Remodeling the Cell Surface Distribution of Membrane Proteins during the Development of Epithelial Cell Polarity

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Abstract. The development of polarized epithelial cells from unpolarized precursor cells follows induction of cell–cell contacts and requires resorting of proteins into different membrane domains. We show that in MDCK cells the distributions of two membrane proteins, Dg-1 and E-cadherin, become restricted to the basal-lateral membrane domain within 8 h of cell–cell contact. During this time, however, 60–80% of newly synthesized Dg-1 and E-cadherin is delivered directly to the forming apical membrane and then rapidly removed, while the remainder is delivered to the basal-lateral membrane and has a longer residence time. Direct delivery of >95% of these proteins from the Golgi complex to the basal-lateral membrane occurs >48 h later. In contrast, we show that two apical proteins are efficiently delivered and restricted to the apical cell surface within 2 h after cell-cell contact. These results provide insight into mechanisms involved in the development of epithelial cell surface polarity, and the establishment of protein sorting pathways in polarized cells.

POLARIZED epithelial cells arise during development through the conversion of nonpolarized precursor cells (reviewed in Saxen et al., 1968; Ekblom, 1989). Specific cell–cell and cell–substratum contacts are required to initiate this developmental process (Klein et al., 1988; Ekblom et al., 1986), which involves the expression of new gene products specific to the epithelium, and the redistribution of proteins that were constitutively expressed in the nonpolarized precursor cells in line with new functional requirements of polarized epithelial cells (reviewed in Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). During this time, the cell surface distribution of proteins becomes restricted to structurally and functionally distinct membrane domains, apical and basal-lateral. The restriction of protein distributions to these membrane domains directly influences the ability of cells to perform vital physiological functions in the vectorial transport of ions and solutes across the epithelium (Berridge and Oschman, 1972; Almers and Stirling, 1984).

The mechanisms involved in the establishment of restricted cell surface distributions of proteins and the formation of membrane domains during the development of polarized epithelial cells are poorly understood. Analysis of the differentiation of polarized epithelial cells in vitro, such as MDCK epithelial cells, provides an experimental approach to this problem (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). In the absence of intercellular contacts single MDCK cells display a uniform distribution of many basal-lateral membrane proteins over the entire cell surface (Balcarova-Ständer et al., 1984), while some apical membrane proteins appear to be localized to the free cell surface (Vega-Salas et al., 1987; Ojakian and Schwimmer, 1988). Induction of contact between MDCK cells through the Ca^{2+}-dependent cell adhesion molecule E-cadherin (Gumbiner et al., 1988) results in the gradual restriction of protein distributions to the apical or basal-lateral membrane domains, and the differentiation of these cells into a structurally and functionally polarized epithelium (Balcarova-Ständer et al., 1984; Vega-Salas et al., 1987).

MDCK cells that have already established a polarized distribution of membrane proteins sort both viral and endogenous glycoproteins in the Golgi complex and then deliver them directly to either the apical or basal-lateral plasma membrane (Rodriguez-Boulan and Sabatini, 1978; Matlin and Simons, 1984; Gottlieb et al., 1986a; Griffiths and Simons, 1986; Stephens and Compans, 1986; Lisanti et al., 1989). The direct delivery of newly synthesized proteins to the appropriate membrane domain provides an explanation for how cells maintain a polarized distribution of cell surface proteins in spite of protein loss from the membrane through endocytosis and degradation. However, it is not known when direct delivery of proteins to specific plasma membrane domains occurs after induction of cell–cell contact, or whether this mechanism plays a role in the establishment of cell surface polarity.

In this study we have investigated how the cell surface distributions of four proteins become restricted to either the apical or basal-lateral membrane during the development of MDCK cell polarity after induction of cell–cell contact. We show that upon induction of cell–cell contact two cell adhesion proteins, DG-1 (Koch et al., 1990) and E-cadherin (Takeichi, 1990), are primarily delivered to the forming api-

cal membrane, but rapidly become restricted to the basal-lateral membrane. Detailed kinetic analysis shows that cell surface polarity of these proteins appears to be generated through the rapid removal of proteins after their delivery to the apical membrane and retention of proteins present in the basal-lateral membrane. The polarized cell surface distributions of these proteins are subsequently maintained by direct delivery of newly synthesized proteins to the basal-lateral membrane which occurs >48 h after the induction of cell–cell contact. In contrast, we show that the polarized distributions of two apical proteins, a membrane protein gp 135/170 (Ojakian and Schwimmer, 1988) and a secreted protein gp 81 (Kondor-Koch et al., 1985; Gottlieb et al., 1986b; Urban et al., 1987), are generated by direct delivery of newly synthesized proteins to the apical membrane immediately after induction of cell–cell contact. These results provide novel insight into how cell surface domains are established and maintained in polarized epithelial cells.

Materials and Methods

Growth and Maintenance of Cells

MDCK cells were maintained in DME/FBS as described previously (Nelson and Veshnock, 1987a). Before all experiments, cells were grown for 2 d at low cell density (see Nelson and Veshnock, 1987a). Cells were trypsinized and plated at confluent density (2.5 × 10^6 cells/filter) on collagen-coated polycarbonate filters (Transwell filters, 0.45 µm; Costar Corp., Cambridge, MA) in medium containing 5 µM Ca^{2+}, and grown overnight at 37°C. Cell–cell contact was induced synchronously across the monolayer by raising the Ca^{2+} concentration of the growth medium to 1.8 mM (Nelson and Veshnock, 1987a).

