.

The Transfected Mammary Cells Undergo Cytodifferentiation When Grown on Porous Supports

The transfected mammary cells, when plated at low density (1.5×10^5/cm^2) on collagen-coated polycarbonate filters, formed after 1 or 2 d in culture a monolayer (Fig. 1 *a*) with tight junctions, but no measurable transepithelial electrical resistance (14). Within 7 d, the cells differentiated and formed a bilayer (Figs. 2 *a* and 3). This coincided with the appearance of a 200–1,000 $\Omega \times$ cm^2 transepithelial resistance (see Fig. 5). The upper layer consisted of flat epithelial cells sealed at their apex by tight junctions (Fig. 3). The lower layer was composed of basal cells that lacked tight junctions, but which established gap junctions with the upper cells (Fig. 3, *b* and *c*). The same results were obtained when the cells were subcloned twice by limiting dilution, indicating that the appearance of two morphologically distinct cell types in culture is not due to the presence of a contaminating cell type in the initial culture. The organization of the cells in the bilayer is reminiscent of the architecture of the alveoli in the mammary gland with the typical secretory and myoepithelial cells, although the shape of the cultivated cells differed from that seen in situ. Secretory and myoepithelial cells are known to express distinct cytokeratin filaments (1, 27, 65). Therefore, we analyzed the distribution of cytokeratins in cells from early (2-d) and late (7–10-d) cultures with an mAb specific for cytokeratin 7, a filament present in luminal but not in myoepithelial cells (1). Cytokeratin 7 was detected in all cells in the early cultures (Fig. 1 *b*), but only in the cells of the upper layer in the late cultures (Fig. 2 *b*). In contrast, actin

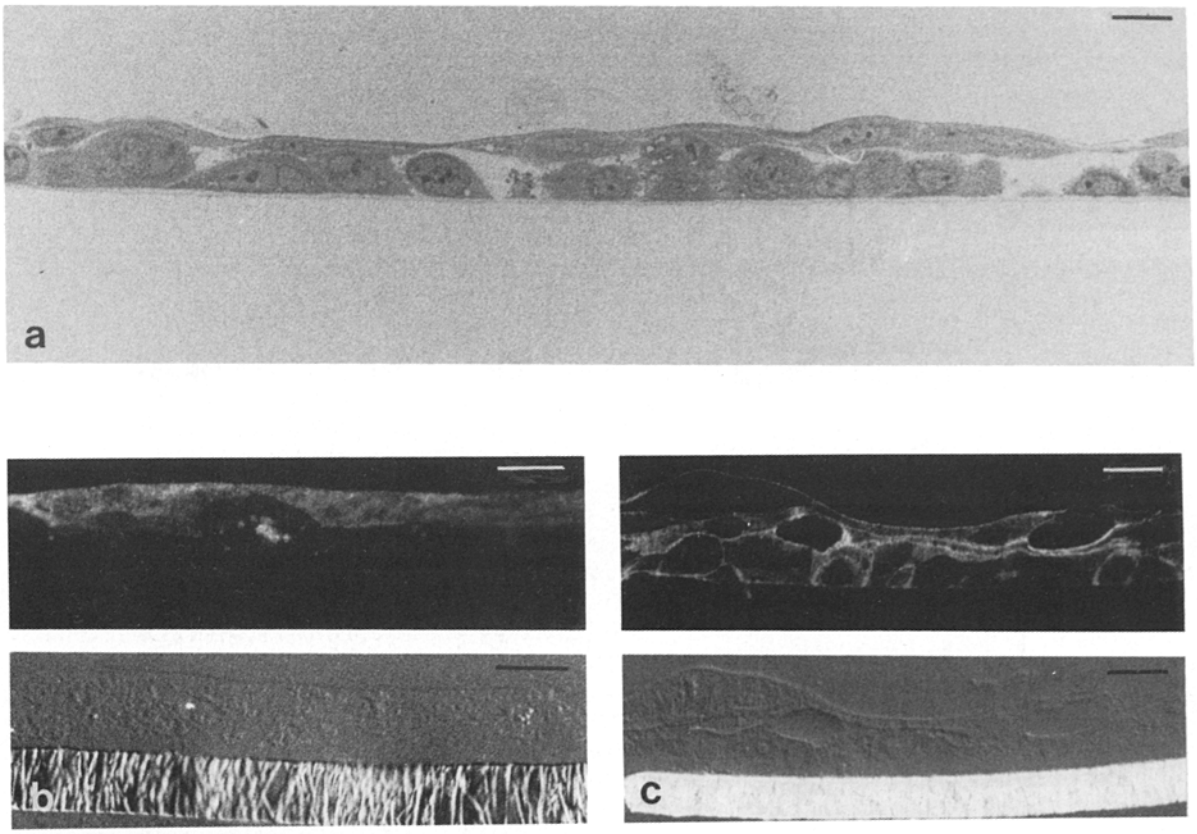

Figure 2. Light micrographs of transfected mammary cells cultured for 10 d. Cloned cells, plated as described in Fig. 1, were cultivated for 10 d and fixed as described above. (*a*) Section stained with toluidine blue. Two cell layers can clearly be seen. The upper cell layer forms a continuous sheet while the basal cells remain separated from each other. (*b*) 0.5-μm frozen section incubated with an anticytokeratin 7 antibody. Labeling is restricted to the upper cells. (*c*) 0.5-μm frozen section incubated with antiactin antibody. Both the upper and basal cells are labeled. The label accumulates at the periphery of the cells beneath the plasma membrane. Same controls as in Fig. 1. Bar, 10 μm.

filaments were evenly distributed in both upper and basal cells (Fig. 2 *c*) and vimentin filaments were not detected in either cell type (data not shown).

