Regulation of Differentiation and Polarized Secretion in Mammary Epithelial Cells Maintained in Culture: Extracellular Matrix and Membrane Polarity Influences

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Abstract. Several previous studies have demonstrated that mammary epithelial cells from pregnant mice retain their differentiated characteristics and their secretory potential in culture only when maintained on stromal collagen gels floated in the culture medium. The cellular basis for these culture requirements was investigated by the monitoring of milk protein synthesis and polarized secretion from the mouse mammary epithelial cell line, COMMA-1-D. Experiments were directed towards gaining an understanding of the possible roles of cell–extracellular matrix interactions and the requirements for meeting polarity needs of the epithelium.

When cells are cultured on floating collagen gels they assemble a basal lamina–like structure composed of laminin, collagen (IV), and heparan sulfate proteoglycan at the interface of the cells with the stromal collagen. To assess the role of these components, an exogenous basement membrane containing these molecules was generated using the mouse endodermal cell line, PFHR-9. This matrix was isolated as a thin sheet attached to the culture dish, and mammary cells were then plated onto it. It was found that cultures on attached PFHR-9 matrices expressed slightly higher levels of β-casein than did cells on plastic tissue culture dishes, and also accumulated a large number of fat droplets. However, the level of β-casein was approximately fourfold lower than that in cultures on floating collagen gels. Moreover, the β-casein made in cells on attached matrices was not secreted but was instead rapidly degraded intracellularly. If, however, the PFHR-9 matrices with attached cells were floated in the culture medium, β-casein expression became equivalent to that in cells cultured on floating stromal collagen gels, and the casein was also secreted into the medium. The possibility that floatation of the cultures was necessary to allow access to the basolateral surface of cells was tested by culturing cells on nitrocellulose filters in Millicell (Millipore Corp., Bedford, MA) chambers. These chambers permit the monolayers to interact with the medium and its complement of hormones and growth factors through the basal cell surface. Significantly, under these conditions α_1-, α_2-, and β-casein synthesis was equivalent to that in cells on floating gels and matrices, and, additionally, the caseins were actively secreted. Similar results were obtained independently of whether or not the filters were coated with matrices.

In conclusion, providing the cells with a culture environment that permits access to the basolateral surface and caters to the polarity requirements of the cell allows optimal cellular differentiation and secretion. Moreover, the simple interaction of any of the basement membrane components, laminin, collagen IV, or heparan sulfate proteoglycan, with the basal epithelial surface, is not in itself sufficient to promote maintenance of differentiation.

Finally, while secretion of α_1-, α_2-, and β-casein from Millicell chambers was polarized to the apical (top) chamber, transferrin was secreted both apically and basolaterally. Cultures of COMMA-1-D cells in Millicell chambers may thus be a useful experimental system for studying pathways of polarized secretion from epithelia.

THE ability of mammary epithelial cells to retain their tissue-specific phenotypes in culture is highly dependent upon the substratum on which the cells are cultured. This was first shown by Emerman and Pitelka (10, 11), who found that collagen gels prepared from rat tail collagen (type I) could support maintenance of morphological differentiation in culture, provided the gels were floated in the culture medium. Cells cultured on plastic or on attached gels failed to express these characteristics. More recently, we and others have expanded upon these original observations and

have found that the culture substratum can influence both the levels of milk proteins and their mRNA molecules, and, additionally, the ability of cells to secrete (2–4, 7, 8, 15, 22, 23, 25, 27–29, 32, 34, 38, 39).

In attempting to understand what factors are contributed by the floating collagen gel that permit tissue-specific differentiation and protein secretion several possibilities can be considered. (*a*) Cells cultured on floating collagen gels assemble a morphologically and biochemically distinct basal lamina at their basal surface. This is not produced by cells on plastic and is only partially formed by cells on attached gels. As basement membrane components have been shown to modulate cellular differentiation in a variety of tissues (12, 13, 19, 21, 31, 33, 35, 36), it is possible that the basally secreted extracellular matrix components directly promote mammary differentiation. (*b*) The floating collagen gels are flexible and contract to approximately one-tenth the diameter of attached gels. Flexibility of substrata, permitting cell shape changes, may be a requirement for optimal differentiation. (*c*) When floated, the basolateral surface of cells becomes exposed to the culture medium. In vivo this is the surface through which nutrients pass into the cell and that carries hormone receptors. It is possible that access to this surface is significantly reduced in cultures maintained on plastic culture dishes, but that the floating gels allow these polarity constraints to be met.

In this paper we report experiments that address the significance of each of these possibilities. We have examined differentiation of the epithelia in terms of (*a*) the quantities of caseins and transferrin made by the cultures, and (*b*) whether or not the proteins are secreted into the culture media. We have also considered whether the cultures are differentiated with respect to the polarity of secretion and discuss some of the cellular factors that influence polarized protein secretion.

Materials and Methods

Cell Culture

COMMA-1-D cells (7) were obtained from Dr. Dan Medina (Baylor College of Medicine, Houston, TX) and were cultured routinely in medium F12/DME with insulin (5 μg/ml), hydrocortisone (2 μg/ml), and FCS (2%). Before initiating experimental cultures, 5 μg/ml prolactin (National Hormone and Pituitary Program, National Institutes of Health, Bethesda, MD) was added for at least 1 wk. COMMA-1-D cells were transferred every 2–3 wk using trypsin or trypsin-EDTA. Medium was changed on the cultures every 2 d. Experiments were carried out using cultures at passages 4–17. Confluent cultures were used for all experiments. Cells were seeded onto plastic tissue culture dishes, type I collagen gels (rat tail tendon collagen), or basement membrane–like matrices produced by cultures of PFHR-9 cells (17, 20).

Cultures of PFHR-9 cells were established, and extracellular matrices isolated from them according to published procedures (19). Cells were grown in either 199 or RPMI containing 10% FCS and split 1:10 every 3 d. When matrices were to be produced, cells were seeded onto fibronectin-coated dishes and cultured to superconfluent densities for 10 d using medium supplemented with ascorbic acid (25 μg/ml). Matrices were prepared using NP-40 detergent to lyse the cells as described previously (19).