Biotinylation and Immunoprecipitation

Cells were rinsed three to five times with ice-cold Hepes-buffered Ringer's buffer (154 mM NaCl, 7.2 mM KCl, 2.25 mM CaCl$_2$, 10 mM Hepes, pH 7.4), and then incubated for 15 min at 4°C in 1 ml Hepes-buffered Ringer's buffer containing 0.2 mg NHS-S-S-biotin (Pierce Chemical Co., Rockford, IL) on a rocking platform. The biotin was first made up as a 100× stock in DMSO, then diluted immediately before use. The filters were rinsed twice with Tris-saline/PMSF (15 mM Tris, pH 7.5, 120 mM NaCl, 1 mM PMSF), then solubilized with CSK buffer (50 mM NaCl, 10 mM Pipes, pH 6.8, 3 mM MgCl$_2$, 0.5% Triton X-100, 300 mM sucrose, 1.2 mM PMSF, 100 µg/ml RNAse, 100 µg/ml DNAse [Boehringer Mannheim Corp., Indianapolis, IN]) for 20 min. The cells were scraped from the filter with a rubber policeman, then sedimented in a Beckman microfuge for 5 min. The soluble supernatant was collected. The cell pellet was triturated in 100 µl SDS immunoprecipitation buffer (15 mM Tris, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1.0% SDS), incubated at room temperature for 5 min, and then brought to 1 ml with CSK buffer.

The soluble and insoluble fractions were immunoprecipitated by the addition of 5 µl Dg-1 antiserum (Pasdar and Nelson, 1989), 20 µl E-cadherin monoclonal antibody supernatant (a gift from Dr. W. Gallin, University of Alberta), or 15 µl E-cadherin antiserum (Nelson et al., 1990) for 1 h each, or by 30 µl gp 135/170 monoclonal antibody (a gift from Dr. G. Ojakian, SUNY, Brooklyn). 75 µl of a 10% suspension of protein A–Sepharose 4B beads (Pharmacia Fine Chemicals, Piscataway, NJ) were included with the immunoprecipitates. Rabbit anti–mouse secondary antiserum was added to all samples immunoprecipitated with mouse monoclonal antibodies. The samples were washed sequentially with high stringency buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 1% [wt/vol] Na-deoxycholate, 0.1% [wt/vol] SDS, 120 mM NaCl, 25 mM KCl) and high salt buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100 0.1% SDS, 1% deoxycholate, 1 M NaCl).

Immunoprecipitated proteins were washed once with cold dH$_2$0, and then removed from the protein A–Sepharose 4B beads by two successive incubations of 10 min each in 200 µl 0.1 M glycine, pH 2.5. The supernatants were pooled and then neutralized by the addition of 40 µl 1M Tris-HCl, pH 7.5, 25 µl 10% BSA, and 5.5 µl 1 N NaOH (Matter et al., 1990a). Biotinylated proteins were precipitated by the addition of 75 µl avidin-agarose

(Pierce Chemical Co.) for 1 h and the samples were washed as above. Protein samples were incubated in SDS sample buffer for 30 min at room temperature before analysis by SDS-PAGE. Metabolically labeled samples were reduced with 200 mM DTT. The polyacrylamide gels were processed for fluorography (Nelson and Veshnock, 1987a) and protein bands were quantitated by scanning densitometry.

Detection of Cell Surface Biotinylated Proteins

Cells were biotinylated on either the apical or basal-lateral membrane and processed for immunoprecipitation as described above. Nonreduced samples were separated by SDS-PAGE, then electrophoretically transferred to nitrocellulose (Towbin et al., 1979). The nitrocellulose filters were blocked overnight in gelatin wash buffer (15 mM Tris-HCl, pH 7.5, 120 mM NaCl, 5 mM NaN$_3$, 1 mM EDTA, 0.1% [vol/vol] Tween-20, 0.1% gelatin) with 5% (wt/vol) BSA, then incubated in gelatin wash buffer containing ^{125}I-streptavidin (~10 µCi/µg) for 1 h or processed with an ABC-alkaline phosphatase kit (Vector Laboratories, Inc., Burlingame, CA). The blots were washed extensively with gelatin wash buffer before autoradiography or color development.

Metabolic Labeling

Cell cultures were initially established in DME/FBS. For metabolic labeling, the medium was changed to DME/FBS without methionine (DME/FBS-Met). After 15 min the medium was removed and 250 µCi [^{35}S]methionine/cysteine (Du Pont NEN, Boston, MA) was added from the basal-lateral side of the Transwell filter in a total volume of 100 µl DME/FBS-Met; 1,000 µl DME/FBS-Met was added to the apical compartment. The cells were incubated for 1 h at 37°C before biotinylation.