The progressive development of transepithelial electrical resistance after plating of cells on type IV collagen reached maximal levels of 200–1,000 $\Omega \times cm^2$ by 7–10 d for both of the clonal cell lines (see Fig. 5). These values were higher than those obtained when cells were grown on type I or III collagen substrates (100–500 $\Omega \times cm^2$). The tightness of the cell layers was also assessed by measuring the diffusion of ^{14}C-inulin from apical to basolateral medium and conversely. The amount transported to each side was negligible. For all the subsequent experiments, we selected clones with the highest transepithelial resistance and level of poly-Ig receptor expression (clone 2).

The Poly-Ig Receptor Is Expressed in Cells of the Upper Layer

Because the transfected mammary cells were able to differentiate in vitro from a monolayer to a bilayer with cells expressing distinct phenotypes, we analyzed morphologically the distribution of the poly-Ig receptor during differentiation. Mammary cells, plated at 3×10^5 cells/cm^2 and cultivated for 2 or 10 d were fixed and processed for immunocytochemistry. Frozen sections were incubated with anti–poly-Ig receptor antibodies [antitail and an antidomain I mAb, or anti-SC polyclonal antibody]. The three antibodies, which recognize different forms of the receptor (56), labeled all cells in the monolayer at day 2 (Fig. 1 *c*), but exclusively the cells from the upper layer at day 10 (Fig. 4, *a–d*). At the periphery of the apical cells, the cytoplasm forms a thin layer that makes it difficult to resolve the two cell surface domains (Fig. 4, *a* and *c*). At higher magnification, however, fluorescence was associated with both the apical and basal plasma membranes, as well as with intracellular structures (Fig. 4 *d*).

The Poly-Ig Receptor Is Associated with Both Cell Surface Domains of Mammary Cells

To determine the amount of receptor associated with each cell surface domain, apical or basolateral plasma membrane proteins were selectively radioiodinated using an enzyme-mediated iodination protocol (23) and then immunoprecipitated with the anti-SC polyclonal antibody or with preimmune antibody as a control (Fig. 6). An 85-kD protein, which corresponds to the intact receptor, was recovered from both apical and basolateral membranes. One third of the total radioiodinated receptor was associated with the apical plasma membrane (Fig. 6). Accessibility of the basolateral membrane of the upper layer cells to the enzymes mediating radioiodination was assessed by light microscope autoradi-