Floating type I collagen gels or floating matrices were generated by gently teasing the cell–matrix unit or cell-collagen gel unit away from the culture dish with a scalpel. Within 24 h the collagen gels had contracted to about one-third of their diameter, while the matrices had contracted to about one-tenth their original diameter.

Chamber cultures, giving improved access of the basolateral surface of cells to the culture medium, were established using Millicell chambers containing a 0.45-μm nitrocellulose filter. Cultures were set up on these either by directly seeding cells onto the filters, or by precoating the filters with PFHR-9–produced matrices.

Radiolabeling of Cultures and Immunoprecipitation

Cultures were radiolabeled in the usual culture medium with 100 μCi/ml [^{35}S]methionine (1,400 Ci/mmol) for 3 h. Thereafter, medium was removed, the cells rinsed with 1 ml of isotonic Tris-saline (50 mM Tris chloride, 0.15 M NaCl), and then scraped into 1 ml of the same buffer for storage at −70°C. The rinse was pooled with the medium and also stored at −70°C. For cells on floating collagen gels and floating matrices, the cells were separated from the media and the washes by centrifugation in a microfuge for 1 min. This procedure also served to remove trapped media from the interstices of the collagen gels. The floating gels and matrices were also collected in 1 ml of Tris-saline buffer.

Before immunoprecipitation, cell samples were sonicated extensively and three 100-μl aliquots were removed for determination of total radioactivity incorporated into acid-insoluble material and estimation of DNA content. The remainder of the samples was adjusted to 1% NP-40, 0.5% sodium deoxycholate, 20 μg/ml aprotinin, and incubated at 4°C for 1 h with intermittent mixing. Insoluble material was removed by centrifugation for 15 min (using a microfuge), and the supernatant fraction was then removed for immunoprecipitation. A 20-μl aliquot of a rabbit antibody against milk proteins was added to each 1 ml of supernatant and incubated at 4°C for 1 h. This was followed by 100 μl of a suspension of protein A–Sepharose (10% wt/vol) for 30 min more. The protein A–Sepharose was pelleted and washed three times with Tris chloride (50 mM) pH 7.2, sodium chloride (0.15 M), NP40 (1%), sodium deoxycholate (0.5%), and aprotinin (20 μg/ml), and finally with Tris chloride (10 mM), pH 6.7. The pellet was then mixed with 2× lysis buffer (50 μl sodium dodecyl sulfate [2%], 50 mM Tris chloride, pH 6.7, glycerol [5%], and β-mercaptoethanol [5%]), boiled for 15 min, briefly centrifuged, and the supernatant fractions then removed. These were loaded onto either 10 or 12.5% polyacrylamide gels and resolved by electrophoresis. Dried gels were subject to autoradiography. The autoradiograms were then scanned using either an LKB Instruments (Gaithersburg, MD) or Bio-Rad Laboratories (Richmond, CA) densitometer to quantify the radioactivity incorporated into each of the bands. This was normalized on the basis of DNA per culture or alternatively on the basis of total acid-precipitable radioactivity incorporated into the cell layer during the labeling period. Both the quantity of media and of cell extracts used for immunoprecipitation were normalized this way. This latter method had an advantage in that small differences in the final specific activities of [^{35}S]methioinine that arose form one culture condition to another (e.g., due to trapping of unlabeled media inside collagen gels or matrices) were reflected in the total acid-precipitable counts incorporated. These variations were thus taken into account by this normalization procedure.

Immunofluorescence Procedures

Monolayers of cells on coverslips or on matrix-coated coverslips were rinsed twice with PBS, fixed with formaldehyde (3.5% vol/vol) for 30 min and rinsed with PBS containing glycine (50 mM). Cells were then made permeable by treatment with acetone (50% acetone/H_2O for 5 min, 100% acetone for 5 min, 50% acetone/H_2O for 5 min) and incubated with PBS containing BSA (0.1%) for 30 min. Coverslips were then placed in PBS plus BSA (0.1%) plus anti-β-casein (1:100 dilution, ascites fluid) and kept at room temperature for 1 h. After washing with PBS plus BSA (0.1%) twice for 15 min, coverslips were placed in PBS plus BSA (0.1%) plus goat serum (5%) for 30 min, and then reacted with fluorescein-conjugated goat anti-mouse Ig (1:200; Tago, Burlingame, CA). Coverslips were finally rinsed three times for 15 min with PBS plus BSA (0.1%), and mounted for viewing using a Zeiss fluorescence microscope equipped with a 63× objective.

Results

The Role of Basement Membrane Components in Promoting Tissue-specific Differentiation and Secretion

When cultures are maintained on floating collagen gels they synthesize and assemble a basement membrane–like structure that contains laminin, IV collagen, and heparan sulfate proteoglycan (28, 29, and unpublished immunocytochemical studies). The deposition of such a basement membrane

