Receptor-mediated Vectorial Transcytosis of Epidermal Growth Factor by Madin-Darby Canine Kidney Cells

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Abstract. Transcellular transport of a variety of ligands may be an important mechanism by which regulatory substances reach their site of action. We have studied the transcellular transport of two 6,000-mol-wt proteins, epidermal growth factor (EGF) and insulin, across polarized Madin-Darby canine kidney (MDCK) cells grown on dual-sided chambers on a nitrocellulose filter substrate. When grown on these chambers, MDCK cells are polarized and express distinct basal and apical surfaces. MDCK cells are capable of unidirectional transport of EGF from the basal-to-apical direction, 50% of bound EGF transported in 2 h. Transport was inhibited by the addition of unlabeled EGF in a dose-dependent manner. Anti-EGF receptor Ab, which inhibited binding, also inhibited transport. No transport in the apical-to-basal direction is noted. Insulin transport is not observed in either direction. Transport correlates with the presence of ligand-specific receptors on the cell surface. Hence, EGF receptors (R_o = 48,000, K_d = 3.5 × 10^{-10} M) are found only on the basal surface of the MDCK cells and neither surface expresses insulin receptors. Characterization of the EGF receptors on MDCK cells, as assessed by affinity, molecular mass, and anti-receptor antibody binding reveals that this receptor has similar characteristics to EGF receptors previously described on a variety of cells. Hence, the EGF receptor can function as a transporter of EGF across an epithelial cell barrier.

TRANSCYTOSIS is the term that has been applied to the transcellular transport of a variety of ligands including immunoglobulins (24, 25), insulin (13), albumin (7), thyroglobulin (9), ferritin (36), nerve growth factor (34), and viral proteins (28). Many of these ligands bind to high affinity receptors known to mediate endocytosis (29, 38). Recent evidence suggests that a number of cell types can, after binding of ligand, direct the ligand-receptor complex to either a lysosomal degradative pathway or can shuttle the intact complex across the cell (37). The mechanism by which transcytosis occurs is poorly understood, but may involve translocation of the endocytotic vesicle after internalization. Such a process would require sorting of endocytic vesicles shortly after internalization. To further examine the process of receptor-mediated transcytosis of hormones, we have examined the ability of a polarized epithelial cell to transport two ligands: epidermal growth factor (EGF)[1] and insulin.

The Madin-Darby canine kidney (MDCK) cells are polarized epithelial cells (4). When grown in culture, the plasma membrane is differentiated into an apical surface with microvilli facing the growth medium and a basolateral surface facing the neighboring cells and the substratum (16, 23, 30). In addition, these structurally and functionally different plasma membrane domains are separated by tight junctions (18). These cells have been used in both studies of immunoglobulin transport and in studies of transport and sorting of cellular and viral proteins (2, 19-22, 27, 28). EGF is a 6,045-mol-wt protein of 53 amino acids with a variety of biological actions (3, 5, 32), including stimulation of epidermal and epithelial cell proliferation. Biological activity requires binding of the ligand to specific plasma membrane receptors which is a well-characterized 170-185-kD glycoprotein with tyrosine kinase activity; after binding, the ligand-receptor complex is internalized and the receptor undergoes a tyrosine phosphorylation. Insulin is a 51-amino acid protein of 5.6 kD which binds to a specific plasma membrane receptor (6, 11). Similarly, binding is followed by tyrosine autophosphorylation of the receptor and the receptor-ligand complex is known to be internalized (6, 12). Receptor-mediated transcytosis (13, 14) of insulin by endothelial cells has been demonstrated and is important in the delivery of insulin to target tissues.

Materials and Methods

Cell Culture

MDCK cells were obtained from the American Type Tissue Collection (Rockville, MD). After three passages in our laboratory, a stock of cells was frozen. Cells used in this study were between passages 3 and 15. Stock cul-

1. *Abbreviations used in this paper*: DSS, disuccinimidyl suberate; EGF, epidermal growth factor.