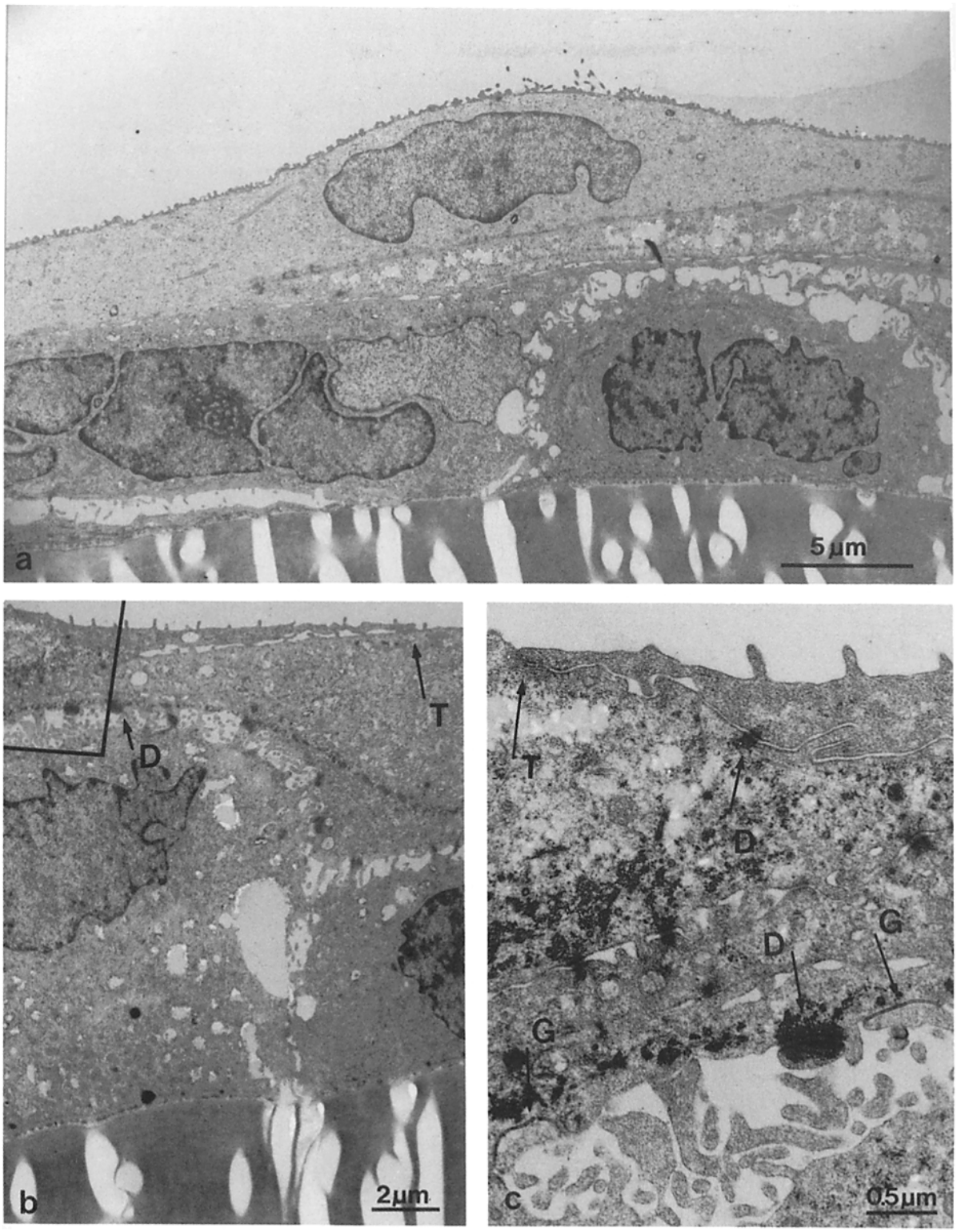

Figure 3. Electron micrographs of transfected mammary cells cultured for 10 d. The transfected cells form a bilayer (*a* and *b*). Microvilli are associated with the luminal membrane of the upper cells and tight junctions (*T*) seal their apices (*b* and *c*). The upper and lower cells are connected by desmosomes (*D*) and gap junctions (*G*). The area of *b*, enlarged in *c*, is outlined.

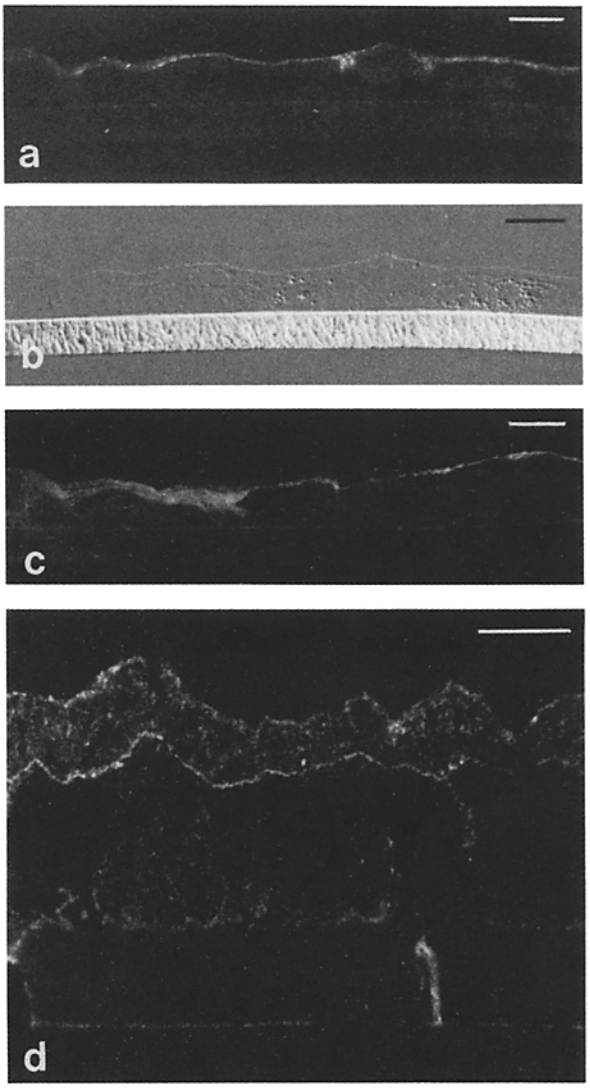

Figure 4. Immunocytochemical localization of the poly-Ig receptor in transfected mammary cells cultivated for 10 d. 0.5-μm frozen sections incubated with anti-poly-Ig receptor antibodies (*a* and *b*, mAb anti-domain I; *c*, mAb antitail; *d*, polyclonal antibody anti-SC). With all three antibodies the labeling is restricted to the upper cells, which can form very thin layers (*a–c*). In areas around the nucleus, the two cell surfaces are discriminated and the label is found associated with both the apical and basal plasma membranes. (*d*) Bars, 10 μm.

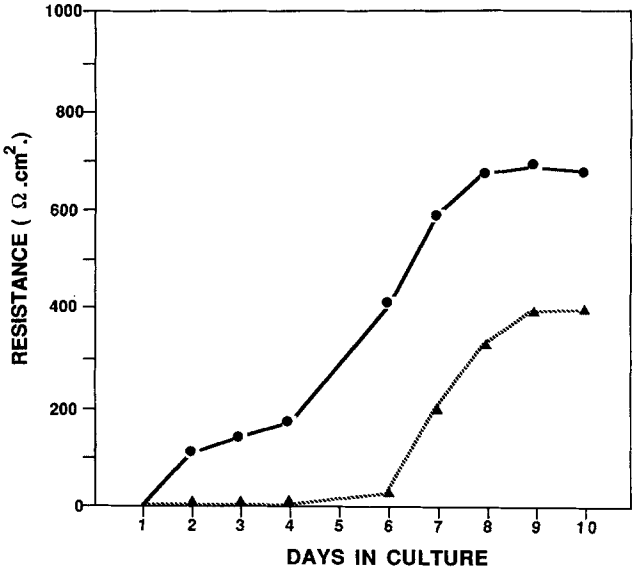

Figure 5. Transepithelial electrical resistance. Transfected mammary cells (clone 2: ● or clone 5: ▲) were plated at 1.5×10^5 cells/cm^{-2} on filters coated with collagen type IV and cultivated for 10 d. The transepithelial resistance was determined each day.

ography of cultures labeled from the basal side. Autoradiographic grains were associated with the basolateral cell surface of the upper cells and the labeling density was similar to that found at the surface of the basal cells (data not shown).