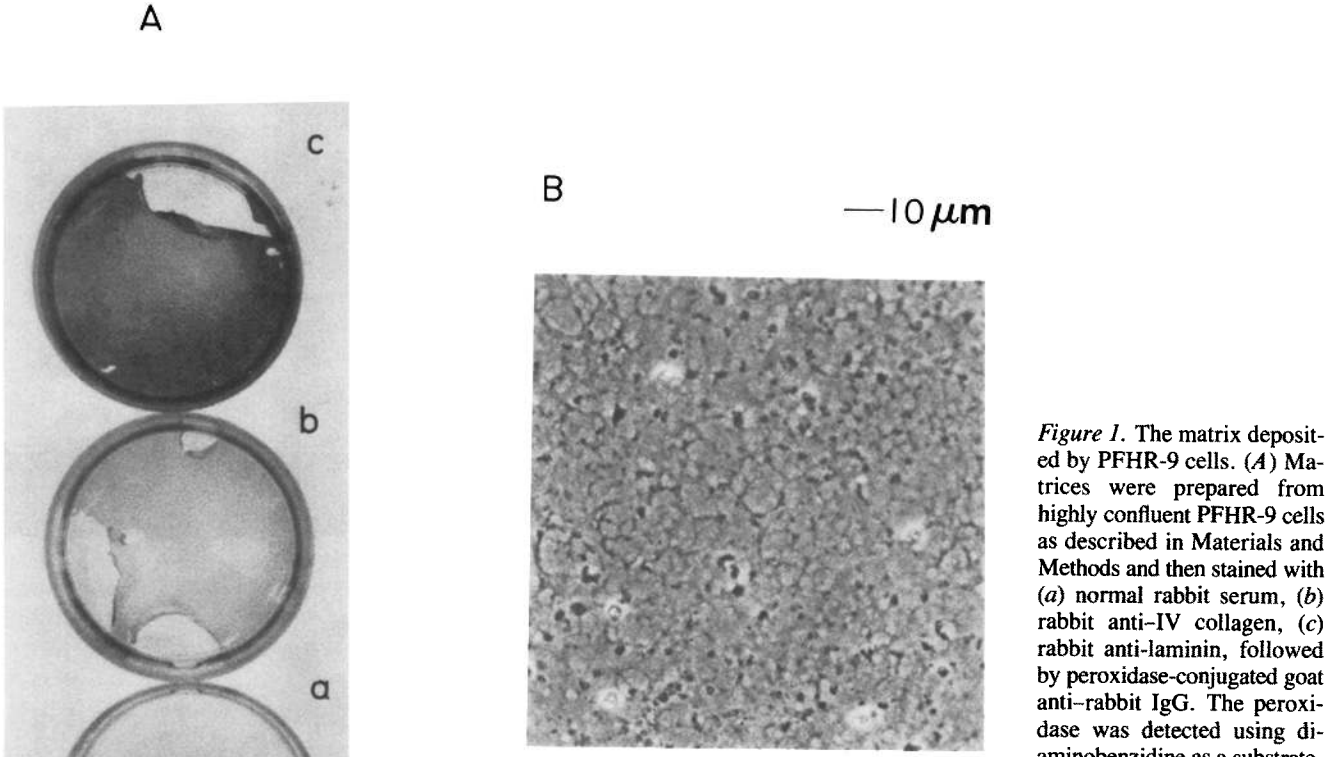

Figure 1. The matrix deposited by PFHR-9 cells. (*A*) Matrices were prepared from highly confluent PFHR-9 cells as described in Materials and Methods and then stained with (*a*) normal rabbit serum, (*b*) rabbit anti-IV collagen, (*c*) rabbit anti-laminin, followed by peroxidase-conjugated goat anti-rabbit IgG. The peroxidase was detected using diaminobenzidine as a substrate. The stained matrices are seen as a sheet of material coating the dish with occasional areas where it has begun to peel away from the edge of the plate. (*B*) A view of the matrix as seen by phase microscopy.

correlates with secretory differentiation in that cells on plastic or attached collagen gels produce incomplete basement membranes and also produce reduced quantities of milk proteins. To test whether basement membrane components do indeed promote milk protein synthesis and secretion, we used an endodermal cell line, PFHR-9, to generate a basement membrane–like matrix on the surface of tissue culture dishes and then seeded COMMA-1-D cells onto this matrix. The PFHR-9 cells have previously been used by others for this purpose (12, 19), and have been shown to produce a matrix rich in collagen IV, laminin, and heparan sulfate proteoglycans (5, 17, 20, 26). The matrices produced by PFHR-9 cells used in these studies are illustrated in Fig. 1. Immunoperoxidase staining using antibodies against collagen IV and laminin were used to reveal the macro structure of the deposited material. Physically, the matrix took the form of a thin sheet that was attached to the culture dish.

COMMA-1-D cells were cultured on PFHR-9–derived matrices in the presence of lactogenic hormones for 10–12 d. Control cultures were maintained on plastic tissue culture dishes under the same conditions and also on collagen gels. Confluent cultures of cells on collagen gels were established after ~4 d, and the gels were then floated in the culture medium for 7–8 d more. β-casein synthesis was monitored as described in Materials and Methods. Experiments revealed that COMMA-1-D cells on plastic synthesized only small quantities of β-casein. While this was increased slightly in cells cultured on PFHR-9–derived matrices, this quantity of β-casein was significantly lower than that found in cells cultured on floating collagen gels (Fig. 2, lanes *a–c* and Table I). This was confirmed by immunofluorescence studies on β-casein distribution in cultures maintained on coverslips and matrix-coated coverslips (Fig. 3). However, it is significant that a few areas of the monolayer on matrix-coated coverslips reacted strongly with anti-β-casein antibody. Phase-contrast microscopy revealed that these were areas of the monolayer that had become slightly detached

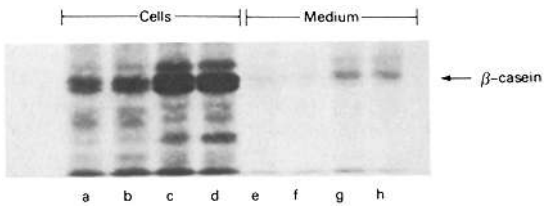

Figure 2. β-Casein synthesized and secreted by COMMA-1-D cells cultured on (lanes *a* and *e*) plastic tissue culture dishes; (lanes *b* and *f*) PFHR-9 matrices (attached); (lanes *c* and *g*) floating collagen gels; (lanes *d* and *h*) PFHR-9 floating matrices. Lanes *a–d* are cell samples; lanes *e–h* are medium samples. Cultures were radiolabeled with [^{35}S]methionine for 3 h, the cell and medium fractions separated and immunoprecipitated as described in Materials and Methods, and immunoprecipitates resolved by SDS-PAGE. Dried gels were subject to autoradiography.