Analysis of Residence Time of Proteins on the Cell Surface

Cells were preincubated in methionine-free medium for 15 min, then labeled from the basal-lateral side of the Transwell filters with [^{35}S]methionine/cysteine for 15 min in medium containing 1.8 mM Ca^{2+} as described above. The medium was discarded and the cells were washed twice in DME/FBS containing a 10,000-fold excess of unlabeled methionine and then incubated for the indicated periods (chase period) before being processed for biotinylation.
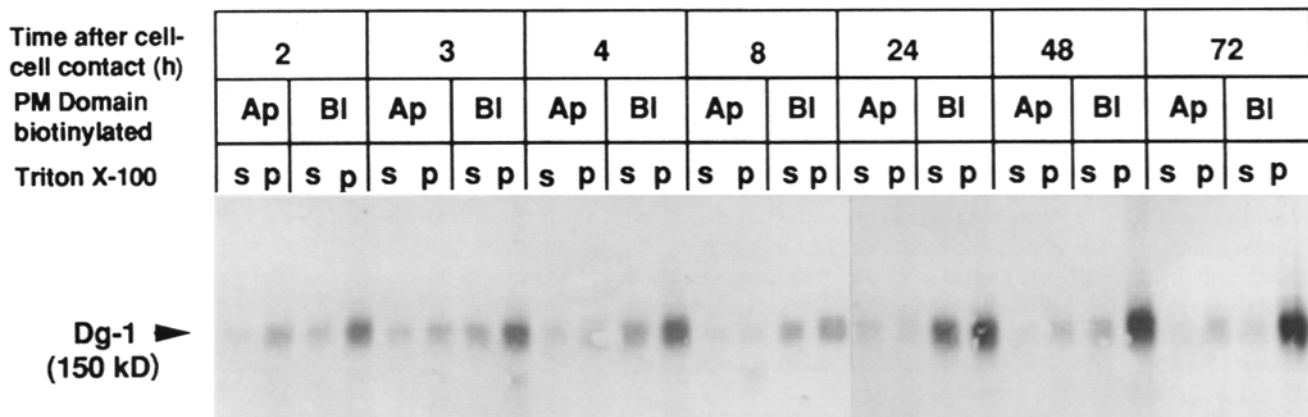
Delivery of Newly Synthesized Secreted Protein (gp81) to the Cell Surface

Cells were incubated in DME/FBS containing 5 µM Ca^{2+} without methionine for 15 min, then labeled in the same medium with 250 µCi [^{35}S]methionine/cysteine for an additional 15 min as described above. The cells were washed with DME/FBS containing 1.8 mM Ca^{2+} and then washed again at the indicated times. 1 ml fresh medium was added to each side for 5 min. 50 µl of apical or basal-lateral medium was collected at each time point, 12.5 µl 4× SDS sample buffer without DTT was added to the aliquot of medium, and the samples were analyzed by SDS-PAGE followed by fluorography.

Results

Induction of Cell–Cell Contact and Cell Surface Biotinylation

MDCK cell cultures were established at confluent density on collagen-coated Transwell polycarbonate filters in DME/FBS containing 5 µM Ca^{2+}. In the presence of 5 µM Ca^{2+}, MDCK cells do not form cell–cell contacts due to inactivation of the Ca^{2+}-dependent cell adhesion molecule E-cadherin (Ozawa et al., 1990), and do not have functional tight junctions (Gonzalez-Mariscal et al., 1985). Raising the Ca^{2+} concentration of the growth medium to 1.8 mM causes a conformational change in E-cadherin (Ozawa et al., 1990) and results in the rapid and synchronous induction of intercellular contact across the monolayer and the formation

Time after cell-cell contact (h)	2				3				4				8				24				48				72			
PM Domain biotinylated	Ap		Bl		Ap		Bl		Ap		Bl		Ap		Bl		Ap		Bl		Ap		Bl		Ap		Bl	
Triton X-100	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p

Dg-1 ► (150 kD)

Figure 1. Distribution of cell surface Dg-1 after induction of cell–cell contact. At the indicated times after induction of cell–cell contacts, cells on pairs of polycarbonate (Transwell) filters were biotinylated on either the apical (*Ap*) or basal-lateral (*Bl*) membrane. Cells were extracted with CSK buffer (see Materials and Methods) and Dg-1 was immunoprecipitated with specific antibodies from the CSK-soluble (*s*) and -insoluble (*p*) pools of proteins. The immunoprecipitates were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose, and biotinylated Dg-1 was detected with an ABC-alkaline phosphatase kit (Vector Laboratories, Inc.). The results of a representative experiment from three separate trials are presented.
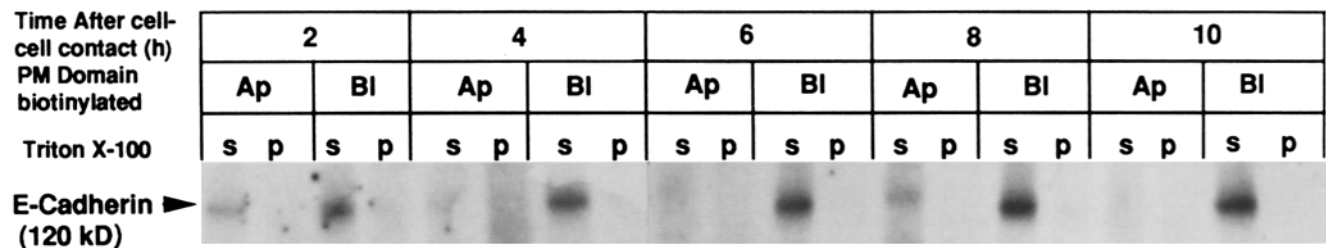
of intercellular junctions (Gonzalez-Mariscal et al., 1985; Gumbiner et al., 1988).