Processing and Intracellular Routing of the Poly-Ig Receptor in Transfected Mammary Cells

Poly-Ig Receptor Biosynthesis. The kinetics of poly-Ig receptor biosynthesis and processing were analyzed by pulse–chase experiments of subcloned transfected mammary cells grown on collagen IV-coated filters. The cells were labeled for 30 min with ^{35}S-cysteine added to the basal medium and chased for up to 4 h. At each time point, the cells were lysed and the labeled proteins were immunoprecipitated with antitail or anti-domain I mAbs. The apical and basolateral media were also collected and immunoprecipitated with the anti-domain I mAb. After the pulse, a coreglycosylated endoglycosidase H-sensitive protein was recovered from cell lysates, which migrated as a 78-kD band on SDS-PAGE (Fig. 7 *A*), which was chased in ~30 min into an 80-kD endoglycosidase H-resistant polypeptide (Fig. 7 *A*). With an additional 30 min, there was a further shift in mobility observed with the antitail mAb, but not with the anti-domain I mAb. This change in mobility on SDS-PAGE is due to the phosphorylation of the receptor (28, 55). Some phosphorylated receptor forms are not recognized by the anti-domain I mAb (Schaerer E., S. Frütiger, G. Huges, C. Tallichet, and J. P. Kraehenbuhl manuscript in preparation).

A protein of 55 kD, whose M_r corresponds to low M_r SC, was recovered from the apical medium, but not from the basal medium (Fig. 7 *B*). The kinetics of release was analyzed by scanning the gels and correcting for the number of cysteine residues present in the receptor and SC. During the chase period, half of the newly synthesized receptor was cleaved as assessed by the loss of immunoprecipitable la-

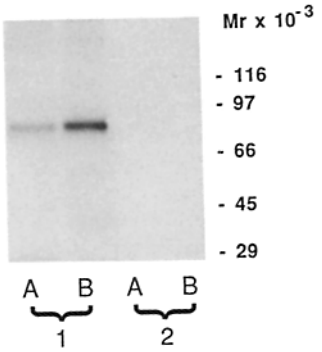

Figure 6. Selective radioiodination of transfected mammary cells. The cells were grown on collagen-coated filters for 8 d and then radioiodinated either from the apical (*A*) or the basolateral (*B*) side at 4°C, lysed, and immunoprecipitated with the anti-SC polyclonal antibody (*1*) or with a preimmune serum (*2*).

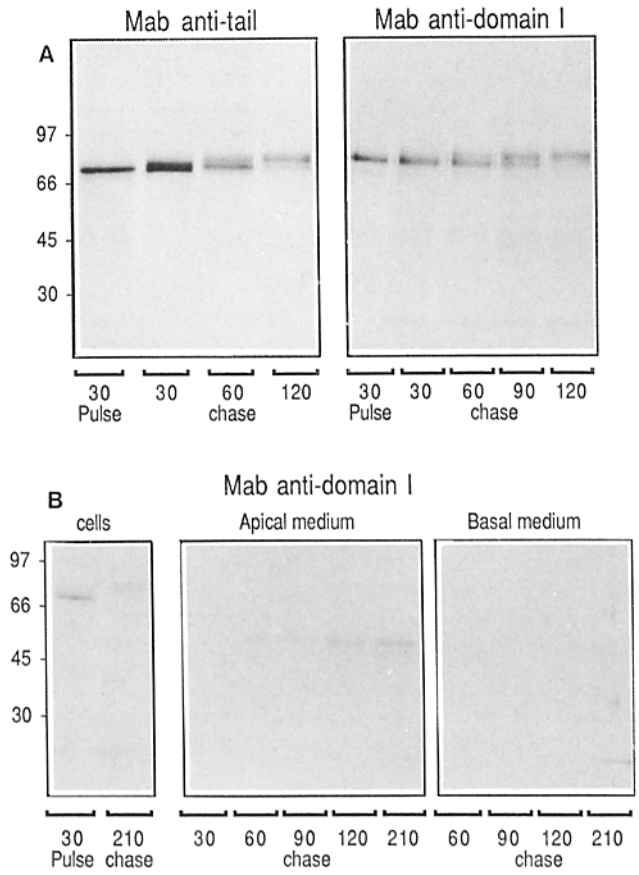

Figure 7. Processing of the poly-Ig receptor in transfected mammary cells. Transfected mammary cells were labeled with ^{35}S-cysteine for 30 min followed by a chase with a 1,000-fold excess of unlabeled cysteine for 30–210 min. (*A*) The cells were lysed and immunoprecipitated either with the mAb antitail or the mAb anti-domain I and analyzed on a SDS-PAGE by fluorography. (*B*) The apical and the basolateral media were collected and immunoprecipitated with the mAb anti-domain I.

beled receptor in the chase sample (Fig. 7 *B*). Half of the cleaved receptor was recovered from the apical medium, and <2% from the basal medium. Since only half of the cleaved receptor appeared as SC in the apical medium, we tested whether the remaining fraction of SC remained associated with the cells as a result of cleavage in an intracellular compartment. There was no 55-kD SC detected in the cell lysate (Fig. 7 *A*) when the immunoprecipitation was carried out with the anti-domain I mAb indicating that cleavage occurred exclusively at the cell surface. Some cleaved receptor could bind nonspecifically to the walls of the Transwell cups.