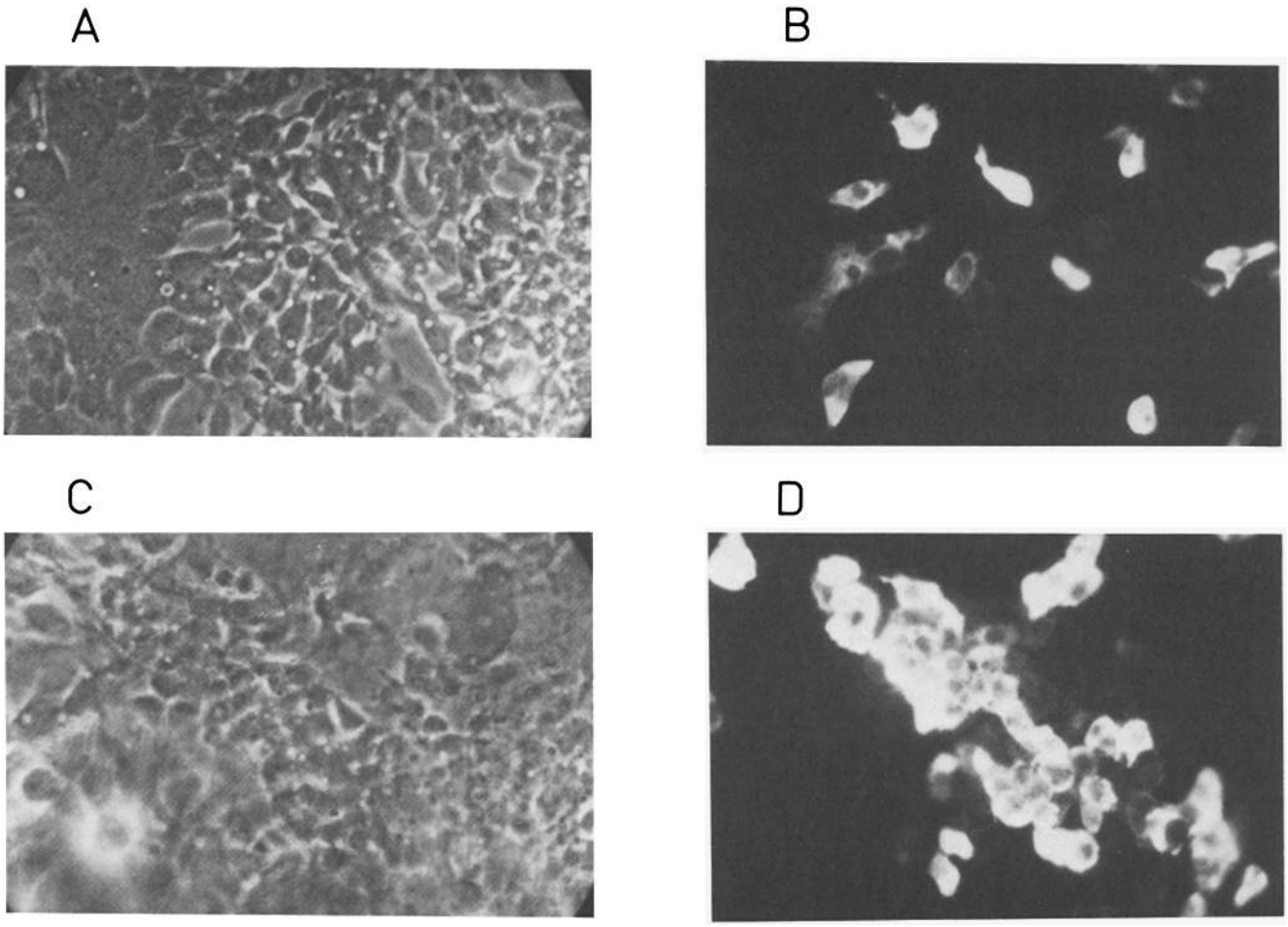

Figure 3. Paired phase and fluorescence micrographs of COMMA-1-D cells stained with anti-β-casein antibodies and fluorescein-conjugated second antibodies. *A* and *B* are photographs of cells cultured on tissue culture plastic, while *C* and *D* are of cells cultured on PFHR-9 matrices. The cell–matrix unit has become slightly detached from the dish in the area photographed (*C* and *D*), making it difficult to focus on the monolayer. However, these partly detached areas are composed of significant numbers of cells expressing β-casein.

from the glass (Fig. 3). However, it was clear that the simple presence of a basement membrane at the basal surface of the cell was not sufficient to promote maximum β-casein expression.

Moreover, as was the case for cells cultured on plastic, cells on PFHR-9 matrices failed to secrete newly synthesized β-casein. This was in contrast to cells cultured on floating gels that actively secreted β-casein into the culture medium (Fig. 2, lanes *e–g*). The failure of cells on PFHR-9 matrices to secrete was confirmed by pulse-chase experiments that showed that none of the newly synthesized casein appeared in the medium even after a 6-h chase in nonradioactive medium (Fig. 4, *A* and *B*). However, the same cells synthesized and efficiently secreted transferrin within this time. Instead of being secreted, some of the newly synthesized β-casein was degraded intracellularly. The cells on the attached matrix thus behaved similarly to cells on plastic, and quite differently from cells on floating gels (Fig. 4, *C* and *D*). We then considered the effect of releasing the cells on PFHR-9–derived matrices from the culture dish in the form of a floating cell–matrix unit. Significantly, under these conditions, the cells synthesized and secreted equivalent quantities of β-casein to cells cultured on floating type I collagen gels (Fig. 2, lanes *d* and *h* and Table I). Floating the cell–matrix unit led to extensive contraction of the monolayer to approximately one-tenth to one-twentieth of its original attached diameter. Additionally, of course, the basolateral surface of the epithelium gained improved access to the medium. Thus it is possible that these two features, rather than the presence of matrix molecules, are responsible for maintenance of secretory differentiation.

It should be noted, however, that although maximum expression of caseins and their secretion was observed only after the cell–matrix unit was released from the culture dish, certain differentiated characteristics were expressed on the attached matrix itself. Notably, cells on matrices formed extremely large domes and accumulated large numbers of fat droplets (Table I).

The Role of Basolateral Exposure to Medium

The possibility that improving access of the basolateral surface to hormones and nutrients could lead to promotion of secretory differentiation was analyzed by culturing the cells