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tures were grown on NUNC 80-cm^2 tissue culture flasks in MEM with Earle's salts, supplemented with 5% FBS. Cells used in experiments were plated directly onto 30-mm surfactant-free 0.45-μm nitrocellulose filters in premounted presterilized plastic chambers (Millicell HA: Millipore/Continental Water Systems, Bedford, MA). Cells were fed by changing media in both the upper and lower chamber compartments and were used in experiments between 5 and 7 d after plating. The number of cells per well were determined by removing cells from the nitrocellulose substrate using 1 ml of 0.1% trypsin in Ca^{++}- and Mg^{++}-free PBS. Trypsinized cells were added to an equal volume of serum containing media and were counted using a hemocytometer (Fisher Scientific Co., Medford, MA). Cell number at confluence ranged between 1.8 and 2.0 × 10^6 cells/well. Monolayers were examined by electron microscopy (kindly performed by Dr. Susan Bonner-Weir). This confirmed that the MDCK cells grew as a single layer of polarized cells on the membrane. To confirm the presence of tight junctions in the MDCK cell monolayer, the effect of 1.0 mM EGTA, which is known to disrupt tight junctions, was examined. 1 mM EGTA was added to both the basal and apical surface and [^{125}I]EGF or [^{14}C]inulin were added in the apical media. At 60 min, in monolayers treated with EGTA, there was 30 times as much inulin and EGF on the basal surface. This demonstrates that EGTA can disrupt tight junctions and that this will lead to apical → basal nonreceptor-mediated leakage of ligand.

Ligands

Receptor grade EGF was purchased from Collaborative Research Inc. (Bedford, MA) and iodinated using chloramine T to a specific activity of 194 μCi/μg. Monoiodinated HPLC-purified A-14 insulin and [^{14}C]inulin were purchased from Amersham Corp. (Arlington Heights, IL).

Binding Studies

Binding of EGF and insulin was performed on either apical and basal surfaces of MDCK cells grown on millicell chambers. For EGF binding, growth media from both surfaces was aspirated and both cell surfaces were washed three times with cold sterile PBS. Hepes buffer (118 mM $NaCl_2$, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM $CaCl_2$, 8.8 mM dextrose, 50 mM Hepes, 1% BSA), pH 7.4, was then added to each side. 50,000 cpm of labeled [^{125}I]EGF was added to either the basal or apical surface. Competition curves were derived by adding variable amounts, ranging from 1 ng/ml (1.7 × 10^{-9} M) to 200 ng/ml (3.4 × 10^{-7} M), of unlabeled EGF to the surface exposed to the labeled ligand. Chambers were incubated at 4°C for 6 h. After three washes with cold PBS, filters were cut out of the chambers using a single-edge razor and counted in a multichannel gamma counter. Data was analyzed using Scatchard analysis. Insulin-binding studies were performed similarly, however, the pH of the Hepes buffer was pH 7.8, and incubations were performed at 15°C.

Ligand Transport

After aspiration of growth media, each side of the chambers was washed three times with PBS, pH 7.4; 1.5 cc of Hepes buffer was added to the outer (basal surface) chamber and 1.0 cc of buffer to the inner (apical surface). These different volumes were used in order to have the same level of fluid in the inner and outer chamber. 50,000 cpm (0.137 ng) of labeled EGF was added to either the apical or basal side of the chambers. Chambers were incubated at the designated temperature and sampled from the side opposite to which the tracer had been added at the designated time points. For sampling, 500-μl aliquots were taken and the volume was replaced with fresh buffer. To determine the specificity of the EGF transport, some studies were performed with varying amounts of unlabeled EGF present. To exclude leakage through a nonconfluent monolayer the transport of 50,000 cpm of [^{14}C]inulin, added to the side containing the labeled ligand, was examined in each chamber studied. Inulin is primarily a marker of the extracellular space and diffuses freely across the nitrocellulose membrane. Insulin transport was studied using the same conditions.

Cross-linking

Disuccinimidyl suberate (DSS) was purchased from Pierce Chemical Co. (Rockford, IL), and was used to cross-link [^{125}I]EGF to MDCK cells. [^{125}I]EGF was added to either the apical or basal surface of MDCK cells grown on two-sided chambers, and incubated for 4 h at 4°C. Chambers were then washed three times with albumin-free Hepes buffer and cells were scraped off the filter using a rubber policeman. Cells were then suspended in 1 ml of albumin-free Hepes in a plastic tissue culture tube. DSS, freshly dissolved in DMSO, was added to the cells to a final concentration of 0.5 mM, and cells were incubated at 15°C for 30 min. Cells were pelleted by centrifugation and the DSS-containing solution was aspirated. The cells were washed three times in 4 ml of solution containing 120 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA. Cell pellets were then solubilized in 200 μl of Laemmli buffer (15) (2% SDS, 10% glycerol, 0.002% bromphenol blue, 10 mM Na_2HPO_4 with 15 mg/ml DTT) (14). Samples were analyzed by polyacrylamide electrophoresis on 7.5% acrylamide gels. Fixed and dried gels were autoradiographed with Kodak X-Omat film for 6 wk.