Transcytosis. Transcytosis was determined by measuring the rate of transport of biosynthetically labeled dimeric IgA and radioiodinated anti-domain I mAb, from the basolateral to the apical medium and vice versa. First, we determined the binding constant of radioiodinated IgA dimer for the receptor expressed at the surface of the transfected cells. Binding experiments were performed at 4°C. The association constant estimated from Scatchard plot analysis was 5×10^8 M^{-1}, in good agreement with published values (25). The rate of transcytosis of IgA dimer (10 pM) from the basolateral to the apical side was four times higher than in the opposite direction (Fig. 8 *A*), which corresponds to ~5,000 molecules transported per cell and per min. The rate of transport of radioiodinated anti-domain I mAb from the basal to the apical side was similar to that of IgA dimer (Fig. 8 *B*). In the presence of a 100-fold excess of unlabeled antibody the rate of transport from the basal to the apical site was in the same range as that of labeled antibody in the opposite direction, suggesting nonreceptor-mediated transcytosis.

Cell Surface Targeting of the poly-Ig Receptor

The poly-Ig receptor was found associated with both surface domains of transfected cells as revealed by immunocytochemistry (Fig. 4) and by immunoprecipitation of radioiodinated plasma membrane proteins (Figs. 6 and 9 *B*). To determine whether newly synthesized receptor was randomly inserted into the plasma membrane or selectively delivered to one cell surface domain, we combined a pulse–chase protocol with selective cell surface trypsinization. To perform such an experiment, it was essential to show that trypsin cleaved the receptor efficiently without perturbing the integrity of cell membranes and junctions. We first tested the tightness of the cell layer after apical or basolateral trypsinization by monitoring transepithelial electric resistance. There was a rapid 20–30% increase in resistance when trypsin was added to the apical medium which was maintained during a 2-h time period. This increase in electrical resistance is probably due to the inactivation by trypsin of apical conductive ion channels. In contrast, trypsin present in the basal medium caused a 60–80% drop in resistance. The residual resistance of 200 $\Omega \times cm^2$, however, remained stable for 2 h (Fig. 9 *A*). Cleavage of the receptor on the side in contact with trypsin was complete after 30 min at 4°C or 10 min at 37°C. On the contralateral side, however, the receptor was fully protected (Fig. 9 *b*).

To detect on which cell surface the newly synthesized receptor appeared first, mammary cells were pulsed for 30 min with ^{35}S-cysteine and then chased for various times in the presence of trypsin (100 μg/ml) in the apical or the basal medium. At each time point, cells and media were collected and immunoprecipitated with antitail mAb (cell lysate) or anti-domain I mAb (media). The immunoprecipitation pattern of a typical experiment is illustrated in Fig. 10. Quantitation was obtained by scanning the fluorograms of three independent experiments. (Fig. 11). In control experiments the half-time of receptor cleavage by endogenous protease was ~120 min, including the 30-min pulse and the 90-min chase time (Fig. 10 *A*). The half-time was reduced by ~30 min when trypsin was added to the apical side and by 60 min with trypsin in the basal medium.

Discussion

The poly-Ig receptor, which mediates transcytosis of mucosal antibodies across glandular and mucosal epithelia, is known to follow a complex pathway in the cell. Its processing and routing has been analyzed in a variety of epithelial cell types (22, 32, 38, 53, 54, 57, 58, 63, 64) including transfected MDCK cells (33, 36, 37). At present, the mechanisms involved in the intracellular sorting and targeting (13) of epithelial plasma membrane proteins including the poly-Ig receptor remain poorly understood. It is not known whether