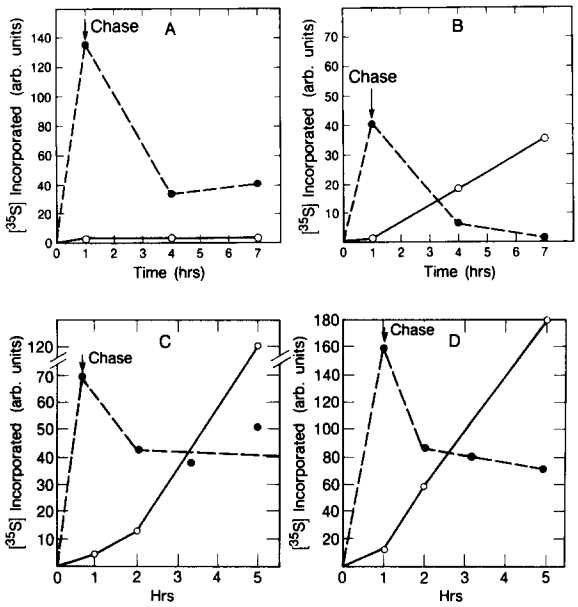

Figure 4. An analysis of the kinetics of secretion of β-casein and transferrin from cells cultured on either PFHR-9 matrices (*A* and *B*) or floating collagen gels (*C* and *D*). Cells were radiolabeled for 1 h with [^{35}S]methionine (100 μCi/ml) in F12 medium containing hormones. The medium was then removed, the monolayers rinsed twice with medium containing excess methionine (10 times the normal quantity), and then chased in unlabeled medium. Cell and medium samples were collected at various times during the chase and immunoprecipitated as described in Materials and Methods. Incorporation into β-casein (*A* and *C*) and the 80-kD protein, transferrin (*B* and *D*), was quantified by densitometric scanning of autoradiograms of gels of immunoprecipitated material. Solid circles denote the intracellular pool of newly synthesized protein while open circles denote secreted β-casein or transferrin. (Note: the abscissa scales [arbitrary units] for matrix and floating gel graphs are not directly comparable.)

Table I. The Effect of PFHR-9 Basement Membranes on Maintenance of Differentiation in COMMA-1-D cells

Culture substratum	Lipid droplets (cells)*	Dome formation‡	β-casein in cells (stained)§	Relative rates of β-casein synthesis‖
	%		%	
Plastic	5–20	++	2–10	1.0
Matrix	30–80	++++	10–40	1.28 ± 0.21
Floating matrix	ND	ND	ND	5.86 ± 0.65
Floating collagen	ND	ND	ND	5.61 ± 1.10

* Lipid droplet formation was monitored using oil red O stain. The proportion of cells stained was determined in cultures at passages 14 and 15. The range of values obtained in two separate experiments is noted.

‡ Dome formation was monitored routinely by phase microscopy. Extremely large domes were found in early passage cultures on matrices (i.e., passage 10 and prior passages) but were reduced in size with continued passaging.

§ The proportion of cells synthesizing β-casein was monitored by immunofluorescence procedures as described in Materials and Methods. The values shown are for two estimations of cultures examined at passages 14 and 15.

‖ The relative rates of β-casein synthesized were determined as described in Materials and Methods. Briefly, cultures were radiolabeled with [^{35}S]methionine for 3 h and newly synthesized casein was immunoprecipitated. After resolution of the immunoprecipitates by SDS-PAGE, β-casein bands were detected by autoradiography and quantified by scanning densitometry. Data was normalized as described in Materials and Methods and the rates of β-casein synthesized determined relative to that in cultures maintained on plastic culture dishes. Data shown is from two experiments using cultures at passages 8 and 10. The values shown are the means and standard deviations of three determinations.

on filters in Millicell units (Millipore Corp., Bedford, MA). These chambers permit separation of apical and basolateral compartments and fully expose the basolateral surface of the cells to the culture medium. Experiments were carried out using plain, uncoated Millipore filters, and also filters that had been coated with PFHR-9 matrix. Cells were grown to confluence in the chambers and then maintained in differentiation medium for an additional week before being radiolabeled. Immunoprecipitation analysis revealed that cells cultured in Millicell units produced significantly more β-casein than did cells on plastic or attached matrix and equivalent quantities to cells on floating gels (Fig. 5). Moreover, the newly synthesized β-casein was actively secreted into the culture medium in both plain chambers and matrix-coated chambers. Similar results were obtained for α1- and α2-casein synthesis and secretion (Fig. 5) and for the synthesis of the milk fat globule membrane protein, xanthine oxidase (data not shown).

Polarity of Secretion from Cells Cultured in Chambers

The behavior of the cells on Millicell chambers offered an opportunity to analyze whether the epithelia were differentiated with respect to the polarity of secretion. Confluent monolayers were established in hormone containing medium in either plain or matrix-coated chambers and radiolabeled for 1 h by including [^{35}S]methionine in the basal chamber. Nonradioactive chase medium was then added to the chambers for 1 or 3 h more and the medium then collected separately from the apical and basolateral compartments (Fig. 6).

The cells secreted milk proteins in a highly polarized fashion with α1, α2, and β-casein being secreted only into the apical compartment. Transferrin was secreted apically, but, interestingly, it also accumulated in lesser quantities in the basal compartment. This observation was quite reproducible and implied that transferrin was being secreted along two pathways: one apically directed and the other basolaterally directed. As transferrin appeared in the apical compartment shortly before it appeared in the basolateral compartment we considered the possibility that it was simply leaking across the monolayer from the apical medium. This was tested experimentally in two ways (*a*) The permeability properties of the monolayers on chambers was examined by including [^{3}H]inulin and [^{14}C]cytochrome *c* in either the top or bottom chamber and following the kinetics of transfer into the opposite chamber. Over a period of 5 h no more than ~10% of the radiolabeled molecules had diffused across the monolayer (Fig. 7). This was consistent with the idea that, at least for the time course of the experimental studies, the monolayer could be considered a relatively impermeable barrier to proteins. (*b*) Medium was collected from the apical compartment of chambers that had been radiolabeled for 1 h and chased for a further hour, and was added to the apical chamber of unlabeled cells that had been previously treated with cycloheximide to inhibit further protein synthesis. No diffusion or transport of radiolabeled transferrin from the apical compartment to the basolateral compartment was observed, even after a 3-h incubation (data not shown). We thus conclude that transferrin is actively secreted both apically and basolaterally, while the caseins are secreted only apically.