Results

Transcellular Transport

As the nitrocellulose filter itself is a barrier to the passive diffusion of small proteins across the chamber, the kinetics of EGF and inulin diffusion across the membrane were examined. Although both molecules have similar molecular masses, the diffusion of inulin was faster than that of EGF. At 2 h (Fig. 1), 45% of the total inulin added and 22% of the total EGF added had diffused across the membrane. Diffusion of both EGF and inulin was somewhat more rapid in the apical-to-basal direction in empty chambers. Insulin diffusion across the membrane at 2 h was 15% of the total [^{125}I]insulin added (data not shown). In contrast, MDCK cell monolayers grown on millicell chambers represented an almost absolute barrier to the diffusion of inulin. At 2 h, <1% of the inulin had diffused across the chamber. When labeled EGF was added to the basal surface of the cell monolayer, ∼3% of the total tracer added appeared on the apical surface within 120 min (Fig. 1). This data suggested that EGF might be actively transported across the cell monolayer.

To characterize the ability of MDCK cells to actively

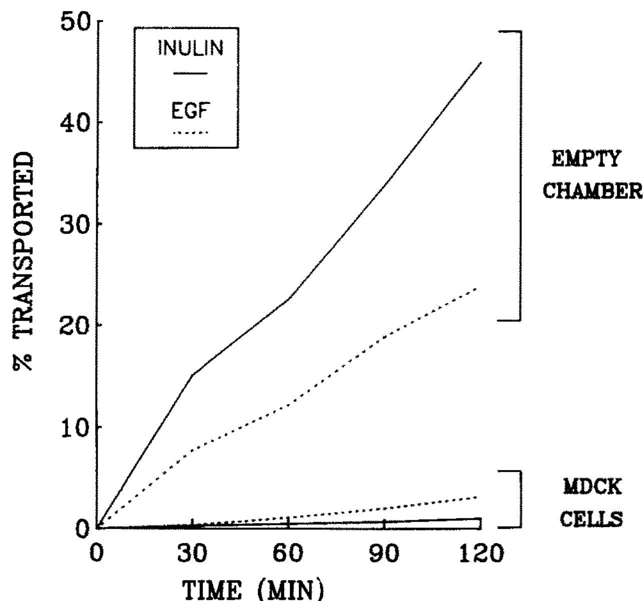

Figure 1. Transport dynamics of chambers. [^{14}C]Inulin or [^{125}I]EGF (∼50,000 cpm) were added to the outer chamber of either empty chambers or Millicell chambers containing a confluent monolayer of MDCK cells. The inner chamber was sampled at 30-min intervals; 0.1-ml samples of transport buffer were taken and the volume was replaced with an equal volume of fresh buffer. Percent transported was calculated by determining total cumulative amount of tracer appearing on the inner chamber as a percentage of the total tracer added.

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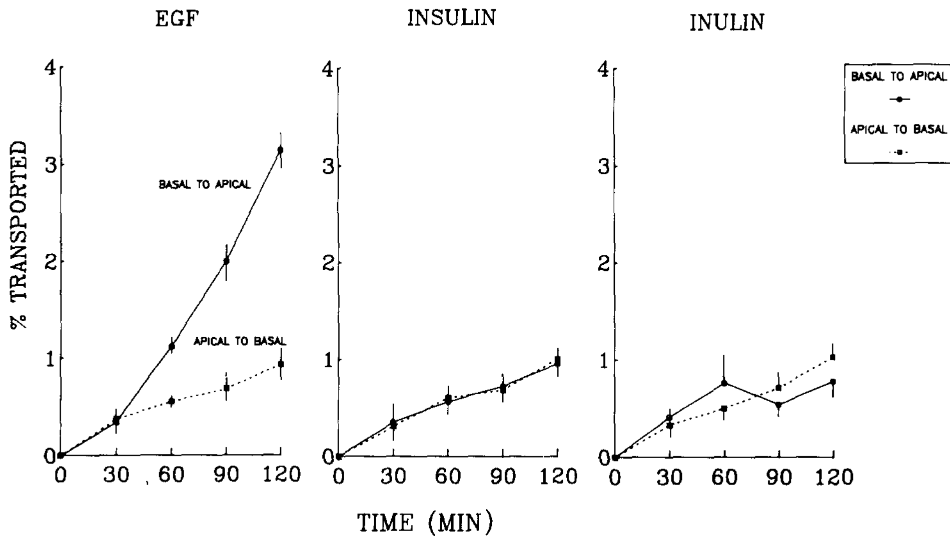

Figure 2. Ligand transport. [14C]-Inulin and either [125I]EGF or [125I]-insulin were added to either the apical or basal surface of MDCK cells grown on chambers. At 30-min intervals samples were taken from the opposite surface, and replaced with an equal volume of fresh transport buffer. Percent ligand transported was determined by calculating total cumulative counts transported as a percent of the total tracer added. Each point represents the mean of three chambers and error bars show SD.