The distribution of proteins on the forming apical and basal-lateral membrane domains was determined quantitatively by labeling either the apical or basal-lateral cell surface of pairs of filters with the membrane-impermeable biotinylating reagent, NHS-S-S-biotin (Sargiacomo et al., 1989). Selective biotinylation of each membrane domain required the formation of tight junctions to prevent diffusion of the reagent from one compartment of the Transwell filter to the other compartment. The presence of tight junctions after induction of cell–cell contact was determined quantitatively by measuring the diffusion of a labeled tracer, [³H]inulin, across the monolayer. Diffusion of [³H]inulin across the cell monolayer decreased to <0.5% of the input amount after 2 h following the induction of cell–cell contact, indicating formation of tight junctions sufficient to block passage of the biotinylating reagent between compartments of the Transwell filter (Hammerton et al., 1991). This was the earliest time that the cell surface distribution of membrane proteins was assessed after cell–cell contact.

Kinetics of Development of Basal–Lateral Cell Surface Polarity

The development of cell surface polarity of Dg-1 and E-cadherin, two well-characterized (basal)-lateral membrane cell adhesion proteins, was determined. At various times after the induction of cell–cell contact, cells on pairs of identical filters were labeled with NHS-S-S-biotin on either their apical or basal-lateral membrane as described above and then extracted. Dg-1 and E-cadherin were selectively immunoprecipitated from cell extracts with specific antibodies, separated by SDS-PAGE, and electrophoretically transferred to nitrocellulose filters (see Materials and Methods). Biotinylated proteins were revealed by probing the nitrocellulose filters with either an avidin–biotin conjugate linked to alkaline phosphatase (Fig. 1), or ¹²⁵I-streptavidin (Fig. 2); similar results were obtained with either reagent.

Analysis of the cell surface distribution of Dg-1 after 2 h following induction of cell–cell contact showed that 50–60% of the total amount of Dg-1 that was biotinylated on the cell surface was restricted to the basal-lateral membrane domain (Fig. 1). After 8 h, >95% of total Dg-1 was found to be re-

Time After cell-cell contact (h)	2				4				6				8				10			
PM Domain biotinylated	Ap		Bl		Ap		Bl		Ap		Bl		Ap		Bl		Ap		Bl	
Triton X-100	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p

E-Cadherin ► (120 kD)

Figure 2. Distribution of cell surface E-cadherin after induction of cell–cell contact. At the indicated times after induction of cell–cell contacts, cells on pairs of polycarbonate (Transwell) filters were biotinylated on either the apical (*Ap*) or basal-lateral (*Bl*) membrane. Cells were extracted with CSK buffers (see Materials and Methods) and E-cadherin was immunoprecipitated with specific antibodies from the CSK-soluble (*s*) and -insoluble (*p*) pools of proteins. The immunoprecipitates were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose, and biotinylated E-cadherin was detected with ¹²⁵I-streptavidin followed by fluorography. The results of a representative experiment from three separate trials are presented.

stricted to the basal-lateral surface. This polarized distribution of Dg-1 on the basal-lateral surface was maintained for the duration of the experiment, up to 72 h (Fig. 1). Analysis of the solubility properties of Dg-1 in buffer containing Triton X-100 showed that the protein accumulated in the basal-lateral membrane in a pool of protein that was insoluble in Triton X-100 (see Fig. 1).

A similar analysis was performed to determine the kinetics of development of cell surface distribution of E-cadherin after cell-cell contact (Fig. 2). 2 h after the induction of cell-cell contact 60-75% of E-cadherin that was biotinylated on the cell surface was restricted to the basal-lateral plasma membrane; within 4 h, >95% of cell surface E-cadherin was detected on the basal-lateral membrane (Fig. 2). This polarized distribution of E-cadherin on the basal-lateral membrane was maintained subsequently for the duration of the experiment. Analysis of the extractability of E-cadherin during this time period revealed that the protein was soluble in buffers containing Triton X-100.

Kinetics of Development of Direct Delivery of Newly Synthesized Membrane Proteins to the Basal-Lateral Cell Surface

We investigated whether vectorial delivery of newly synthesized proteins to the forming basal-lateral membrane contributed to the rapid development of cell surface polarity of Dg-1 and E-cadherin. A previous study reported that E-cadherin is delivered directly to the basal-lateral membrane domain in fully polarized MDCK cells (Le Bivic et al., 1990); the delivery of newly synthesized Dg-1 to different membrane domains in polarized epithelial cells has not previously been examined. At different times after the induction of cell-cell contact, cells on pairs of identical filters were metabolically labeled with [^{35}S]methionine/cysteine for 1 h. At the end of this labeling period, the apical and basal-lateral cell surfaces, respectively, were biotinylated on pairs of filters with NHS-S-S-biotin for 15 min at 4°C. A time line is shown in Fig. 3 A to illustrate this experimental protocol. This protocol was used to label the first wave of newly synthesized protein that had been delivered directly from the Golgi complex to each plasma membrane domain. It should be noted that both newly synthesized Dg-1 (Pasdar and Nelson, 1989) and E-cadherin (Shore and Nelson, 1991) begin to arrive at the plasma membrane ~1 h after synthesis. Cells were extracted and biotinylated proteins were affinity isolated by first immunoprecipitating proteins with specific antibodies, followed by precipitation of the biotinylated pool of those immuno-isolated proteins with avidin-agarose. Proteins were identified by SDS-PAGE and fluorography, and the relative amounts of biotinylated proteins were quantitated by scanning densitometry.