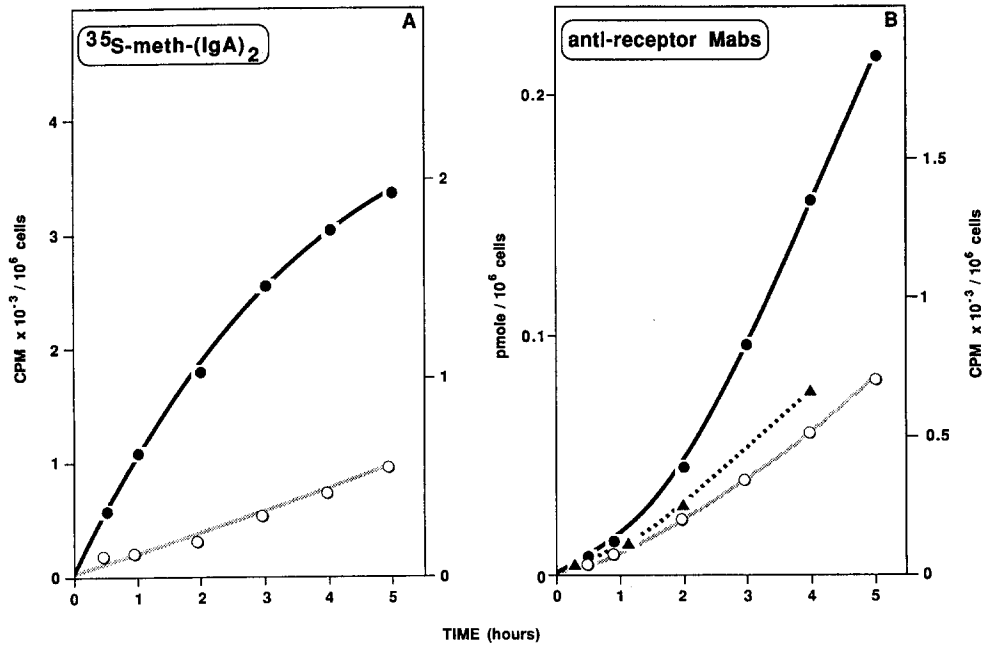

Figure 8. Transcytosis of dimeric IgA or antireceptor antibodies. (*A*) Transfected mammary cells (clone 2) were cultivated for 10 d on collagen-coated filters. 35 μg of ^{35}S-methionine-labeled dimeric IgA antibodies (6 × 10^4 cpm/mg) in 1 ml of medium were added to the apical or the basal side. The medium on the opposite side was collected at the indicated times. The proteins were precipitated with 10% TCA and counted by liquid scintillation. (●) Dimeric IgAs were added on the basal side and counted on the apical side. (○) Dimeric IgAs were added on the apical side and counted in the basal medium. (*B*) ^{125}I-labeled mAb anti-domain I antibodies (3.4 × 10^6 cpm/mg) were added either to the apical or the basolateral medium of transfected mammary cells (clone 2) grown for 10 d on filters. The apical or the basolateral medium was collected at increasing time intervals. The proteins were precipitated with 10% TCA and counted. (●) ^{125}I-mAb anti-domain I added to the basolateral side and counted in the apical medium. (○) ^{125}I-mAb anti-domain I added to the apical side and counted in the basolateral medium. (▲) ^{125}I mAb anti-domain I with a 100-fold excess of unlabeled anti-domain I mAb added to the basal side.

such mechanisms are identical in all epithelial cell types. For secretory proteins, for instance, it has been established that polarized transport of the same recombinant protein varies in diverse epithelial cell types (17, 24, 45). To define the rules that govern sorting and targeting of epithelial plasma membrane proteins one has to compare the routing of the same membrane proteins transfected in different epithelial cell types. Therefore, we have expressed the poly-Ig receptor into an immortalized rabbit mammary cell line (14), and compared its processing and polarized transport to that reported in transfected MDCK cells (33, 37).

Immortalized Mammary Cells Undergo Cytodifferentiation in Culture

The rabbit mammary cell line used in this study does not express milk-specific genes (14) nor the poly-Ig receptor, suggesting that immortalization inhibited the terminal differentiation of these cells in vitro or that ductal rather than alveolar secretory cells were immortalized upon microinjection of SV40 DNA. In culture, immortalized mammary cells initially form a monolayer with tight junctions but low transepithelial electrical resistance (14). Increase in transmural resistance parallels the development of a bilayer. The two cell types which constitute the bilayer differ functionally and structurally. The upper cells have features of luminal mammary cells, including expression of cytokeratin 7 (27, 65, 68) and the presence of tight junctions separating the cell surface into apical and basolateral domains. The basal cells, which do not express cytokeratin 7 nor vimentin, could possibly represent a predifferentiated form of myoepithelial cells. It has been proposed that the phenotype of the myoepithelial or basal cells is fully expressed when the cells are attached to a basal lamina and covered by a layer of luminal cells (40). In our study, the morphology of the two cell types differs from that observed in situ. Analysis of the polarized transport of the poly-Ig receptor requires tight cell layers, which are obtained by growing the cells on collagen-coated filters. On rigid supports, primary mammary cells exhibit the same morphological features (20) as immortalized cells (Figs. 1 and 2). It is known that for primary mammary cells drastic changes both in cell shape and function follow the formation of floating gels by releasing attached collagen gels (20, 62). The same changes in cell shape but not in function were observed when immortalized mammary cells were grown on floating collagen gels. Under such conditions the organization of the bilayer was reminiscent of the mammary gland.

Transfection of the immortalized mammary cells did not alter their capacity to undergo cytodifferentiation. Expression of the poly-Ig receptor paralleled that of cytokeratin 7. In immature cultures the receptor was found in all cells forming the monolayer, while in the bilayer expression was restricted to the upper cells. It is surprising that expression in transfected mammary cells of a cDNA driven by SV40 promotor and enhancer elements is regulated in a cell-specific manner. It is known, however, that tissue-specific transacting transcriptional factors interact selectively with distinct *cis* elements of the SV40 promotor (9, 39, 52), and the distribution of such factors may vary from one mammary cell type to the other.