No differences in the polarity of milk protein secretion

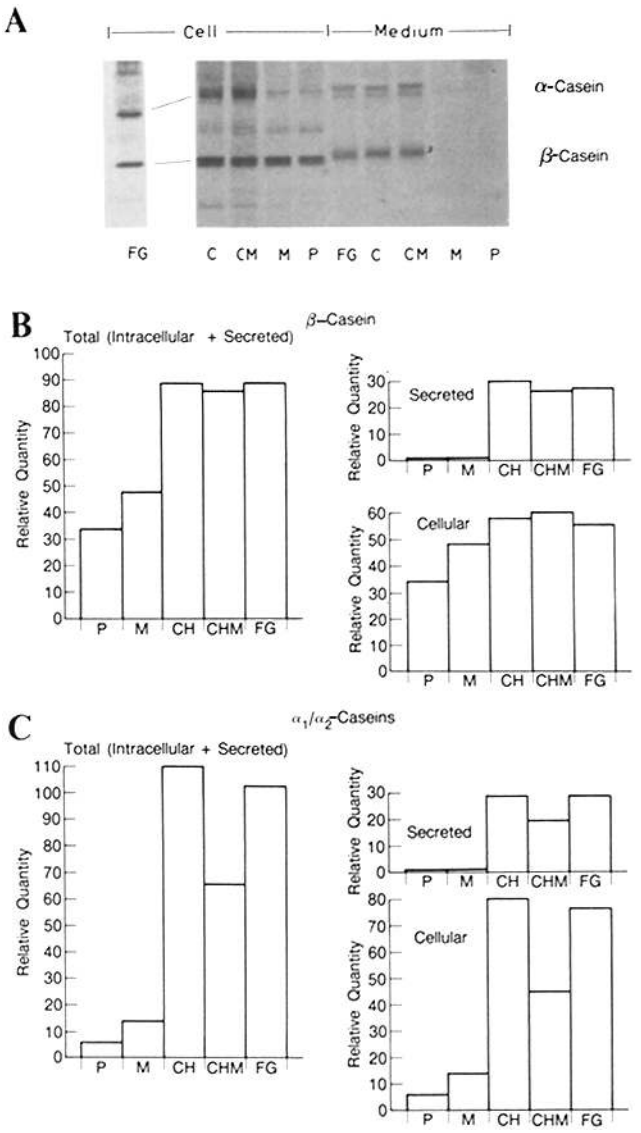

Figure 5. A comparison of α_1-, α_2-, and β-casein synthesis and secretion from cells cultured on nitrocellulose filters in Millicell units, with cells on plastic, PFHR-9 matrices, and floating collagen gels. Cultures were established on the various substrata as described in the text and radiolabeled with [^{35}S]methionine for 3 h. Thereafter cell and medium samples were collected separately and α_1-, α_2-, and β-casein immunoprecipitated as described. The autoradiogram shown in *A* shows data from one experiment and the lower histograms (*B* and *C*) quantify the data obtained in three experiments. (Mean values are plotted. Deviation about the mean was no greater than ±10%.) *P*, plastic; *M*, PFHR-9 matrix; *CH*, Millicell chamber; *CHM*, Millicell chamber with filter coated with matrix produced by PFHR-9 cells; *FG*, floating collagen gel. Histogram *B* shows results obtained for α_1- and α_2-caseins and histogram *C* shows results for β-casein. Data for the secreted and intracellular pools is plotted separately to demonstrate the effects of substratum on secretion.

were seen between cells cultured on plain filters or cells cultured on matrix-coated filters (Fig. 6). However, in cells cultured on matrix, some additional unidentified high molecular weight proteins were secreted exclusively basally.

Discussion

Three significant observations emerge from this study. (*a*) The provision of a basement membrane–like matrix at the basal surface of cells is not, in itself, sufficient to promote either maximum β-casein expression or its secretion from the cell. (*b*) Exposing the basolateral surface of the epithelium to the culture medium, and thus meeting the polarity constraints of the cells, promotes both synthesis and secretion of milk proteins. (*c*) Protein secretion from the cells is highly polarized with the α1, α2, and β-caseins secreted apically. Transferrin, however, is secreted both apically and basolaterally.

Our data clearly showed that cells cultured on matrices produced by PFHR-9 cells expressed quantities of β-casein similar to those of cells cultured on plastic, and failed to secrete the newly synthesized molecules whenever the matrices were maintained as inflexible entities associated with the culture dish. Thus the simple presence of a matrix composed of laminin, collagen (IV), and heparan sulfate proteoglycan was not a sufficient signal to promote either optimal expression or secretion of differentiated products. Noteably, certain differentiated characteristics, such as fat droplet formation, were increased in cells on attached matrices, but release of the cell–matrix unit from the culture dish was necessary for both maximum casein synthesis and secretion. Under these conditions floating matrices supported casein expression as well as floating collagen (I) gels did.

The apparent difference in the effects of matrix components on lipid metabolism and casein synthesis and secretion is consistent with our earlier work demonstrating that the differentiation program of mammary epithelial cells was not coordinately regulated in culture. Studies of the effects of matrix components on fat synthesis are currently under investigation and will permit a better analysis of the relative roles of cell–matrix interactions and cell polarization on lipid differentiation in the cultured epithelia.

An important conclusion of the work presented here is that the stromal type I collagen that is used in making collagen gels is not specifically required for maintenance of differentiation, and can be replaced by basement membrane components. While this conclusion does not support an earlier suggestion that type I collagen did indeed play a regulatory role in mammary epithelial cell differentiation (37), it is important to distinguish between requirements for maintenance of secretory differentiation and promotion of cellular differentiation as occurs during pregnancy and lactation. That stromal–epithelial interactions, in particular type I collagen binding to cellular heparan sulfate proteoglycan, may play a role in development has been suggested by Bernfeld and colleagues (1, 18). Moreover, differentiation of cultured mammary epithelial cells from virgin mice into lactating cells requires culture of cells within stromal collagen gels and adherence to a strict regime of hormonal additions to the culture medium (8).

Significantly, the response of the COMMA-1-D cells to the matrix produced by PFHR-9 cells seems to be different from that of rat and mouse primary cultures to a basement membrane–like matrix isolated from the Engelbreth-Holm-Swarm (EHS)[1] tumor (2–4, 27). The accumulation of α-

1. *Abbreviation used in this paper*: EHS, Engelbreth-Holm-Swarm.