Fig. 3, B and C, shows the relative proportion of total newly synthesized Dg-1 that was delivered to either the forming apical or basal-lateral plasma membranes at different times after the induction of cell-cell contact. 8 h after the induction of cell-cell contact, when cell surface polarity of this protein was already established (see Fig. 1), only 26% of newly synthesized Dg-1 on the cell surface had been delivered to the basal-lateral membrane; the remainder (74%) had been delivered to the apical membrane (Fig. 3 C). At later times we detected a four- to fivefold increase in the

overall amount of newly synthesized Dg-1 delivered to the cell surface (Fig. 3 B). 24 h after cell-cell contact, the percentage of Dg-1 delivered to the basal-lateral membrane remained at ~20% (Fig. 3, B and C). Subsequently, the percentage of newly synthesized Dg-1 delivered directly to the basal-lateral membrane increased. 36 h after induction of cell-cell contact, 71% of newly synthesized Dg-1 that arrived at the cell surface was delivered directly to the basal-lateral membrane, and by 72 h the percentage had increased to >95% (Fig. 3, B and C).

The kinetics of development of direct delivery of E-cadherin to the basal-lateral membrane were similar to that of Dg-1. Fig. 3, B and D, shows that 12 and 24 h after the induction of cell-cell contact 72 and 66%, respectively, of newly synthesized E-cadherin that arrived at the cell surface had been inserted directly into the apical membrane; note that at these times the cell surface distribution of E-cadherin was already restricted to the basal-lateral membrane (see Fig. 2). The percentage of total newly synthesized E-cadherin that was delivered directly to the basal-lateral membrane increased to 75% by 36 h (Fig. 3, B and D), and to 94% by 72 h after the induction of cell-cell contact (Fig. 3, B and D).

Residence Time of Basal-Lateral Membrane Proteins Delivered to the Apical and Basal-Lateral Cell Surfaces
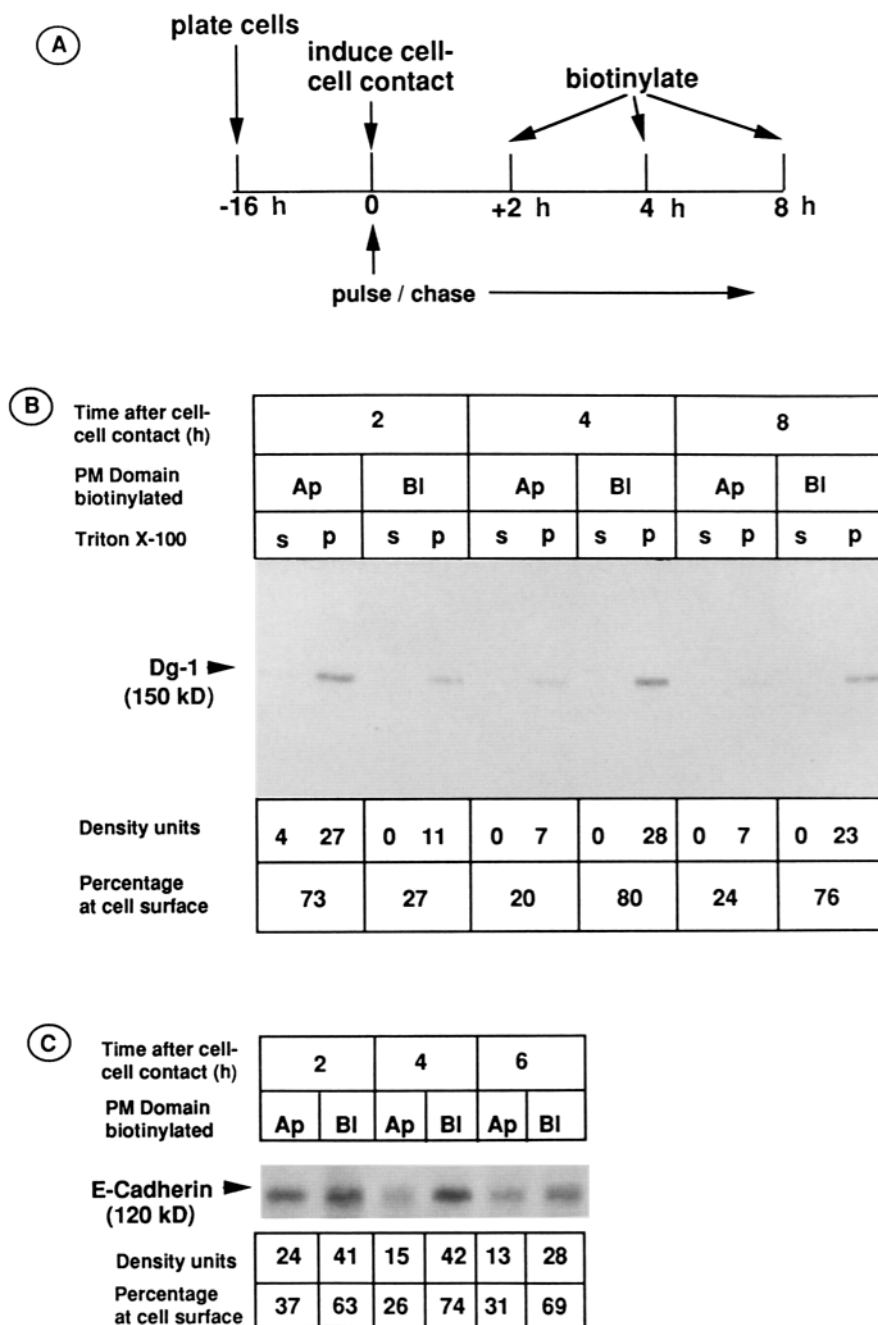
The data presented above show that cell surface polarity of two basal-lateral membrane proteins develops within a few hours after induction of cell-cell contact. However, direct delivery of newly synthesized Dg-1 and E-cadherin to the basal-lateral membrane does not happen until 36 or more hours after the induction of cell-cell contact. Thus, direct delivery of newly synthesized proteins to the basal-lateral membrane cannot account for the rapid establishment of cell surface polarity of these proteins.