Polarized Transport of the Poly-Ig Receptor in the Upper Cells of the Bilayer

From earlier studies in whole animals or in perfused organs, it was recognized that transport of polymeric immunoglobu-

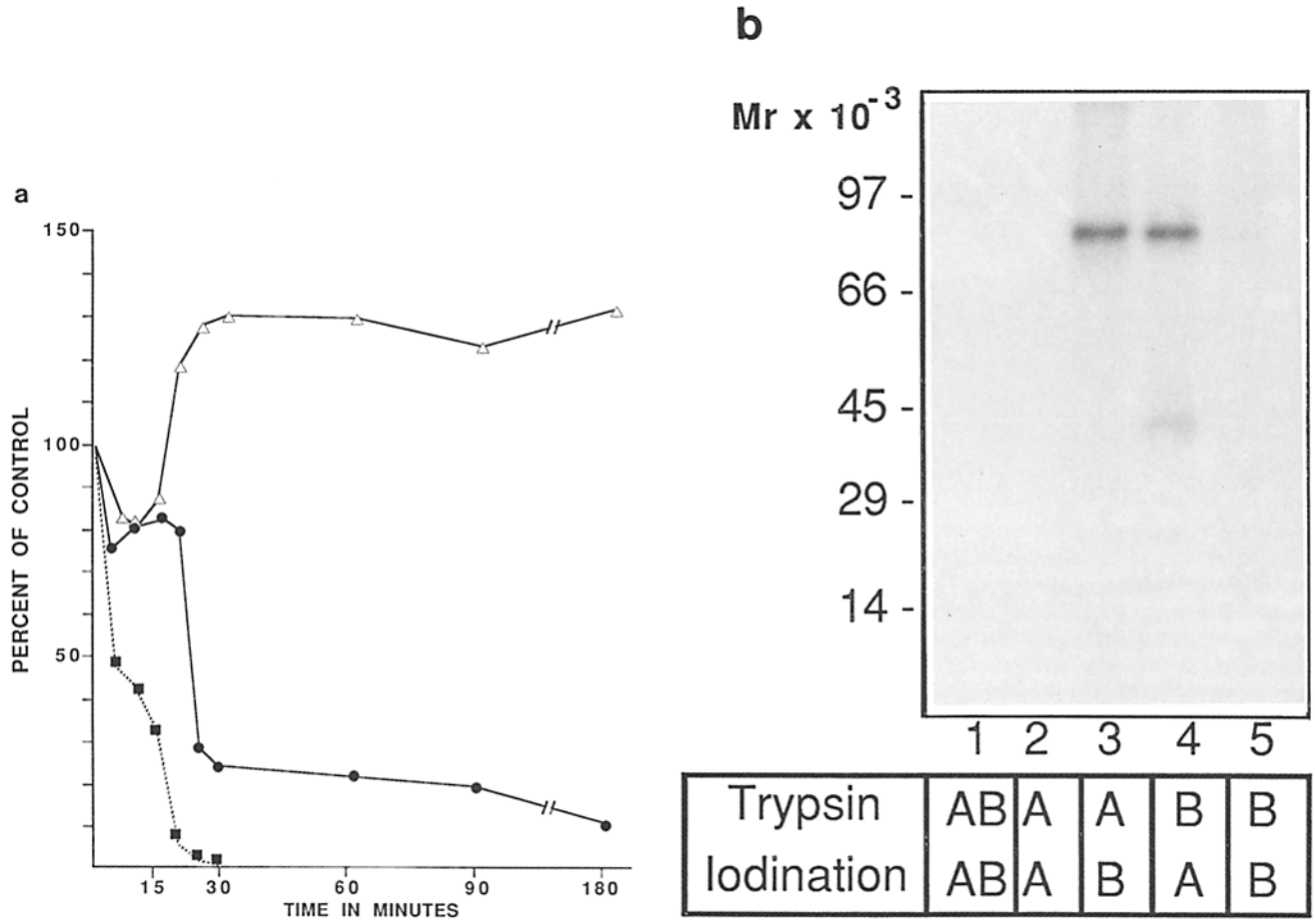

Figure 9. Selective cell surface trypsinization of transfected mammary cells. (*a*) Effect of trypsinization on the transepithelial electrical resistance of transfected mammary cells grown for 10 d on collagen-coated filters. Trypsin (100 mg/ml) was either added to the apical (△), the basal (●), or to both sides (■). The transepithelial resistance of the cells before addition of trypsin was 625 ± 16 Ω × cm² (n=6). (*b*) Cell surface radioiodination of transfected mammary cells (clone 2) grown on filters for 10 d. Trypsin (100 mg/ml) was added either to the apical (*A*) or the basolateral (*B*) side for 30 min at 4°C before cell surface radioiodination from the apical or the basolateral side. The cells were lysed and immunoprecipitated with mAb anti-domain I. The immunoprecipitates were resolved on a 5–13% SDS-polyacrylamide gel, and the gels were processed for autoradiography. The lower M_r band seen in lane *3* is also present in control experiments. We do not know whether the polypeptide is a receptor degradation product or an unrelated protein associated with the basolateral poly-Ig receptor.

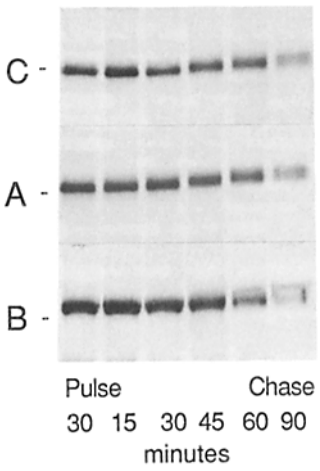

Figure 10. Selective cell surface trypsinization of biosynthetically labeled transfected mammary cells. Transfected mammary cells (clone 2), grown on collagen-coated filters for 10 d, were pulsed with ^{35}S-cysteine for 30 min and chased for different time in the absence (*C*) or presence of trypsin, added to the apical (*A*) or the basolateral (*B*) side. Immunoprecipitation with the mAb antitail antibody. The immunoprecipitate was resolved on 5–13% SDS-polyacrylamide gel, and the gels were fluorographed.

lins, mainly IgAs, is vectorial (4). Indeed SC, the ectodomain of the poly-Ig receptor, is bound to its ligand only in secretions and not in the interstitial fluid where the ligand is produced. This implies that (*a*) the receptor has to be cleaved during transcytosis or at the luminal cell surface to release SC or SC–ligand complexes into secretions (26); (*b*) the protease which cleaves the receptor has to be asymmetrically distributed in the cell (38); and (*c*) the receptor is likely to serve only once unless the cleaved receptor remains bound to the membrane anchor (54).