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transport hormones across the cell monolayer, basal-to-apical and apical-to-basal transport of inulin, EGF, and insulin across the MDCK monolayers were evaluated. 2 h after the addition of [14C]inulin to either the basal or apical surface of the chamber, a total of 1% of the total inulin added appeared in the contralateral surface (Fig. 2). As inulin is a marker of the extracellular space and is not actively transported via a receptor-mediated process, the appearance of inulin on the contralateral surface was considered passive diffusion across the cell monolayer. When EGF was added to the basal surface, the appearance of labeled growth factor on the apical surface was time dependent.

Over 120 min, the amount of tracer increased in a linear fashion, after an initial 30-min lag period. At 120 min, 3.2% of the total tracer added to the basal surface had appeared on the apical surface. In contrast, when EGF was added to the apical surface, less than 1% of the total tracer added appeared in the lower chamber. Whereas the amount of EGF transported in the basal-to-apical direction was three times higher than the diffusion of inulin in the basal-to-apical direction, the amount of EGF transported in the apical-to-basal direction was equal to the amount of inulin diffusing in the apical-to-basal direction. Hence, active transport of EGF was unidirectional in MDCK cells grown in dual-sided chambers. We next examined the ability of MDCK cells to transport insulin. The appearance of insulin on the contralateral side paralleled the appearance of inulin, and did not exceed 1% of the total tracer added at 120 min. Hence, insulin was not transported in either direction by MDCK cells (Fig. 2) when added to either the basal or apical surface.

To determine the mechanism by which EGF might be preferentially transported in the basal-to-apical direction, the ability of MDCK cells to bind EGF and insulin to the basal and apical surfaces was evaluated. EGF added to the basal surface of the MDCK cells bound in a specific and saturable fashion. At 4°C, binding was maximal within 4 h and was 6.5% of the total tracer added. Binding could be inhibited by the addition of increasing amounts of cold unlabeled EGF at concentrations between 1 ng/ml (1.7×10^{-9} M) and 200 ng/ml (3.4×10^{-7} M) as seen in Fig. 3. Using Scatchard analysis (31) a linear Scatchard curve was generated. MDCK cells were found to have 48,000 EGF receptors with a K_d of 3.5×10^{-10} M on the basal surface. Substantially less binding was detectable on the apical surface of the MDCK cells. Less than 1% of the total tracer added bound to the cells; while some inhibition of binding was seen with a large excess of tracer EGF, the total binding of 0.8% was so low that a meaningful Scatchard could not be generated. Nonspecific binding of 125EGF to empty chambers was 1.0–1.5% of the total tracer added. This binding is equal to the nonspecific binding of EGF (not inhibitable by 200 ng/ml of unlabeled

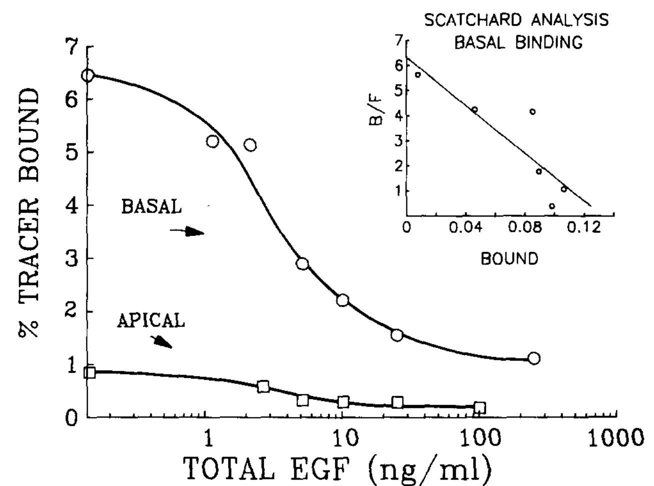

Figure 3. EGF-binding studies. 50,000 cpm of iodinated EGF were added to either the apical or the basal surface of MDCK cells. For the competition curve, binding studies were performed in the presence of cold EGF ranging between 1 and 200 ng/ml. Wells were incubated for 6 h at 4°C. After incubation, both the apical and basal surfaces were washed with cold PBS. Filters were detached from chambers using a razor, and counted in a gamma counter. Approximately 6% of the tracer bound to the basal surface. This binding was 50% inhibited by 5 ng/ml of cold EGF. Binding to the apical surface was significantly lower; less than 1% of the tracer bound. Analysis of Scatchard plot in the inset revealed 48,000 receptors per cell with a K_d of 3.5×10^{-10} M on the basal surface of the cells. Binding on the apical surface was so low that a meaningful Scatchard could not be derived.