We investigated the possibility that cell surface polarity of these proteins developed as a consequence of the removal of proteins delivered to the apical membrane and retention of proteins delivered to the basal-lateral membrane. To determine the fate of proteins delivered to different membrane domains, we measured the length of time (termed residence time) that newly synthesized proteins remained on a given cell surface domain after delivery from the Golgi complex. Cells were metabolically labeled for 15 min with [^{35}S]methionine/cysteine at the time that cell-cell contact was induced. Then, cells were chased in medium containing an excess of unlabeled methionine for different lengths of time. At various times during the chase period, the cell surfaces of identical pairs of filters were biotinylated for 15 min at 4°C on either the apical or basal-lateral plasma membrane as described above. Fig. 4 A shows a scheme of this experimental protocol. Dg-1 and E-cadherin were immunoprecipitated from cell extracts with specific antisera, and the biotinylated pools of each protein were affinity isolated with avidin-agarose and identified by SDS-PAGE and fluorography. The amounts of biotinylated protein were quantitated by densitometric scanning, and the values obtained were expressed either as a percentage of the total cell surface protein located on the apical or basal-lateral membrane, or as a total amount expressed in density units (U).

After 2 h following induction of cell-cell contact and
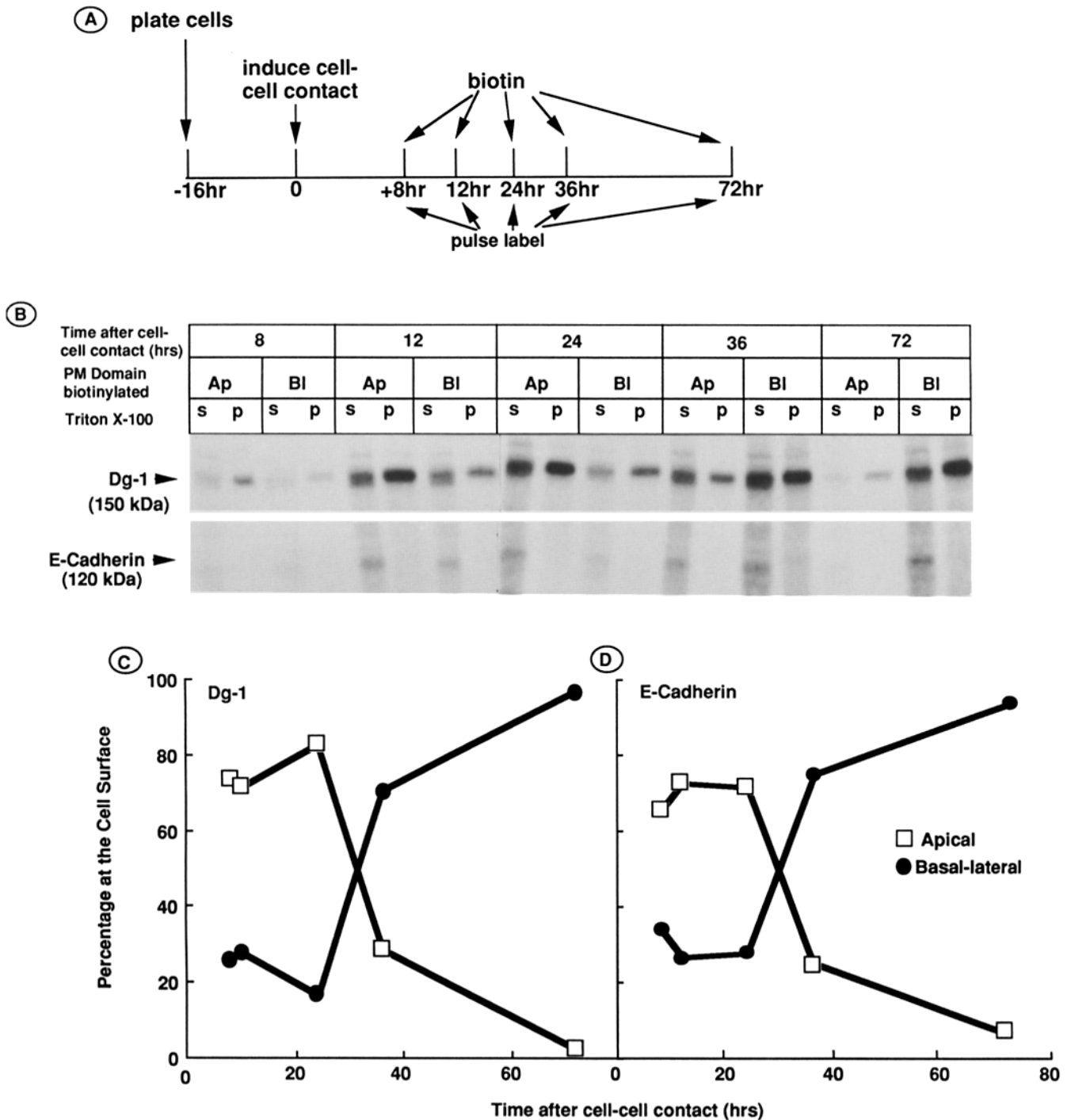
Figure 3. Delivery of newly synthesized Dg-1 (*B, C*) and E-cadherin (*B, D*) to the cell surface after induction of cell–cell contact. (*A*) Scheme of the experimental protocol used to determine the delivery of newly synthesized proteins to the cell surface. Confluent monolayers of cells were established on polycarbonate (Transwell) filters in DME/FBS containing 5 μM Ca^{2+} to inhibit cell–cell contact. Cell–cell contact was subsequently induced by raising the Ca^{2+} concentration of the growth medium to 1.8 mM. At different times after induction of cell–cell contact (*A, B*), cells on pairs of filters were pulse-labeled with [^{35}S]methionine/cysteine for 1 h and then biotinylated on either the apical (*Ap*) or basal-lateral (*Bl*) membrane. Cells were extracted with CSK buffer (see Materials and Methods) and the soluble (*s*) and insoluble (*p*) pools of protein were sequentially precipitated with specific antibodies to Dg-1 (*B, C*) or E-cadherin (*B, D*) and then avidin–agarose. The immunoprecipitates were processed for SDS-PAGE and fluorography (*B*), and the amount of protein was determined by scanning densitometry (*C, D*). The results of a representative experiment from four separate trials are presented.