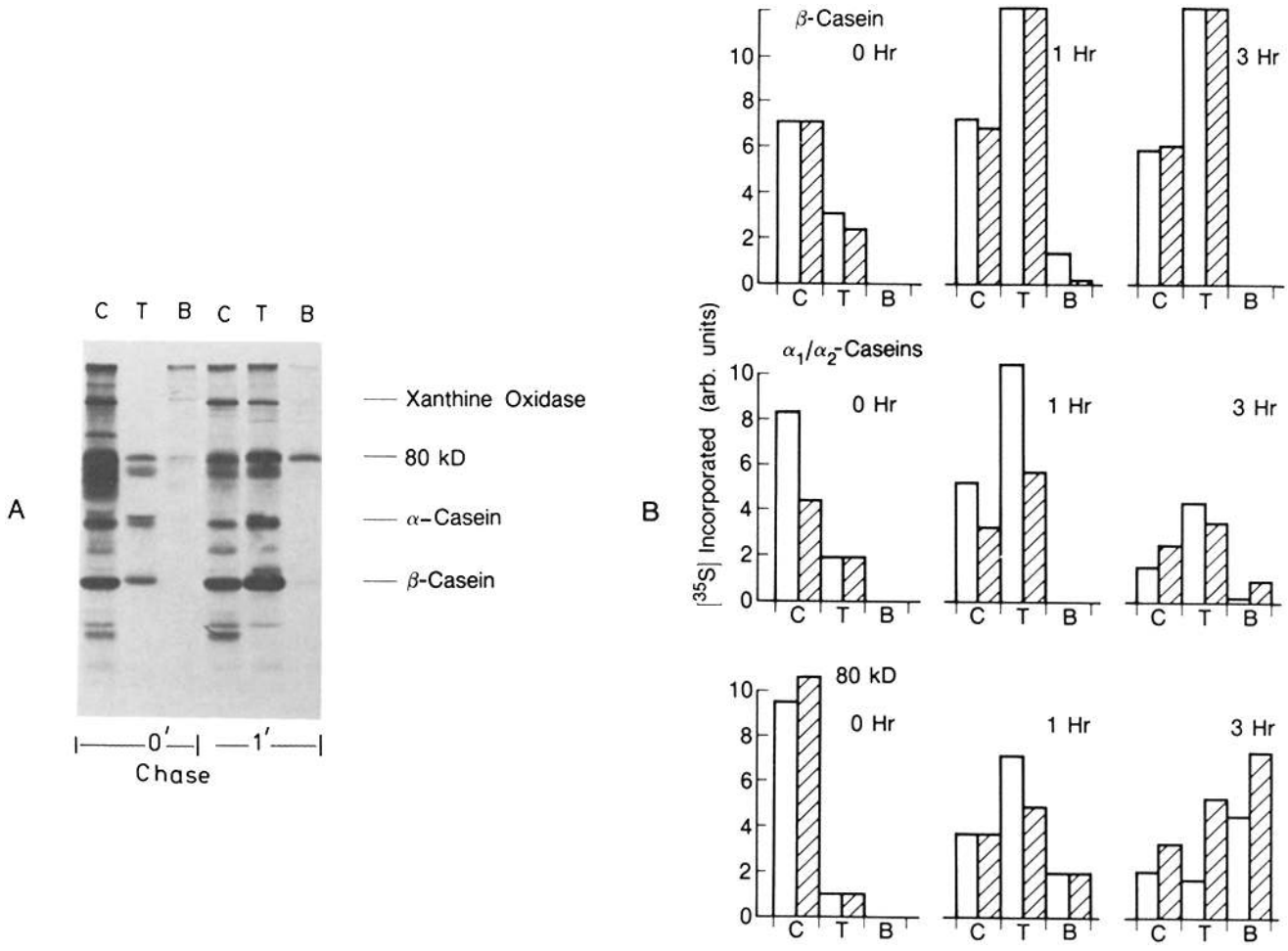

Figure 6. Polarity of protein secretion from cells cultured on nitrocellulose filters in Millicell chambers. Cells were cultured on either plain nitrocellulose filters in chambers or on filters coated with PFHR-9 matrix as described in Materials and Methods. Cultures were radiolabeled for 1 h with [^{35}S]methionine (250 μCi/ml) in F12 medium containing hormones in the basal compartment of the chambers. After this time, medium in the top (*T*) and bottom (*B*) compartments was separately harvested, the monolayers rinsed twice with PBS, and reincubated in unlabeled F12/DME medium for 1 or 3 h more. At each of these chase periods top and bottom compartments were separately harvested. Radiolabeled proteins from cell (*C*), top (apical) medium (*T*), and bottom (basolateral) medium (*B*), were immunoprecipitated and the immunoprecipitates resolved by SDS-PAGE. The autoradiogram shown in *A* shows sample data obtained for cells cultured on plain filters and collected either after 1 h labeling (0 chase) or after 1 h chase in unlabeled medium. Histogram *B* quantifies data obtained for cells on either plain filters (*open bars*) or matrix-coated filters (*striped bars*) over a 3-h chase period and demonstrates that while the α_1-, α_2-, and β-caseins are secreted almost exclusively apically, the 80-kD protein (transferrin) is secreted both apically and basolaterally. No significant effects of coating the filters with matrix were noticed.

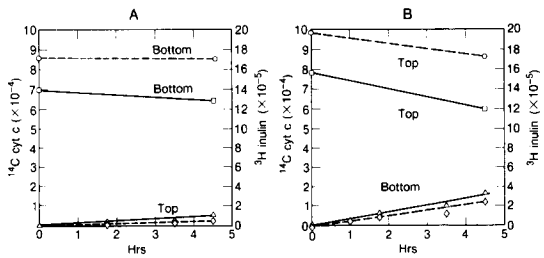

Figure 7. Permeability properties of COMMA-1-D monolayers cultured on nitrocellulose filters in Millicell chambers. Established monolayers of cells on plain filters were radiolabeled with a mixture of [^{14}C]cytochrome *c* and [^{3}H]inulin in either the bottom (*A*) or top (*B*) compartment, and reincubated at 37°. Samples were then taken from the bottom and top compartments at various times thereafter and the quantity of radioactivity in each compartment determined. (*Open circles*, *open diamonds*) inulin; (*open squares*, *open triangles*) cytochrome *c*.

lactalbumin and α-casein mRNA by primary cultures of rat mammary epithelial cells is stimulated significantly by a matrix of EHS basement membrane components coated onto tissue culture dishes. Additionally, laminin alone seems to promote α-lactalbumin and α-casein accumulation even when added to the medium of cells cultured on plastic. Likewise, casein mRNA and protein accumulates in primary mouse mammary cultures maintained on EHS-derived matrix molecules. A notable difference between the PFHR-9 matrix and the EHS-derived matrix is in their physical characteristics. While the PFHR-9 matrix has the consistency of a thin film of material, the EHS-derived matrix has the consistency of a gel with considerable flexibility. As such it is probably more analogous to the floating PFHR-9 matrix than the attached matrix. Moreover, primary cultures of mouse cells do not plate out on the surface of the gel but instead reorganize to develop glandular-like structures with exposed

basolateral surfaces. Thus, the EHS-derived matrix may function, at least partially, by meeting the polarity needs of the epithelia.