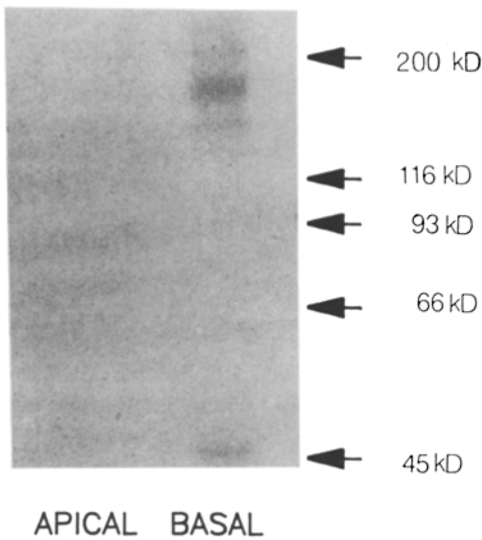

Figure 4. Cross-link of EGF to receptor. 151,000 cpm of ^{125}I-labeled tracer was added to either the basal or apical surface of MDCK cells grown on chambers. Cells were then incubated for 4 h at 4°C. Chambers were washed with cold PBS and scraped from the chamber using a rubber policeman. Cells were suspended in Hepes binding buffer (without BSA) containing the cross-linked reagent DSS for 30 min. Cells were collected by centrifugation, solubilized in Laemmli buffer. Samples were subjected to PAGE over 7.5% gels. After fixing and drying, gels were autoradiographed. On the left is the sample taken from cells exposed to EGF on the apical surface. The right lane shows sample from the basal surface. In this lane a 185-kD band corresponding to the molecular mass of the EGF receptor is visualized.

EGF) to the basal surface of MDCK cells, which we attribute to trapping of label in the nitrocellulose filter.

The presence of EGF receptors on the basal but not apical surface of MDCK cells was also confirmed by cross-linking radiolabeled EGF to its receptor using the reagent DSS. Iodinated EGF was added to either the basal or apical surface of the MDCK cells and exposed to cross-linking reagents as described in Materials and Methods. As can be seen in Fig. 4, labeled EGF added to the basal surface could be cross-linked to a 185-kD protein. This is the same as the molecular mass of the EGF receptor in a number of cells. We failed to detect binding of insulin to either the basal or the apical surface of the MDCK cells confirming the absence of insulin receptors on MDCK cells as has been previously reported (10).

Specific transport of EGF by MDCK cells in the basal-to-apical direction correlated with the presence of high affinity EGF receptors on the basal but not the apical surface of the cells. MDCK cells do not express insulin receptors on either the basal or the apical surface and no transport of insulin was noted in either direction. These data suggest that the transport of EGF is a receptor-mediated process.

We, therefore, expected that specific transport of EGF in the basal-to-apical direction should be inhibited by the addition of cold EGF. Transport studies in the presence of 0.85 nM, 17 nM, and 110 nM of unlabeled EGF were performed. As can be seen in Fig. 5, increasing amounts of unlabeled EGF in the transport buffer led to decreased transcytosis of labeled EGF. At 110 nM, active transcytosis of EGF was completely inhibited. We also evaluated the abilty of a large excess of cold EGF to affect transcytosis of EGF in the basal-to-apical direction. 110 nM cold EGF had no effect on the transcytosis of labeled EGF in the apical-to-basal direction.

Antibodies directed against the EGF receptor can inhibit binding of EGF to the receptor. One such antibody was available to us (kindly provided by Dr. S. Cohen). This antibody inhibited binding of EGF to MDCK cells at a 1:100 dilution. At the same dilution, it also inhibited the transport of EGF across the MDCK cell monolayer as can be seen in Fig. 6.

Next, the temperature dependence of the transcytosis was evaluated. Tracer EGF was added to cell monolayers on Millicell chambers at 4°C. Immediately after addition, chambers were incubated at 4, 15, or 37°C. As can be seen in Fig. 7, transport of EGF was markedly decreased at 15°C and even further decreased at 4°C.