(A) plate cells / induce cell-cell contact / biotinylate

-16 h — 0 — +2 h — 4 h — 8 h

pulse / chase ————→

(B)

Time after cell-cell contact (h)	2		2		4		4		8		8	
PM Domain biotinylated	Ap		Bl		Ap		Bl		Ap		Bl	
Triton X-100	s	p	s	p	s	p	s	p	s	p	s	p
Dg-1 (150 kD)												
Density units	4	27	0	11	0	7	0	28	0	7	0	23
Percentage at cell surface	73		27		20		80		24		76	

(C)

Time after cell-cell contact (h)	2		4		6	
PM Domain biotinylated	Ap	Bl	Ap	Bl	Ap	Bl
E-Cadherin (120 kD)						
Density units	24	41	15	42	13	28
Percentage at cell surface	37	63	26	74	31	69

Figure 4. Residence time of newly synthesized Dg-1 (*B*) and E-cadherin (*C*) on the cell surface after induction of cell–cell contacts. (*A*) Scheme of the experimental protocol used. Confluent monolayers of cells were established on polycarbonate (Transwell) filters in DME/FBS containing 5 μM Ca^{2+} to inhibit cell–cell contact. Cell–cell contact was subsequently induced by raising the Ca^{2+} concentration of the DME/FBS to 1.8 mM. At the time of induction of cell–cell contacts, cells were pulse-labeled for 15 min with [^{35}S]methionine/cysteine and then incubated for different lengths of chase time in the absence of labeled amino acids. At those times (*B, C*) cells on pairs of filters were biotinylated on either the apical (*Ap*) or basal-lateral (*Bl*) cell surface, extracted with CSK buffer (see Materials and Methods), and the soluble (*s*) and insoluble (*p*) pools of protein were immunoprecipitated with either Dg-1 (*B*) or E-cadherin (*C*) antibodies followed by avidin-agarose. The precipitated proteins were processed for SDS-PAGE and fluorography (*B, C*). Proteins were quantitated by scanning densitometry, and the amounts are expressed either as a percentage of the total on the apical and basal-lateral membrane, or as a total amount (density units). Results of a representative experiment from three separate trials are presented.

metabolic labeling, 27% of newly synthesized Dg-1 expressed on the cell surface was detected on the basal-lateral membrane (Fig. 4 *B*). After 4 h of chase, that proportion increased to 80% (Fig. 4 *B*). It is possible that this increase is due to delayed delivery of labeled Dg-1 to the cell surface. However, this is unlikely since all Dg-1 that is synthesized under these culture conditions is delivered to the cell surface within 90 min of the end of the pulse label period (see Pasdar and Nelson, 1989).

Analysis of the detectable amounts of Dg-1 on the apical and basal-lateral surfaces at different times during the chase period provides insight into the source of the increase in the amount of Dg-1 in the basal-lateral membrane during this chase period. Between 2 and 4 h, the amount of newly syn-

thesized Dg-1 detected in the basal-lateral membrane increased from 11 to 28 U (Fig. 4 *B*). Concomitantly, the amount of newly synthesized Dg-1 detected on the apical membrane decreased from 27 U, which represented the initial amount of newly synthesized Dg-1 on the apical membrane 2 h after synthesis, to 7 U after 4 h; the estimated residence time $t_{1/2}$ of Dg-1 in the apical membrane was 30–60 min (Fig. 4 *B*) as determined by regression analysis. The removal of Dg-1 from the apical membrane domain and concomitant accumulation of Dg-1 in the basal-lateral membrane can account for the rapid development of cell surface polarity of Dg-1 in the absence of direct delivery of newly synthesized protein to the basal-lateral membrane.