One of the most important observations of this study is that exogenous matrices are not necessary to promote secretory differentiation. Instead, exposure of the basolateral surfaces of cells to the culture medium seem to be sufficient. Cells maintained on filters in Millicell chambers produced quantities of milk proteins similar to those of cells on floating collagen gels. Additionally, the proteins were actively secreted from the cultures. Coating the filters with matrix produced by PFHR-9 cells had no additional promoting effect on either α_1-, α_2-, or β-casein expression or on their secretion, demonstrating that the exogenous matrix was not necessary. Additionally, as the filters are clearly an inflexible substrate, it would appear that contraction of the monolayer, as occurs when cells are on floating collagen gels or matrices, is not essential. Several factors may contribute to promotion of differentiation and secretion on Millicell chambers. A major consideration is that hormone receptors are likely to be basolaterally located in the plasma membrane and will thus be accessible to the lactogenic hormones, insulin, hydrocortisone, and prolactin, when the basolateral surface is exposed to the medium. This would, of course, also be true for cells on floating collagen gels and matrices that also promote differentiation and secretion. In contrast, access of hormones to receptors would be significantly impeded in confluent cultures maintained on plastic or attached PFHR-9 matrices.

Although the distribution of receptors in the plane of the plasma membrane has not yet been determined, the polarity of other membrane proteins in cultured cells has been considered. For several basolateral proteins in other epithelial cells (Madin–Darby canine kidney), it appears that the development of membrane polarity is a function of cell density (16, 30). At low cell density basolateral proteins are not polarized but become exclusively basolaterally located as the cultures become confluent and as tight junctions form between adjacent cells. Our own studies on the polarity of apical membrane proteins in cultured mammary carcinoma cells have shown that for apical antigens, polarity is established even at low cell densities (Parry, G., J. Beck, L. Moss, and G. Ojakian, manuscript submitted for publication). Thus it is highly likely that, under the confluent conditions used for all experiments reported here, the membrane receptors are indeed polarized, and that chamber cultures cater to the polarity requirements of the cells.

A second factor which is likely to be important in the promotion of differentiation in chamber cultures is matrix components that are synthesized by the cells themselves. Preliminary experiments that we have carried out indicate that the chamber cultures do indeed synthesize matrix components, and experiments are underway to assess their individual roles. Clearly, however, any effects of the matrix components are manifest only under conditions when cell polarity constraints are met.

The biochemical differentiation of mammary epithelial cells observed in this study contrasts with earlier observations of the morphology of cultures maintained on filters (9). Primary cultures of cells from late pregnant mice exhibited a flattened morphology when cultured on filters, and were not very different from cells cultured on plastic. In contrast, cells on floating collagen gels were columnar and looked very much like secretory epithelial cells in vivo. Two possibilities could explain this discrepancy. First, it is possible that biochemical differentiation does not correlate with morphological differentiation and there is no relationship between cell shape and secretory differentiation; alternatively, the COMMA-1-D cell line may be responding differently from the primary cultures. A noteable difference between primary cultures and COMMA-1-D cells is that the latter grow in culture while primary mammary epithelial cells do not. Thus it is possible that, under the conditions used for these experiments, the density of COMMA-1-D cells on the filters was different from that used earlier with primary cultures. Should the density of cells in culture be an important factor in influencing secretory differentiation in Millicell chambers, then the differences in response to Millipore membranes may be accounted for. A similar explanation could also be applicable to our earlier result that primary cultures on floating but inflexible glutaraldehyde-fixed collagen gels did not maintain differentiated characteristics in culture, even though exposure to basolateral surfaces was provided.

Our results with chamber cultures, however, are quite consistent with observations made earlier with cultures of thyroid cells. As is the case for mammary epithelial cells, differentiation of thyroid cells in response to thyroid-stimulating hormones is dependent on cells being cultured on floating collagen gels. Polarity concerns, namely access of thyroid-stimulating hormone to the basolateral surface of cells, were solely responsible for the need for floating collagen gels (37).

Studies on the polarity of secretion from the cultures demonstrated apical secretion of the caseins and transferrin, but interestingly, transferrin was also secreted basolaterally. Control experiments demonstrated that secretion through both the apical and basolateral surfaces was real and was not due to leakiness across the monolayer or to active transport of transferrin from the apical to the basolateral compartments. The experiments thus reveal the apparent existence of both apical and basolateral exocytotic pathways in the mammary epithelial cell. The α1, α2-, and β-caseins are clearly sorted intracellularly into the apical pathway while transferrin may not be sorted. These studies thus support our earlier view that transferrin and casein are secreted by independent routes from the mammary epithelial cells (22, 24). As such this experimental cell system may prove to be valuable in studying some of the sorting mechanisms that are responsible for either polarized secretion or divergent secretory pathways in epithelia.

In this context, however, it should be noted that while our results on the polarity of secretion are qualitatively clear, we have yet to determine transit times for milk protein diffusion across the cell-free Millipore filter. This would, of course, influence the rate of accumulation of secreted proteins in the basal chamber, and the polarized secretion rates observed in the experiments reported here may not quantitatively reflect what the cells are doing. Determination of these diffusion rates is currently underway, and this will permit optimization of the experimental system for polarity studies. However, Millipore chambers similar to those used in this study have been used in an earlier study of polarized secretion from Sertoli cells (14) and their effectiveness for polarity studies has been clearly demonstrated.

Finally, the fact that transferrin is secreted both apically and basolaterally has important physiological implications. The apically secreted transferrin may function as an iron carrier in milk or may have a bacteriostatic function. However, the basolaterally secreted material may function as an autocrine growth factor or even as a modulator of cellular differentiation.

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