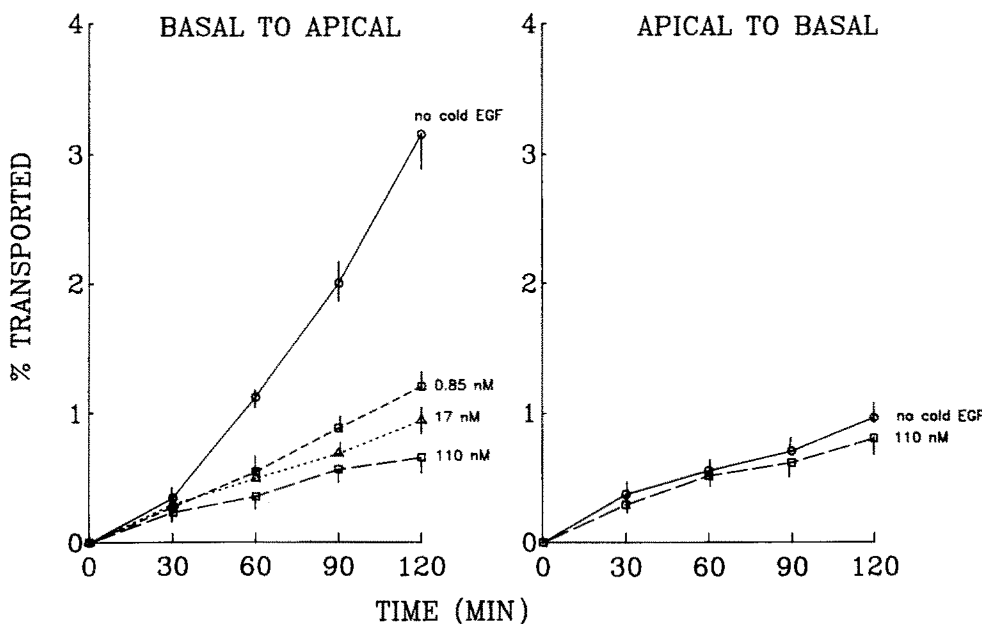

Figure 5. Effect of cold EGF on transport. Transport studies were performed as described in Materials and Methods in the presence of various concentrations of unlabeled EGF. (*Left*) Addition of cold EGF at a concentration of 0.85 nM (5 ng/ml) led to a substantial decrease in transport of tracer EGF. More inhibition was noted in the presence of 17 and 100 nM EGF. In contrast, addition of cold EGF at a concentration of 110 nM had only a minimal inhibitory effect on transport in the apical-to-basal direction, and this difference was not statistically significant (*right*).

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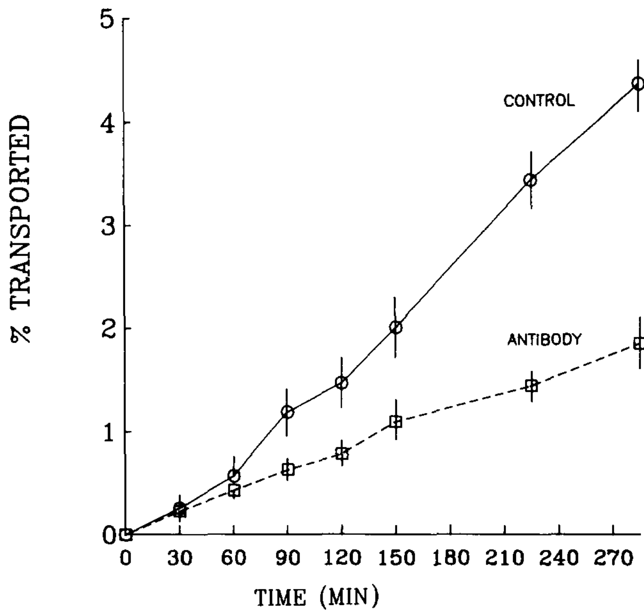

Figure 6. Effect of anti-receptor antibody on EGF transcytosis. Transport of tracer EGF was examined in the presence and absence of anti-EGF receptor antibody. Antibody, at a dilution of 1:100, was added to the basal surface of cells and preincubated for 60 min at 37°C. Tracer EGF was then added and samples were taken at 30-min intervals over 4.5 h. Each point represents the mean of triplicate wells and bars show the SD. Anti-receptor antibody inhibited transport by 70%.