It has been postulated and generally accepted that unidirectional transport of polymeric IgA, as well as vectorial secretion of SC, is a direct consequence of intracellular sorting and selective surface targeting of the receptor. Accordingly, all newly synthesized receptors are initially directed to the basolateral plasma membrane in order to in-

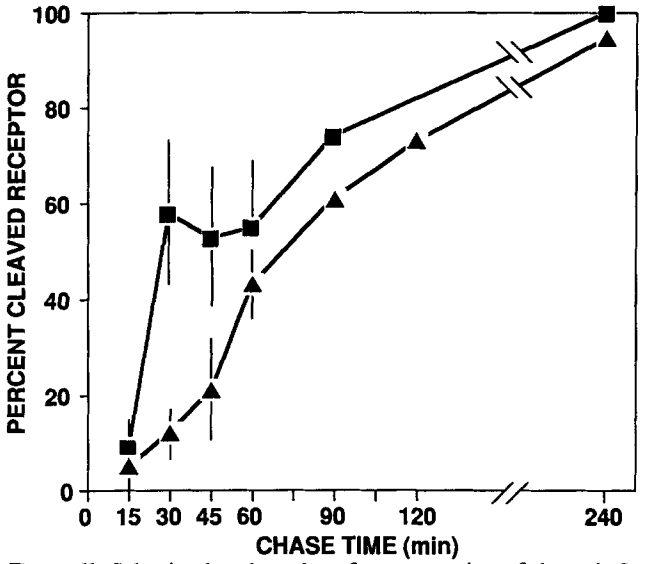

Figure 11. Selective basolateral surface expression of the poly-Ig receptor. The fluorograms of Fig. 10, as well as those of two additional experiments were scanned and the mean values are given with the SEM. The amount of receptor that disappears after a 30-min pulse and a 4-h chase period is taken as 100% of receptor cleavage and all points were normalized to this value. (▲) Apical trypsinization. (■) Basal trypsinization.

teract with the ligand. This model predicts that any free SC that is detected in secretion represents a receptor that failed to bind the ligand at the basolateral cell surface. Random cell surface insertion of the receptor, however, could also explain the presence of free SC in secretion. In this model, vectorial transport of the ligand is due to the asymmetric distribution of the receptor-cleaving protease restricted to the apical plasma membrane or vesicles. Only those receptors that are inserted into the basolateral membrane are able to interact with the ligand and mediate its transcytosis.

To address the question of random versus selective cell surface insertion of the poly-Ig receptor, there is a need for cell culture systems in which each cell surface domain is freely accessible to manipulation. The use of permeable supports presents the opportunity to identify molecules expressed at the cell surface and to tag newly synthesized plasma membrane proteins when they appear at the cell surface. First, we have determined that biosynthesis and processing of the receptor in transfected mammary cells grown on collagen-coated filters mimics that reported in normal rabbit mammary gland (53) and rat liver (57, 63, 64). Second, we have demonstrated biochemically that newly synthesized receptors are initially directed to the basolateral surface where they arrive in ~1 h. Whether the receptor as it leaves the Golgi complex expresses a dominant signal for basolateral delivery, or follows a basalateral default pathway by masking a dominant signal for apical transport, as proposed for liver plasma membrane proteins (2), remains to be established. If sorting and addressing information is associated with the ectodomain of the receptor, we know from our study performed in mammary cells (low M_r receptor) and the results in MDCK cells (high M_r receptor) that such signals are not associated with Ig-like domains II and III which are missing in the low M_r receptor. Finally, we have shown that after internalization and transcytosis, the receptor appears at the apical membrane within half an hour. Cleavage at the apical membrane and secretion of SC into the apical medium requires additional time, however, and this explains the presence of intact receptor detected in the apical membrane both morphologically and biochemically. Recently, it has been shown that the density of functionally intact poly-Ig receptors in transfected MDCK cells was fivefold higher on the apical than on the basolateral membrane (5). This observation, in agreement with our data, contradicts an early morphological description, in which no receptor was found associated with the apical membrane in transfected MDCK cells (33). Our results differ from those reported for transfected MDCK cells (33) in that the half-time of receptor cleavage is much shorter in mammary cells and closer to the in vivo situation (58). Since the cellular location of receptor cleavage was not determined in transfected MDCK cells, it is difficult to compare the results between the two cell types.

In conclusion, expression of the poly-Ig receptor in transfected mammary cells becomes restricted to one of the two cell types which differentiate in culture. In the luminal cells, the newly synthesized receptor is first targeted to the basolateral membrane before reaching the luminal cell surface where cleavage occurs with the subsequent release of SC into the medium. The difference in receptor processing between mammary and kidney cell lines suggests a distinct cellular machinery.

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