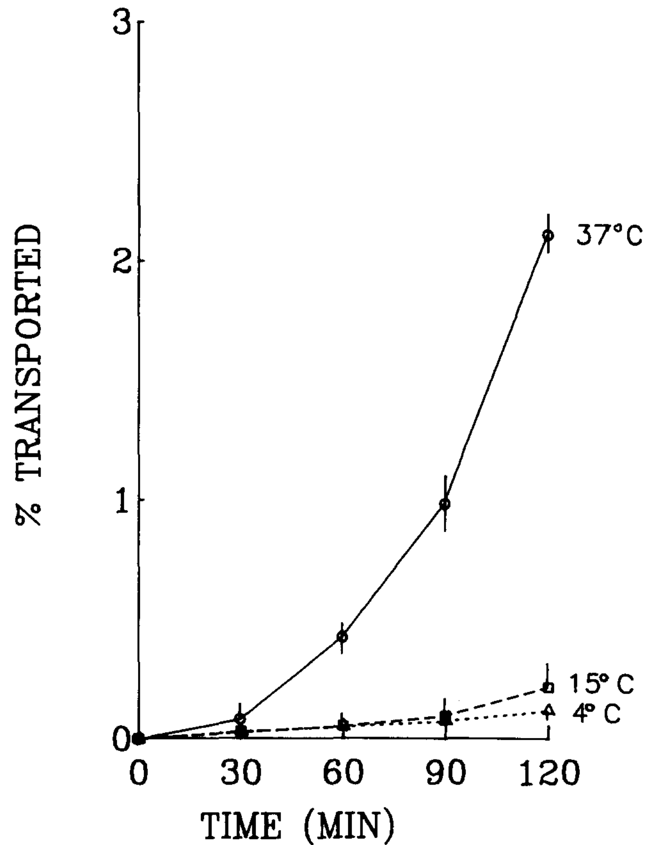

Figure 7. Temperature dependence of EGF transport. Transport of EGF from the basal to the apical surface was evaluated at 37, 15, and 4°C. Samples were taken as described. Each point represents the mean from triplicate wells and bars show the SD. Transport was temperature dependent. Cooling samples to 15°C almost completely inhibited the translocation from the basal to the apical surface.

To ensure that EGF transported across MDCK cells was intact, we examined tracer EGF using Sephadex column chromatography over a G-25 column. As can be seen in Fig. 8, the elution pattern of EGF tracer collected from the upper chamber after a transport experiment did not differ markedly from the elution pattern of control EGF tracer that had not been exposed to MDCK cells. In each case, intact EGF eluted in fractions 13-16 and degraded EGF eluted in the void volume. To calculate the percent of tracer degraded, we compared the area under the curve of the two peaks. For control EGF, the tracer was 89% intact and for the transported EGF the tracer was 78% intact. Hence, the tracer transported by the MDCK cells remains essentially intact.

We also compared the relative quanitities of tracer EGF bound and transported by the basal surface of the MDCK cells. [^{125}I]EGF was bound to the basal surface of MDCK cells for 2.5 h at 4°C. Unbound tracer was removed by washing three times with 1 ml of Hepes buffer. Fresh buffer was then added to each chamber and chambers were transferred to 37°C and sampled as described. EGF appeared at the apical surface within 15 min of warming. The amount of EGF transported, as can be seen in Fig. 9, was ~30% of the total tracer specifically bound to MDCK cells.

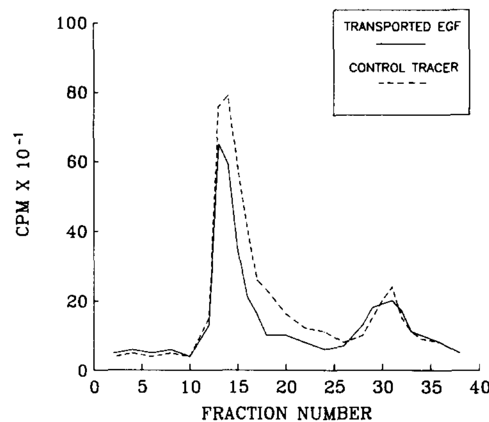

Figure 8. Analysis of degradation of transported tracer. Transport studies were performed under usual conditions except that 500,000 cpm was added to the basal surface. After a 2-h incubation at 37°C, counts were collected from the apical surface and subjected to column chromatography over a Sephadex G-50 column. The elution pattern of transcytosed EGF was compared with the elution pattern of tracer EGF not exposed to cells. In both cases, intact EGF eluted between fractions 12 and 15 and degraded EGF eluted in the void volume, fractions 26-35. Analysis of the area under the curves revealed that 89.5% of the control tracer and 78% of the transcytosed tracer eluted as intact EGF.

Discussion

Increasingly, transcellular transport of a variety of ligands is being recognized as an important mechanism by which a number of regulatory substances may reach their site of action (7, 9, 13, 24, 25, 34, 36). In specialized tissues such as the brain and the thyroid, transcytosis is the mechanism by which hormones and metabolic factors may enter and/or be

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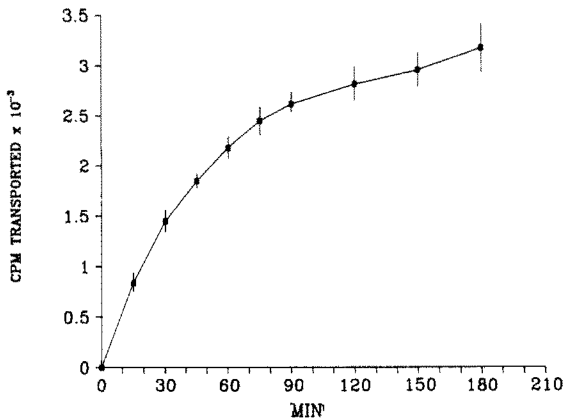

Figure 9. Relative proportion of bound EGF that is transported. Tracer EGF was added to the basal surface of MDCK cells and allowed to bind for 2.5 h at 4°C. At the end of this incubation, the basal surface was washed three times with cold PBS to remove unbound EGF. Chambers were warmed to 37°C and sampled at designated intervals over a 3-h period. Total EGF bound in this experiment was 10,222 cpm with a range of 587 cpm. Total transported at 3 h was 3,180 with a range of 400 cpm.

cleared from the systemic circulation (9, 36). While transcellular transport is likely to be an important physiologic process, the general nature of this phenomenon and the mechanism by which it occurs is not yet understood.

In this report, EGF was shown to be transported across the MDCK cells unidirectionally via a receptor-mediated process demonstrating that the EGF hormone receptors can function as transporters of hormones across an epithelial cellular barrier. The EGF receptors in the MDCK cells had similar binding affinities and molecular size as those previously reported in a variety of cells and can be recognized by antibody made against EGF receptors of another cell line (3, 5, 32). Similarly, we have previously shown that insulin may be transported in endothelial cells and that the endothelial cell insulin receptor has a similar molecular mass and subunit structure and tyrosine kinase as the adipocyte insulin receptor (6, 11, 12).

After binding of a ligand to its membrane receptor, ligand–receptor complexes are internalized rapidly. Once internalized via endocytotic vesicles, there appear to be a number of possible pathways for processing of the hormone–receptor complex. These pathways include targeting to lysosomes, recycling (33, 35), and transcytosis. Analysis of these pathways is complex as the hormone–receptor complex may remain intact or may dissociate. In this study, we have demonstrated that epidermal growth factor can be shunted via a transcytotic pathway. Similarly, when insulin and insulin-like growth factor are added to endothelial cells most of the internalized ligand is transported across the cell while a minor component is degraded. Multiple pathways may not be unique for these types of cells and may exist in all cells; however, the quantitative dynamics of hormone traffic in the different pathways may vary greatly. For example, the existence of a dual pathway for the processing of insulin in adipocytes has been demonstrated (17); however, in contrast to endothelial cells most of the internalized insulin is degraded and only a small fraction of the hormone is recycled undegraded. In addition, the sorting pathway is dependent on both the cell type and the ligand; e.g., we have shown that a large percent of insulin-like growth factor-II was degraded in endothelial cells, whereas insulin was not (8). While it has been postulated that serine phosphorylation of receptors may signal internalization (1, 26), the mechanism by which endocytic vesicles might be channeled via different pathways is unknown and it is unclear if the pathways involve continuous coupling of the hormone to the receptor.

EGF receptors are internalized rapidly and can be targeted for recycling or degradation or transcellular transport. Using the data from Scatchard analysis (Fig. 3) and the amount of EGF transported (Fig. 9) we calculated that the rate at which EGF is transported over the initial 30 min of the experiment is 1.5×10^7 EGF molecules per min. This amount of transport is certainly compatible with a receptor-mediated mechanism as there are 9.6×10^{10} receptors per well available for transport.

In addition to demonstrating that EGF transcytosis in MDCK cells is a receptor-mediated process, these data also show that the transport process is unidirectional, as has been seen for insulin in endothelial cells (13) and IgA secretory protein in MDCK cells (24, 25). The mechanism appears to be an uneven distribution of EGF receptors between the basal and apical plasma membrane. Binding and cross-linking studies revealed that most, if not all, of the EGF receptors are located on the basal surface suggesting that MDCK cells sort EGF receptors to the basal surface, a finding which is consistent with the known polarity of MDCK cells grown on dual-sided chambers (16, 23, 30). Such a mechanism for unidirectional transport might operate in other cells, such as endothelial cells. It is unclear if during transcytosis the hormone–receptor complex dissociates intracellularly and if receptor fusion at the apical surface is necessary.

Hence, specific transport of EGF by MDCK cells in the basal-to-apical direction correlated with the presence of high affinity EGF receptors on the basal but not the apical surface of the cells. MDCK cells do not express insulin receptors on either the basal or the apical surface and no transport of insulin was noted in either direction. These data suggest that the transport of EGF is a receptor-mediated process and the mechanism for the unidirectional transport is due to sorting of EGF receptors to the basal surface.

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