The residence time of newly synthesized E-cadherin on

different membrane domains was also analyzed. 2 h after metabolic labeling and cell–cell contact, 63% of newly synthesized E-cadherin detected on the cell surface was localized to the basal-lateral membrane (Fig. 4 C). After 4 h 74% was located on the basal-lateral membrane (Fig. 4 C). The detectable amounts of newly synthesized E-cadherin were analyzed on each cell surface domain during the chase periods. Results showed that protein is lost rapidly from the apical membrane (estimated residence time $t_{1/2}$ <2 h by regression analysis), but there was no concomitant increase in the amount of protein in the basal lateral membrane (Fig. 4 C). The amount of E-cadherin in the basal-lateral membrane also began to decrease after 6 h of chase (Fig. 4 C).

These results show that restricted distributions of Dg-1 and E-cadherin in the basal-lateral membrane appear to be generated after delivery of newly synthesized protein to the cell surface by rapid removal of protein from the apical membrane and, in the case of Dg-1, delivery of that protein to the basal-lateral membrane. In addition, the accumulation of proteins in the basal-lateral membrane is due to longer residence time compared with proteins that were inserted into the apical membrane.

Kinetics of Development of Cell Surface Polarity of a Marker Protein of the Apical Membrane

The development of apical cell surface polarity was analyzed using a monoclonal antibody specific for a marker protein of the apical membrane, gp 135/170 (Ojakian and Schwimmer, 1988). Within 2 h of cell–cell contact, the earliest time that could be analyzed (see above), we detected 80% of the total cell surface gp 135/170 on the apical membrane (Fig. 5). The polarized cell surface distribution of gp 135/170 was maintained 8 h after cell–cell contact (Fig. 5). This result indicates that cell surface polarity of gp 135/170 is established at the time of, or shortly after the induction of cell–cell contact. It is noteworthy that in the absence of cell–cell contact gp 135/170 is localized to the cell surface not in contact with the substratum (Ojakian and Schwimmer, 1988). It is probable, therefore, that cell surface polarity of gp 135/170 was established before induction of cell–cell contact.

Development of Direct Delivery of Newly Synthesized Proteins to the Apical Cell Surface

Given that the cell surface polarity of gp 135/170 was established at the time of, or shortly after cell–cell contact, we sought to determine whether newly synthesized apical proteins were directly delivered to the apical cell surface at this time. Within the first few hours after induction of cell–cell contact, we detected little or no delivery of newly synthesized gp 135/170 to the cell surface. However, 10 h after the induction of cell–cell contact, we detected delivery of newly synthesized gp 135/170 to the cell surface; 67% of the protein that arrived at the cell surface was delivered directly to the apical membrane (Fig. 6, A and B). The increase in appearance of newly synthesized gp 135/170 at 10 h reflects an increase in total cellular protein synthesis that occurs after 10–12 h of cell–cell contact (see also Fig. 3). Within 24 h after cell–cell contact, 76% of cell surface gp 135/170 was delivered directly to the apical membrane (Fig. 6, A and B).

To determine whether apical proteins were delivered directly to the plasma membrane at the time of cell–cell con-
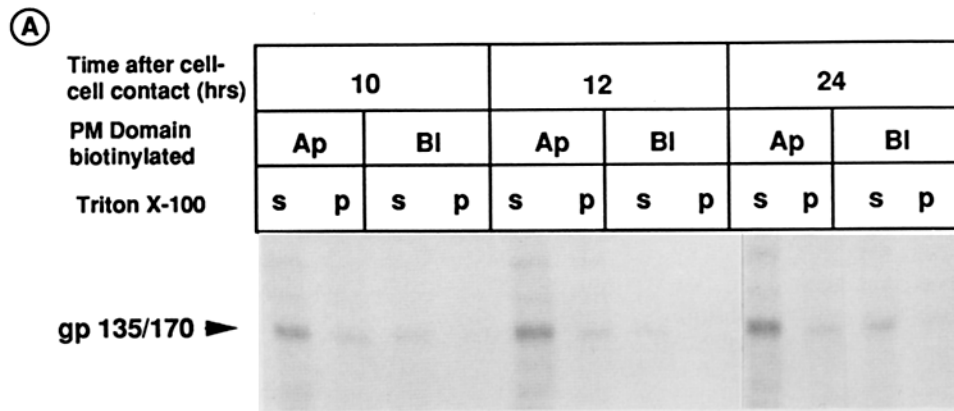

Time after cell-cell contact (hrs)

	2		4	
PM domain biotinylated	Ap	Bl	Ap	Bl
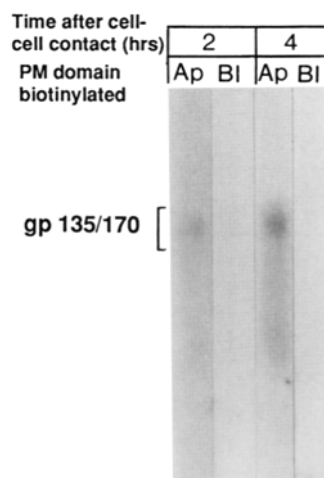
gp 135/170

Figure 5. Distribution of cell surface gp 135/170 after induction of cell–cell contact. At the indicated times after induction of cell–cell contacts, cells on pairs of polycarbonate (Transwell) filters were biotinylated on either the apical (*Ap*) or basal-lateral (*Bl*) membrane. Cells were extracted with CSK buffer (see Materials and Methods) and gp 135/170 was immunoprecipitated with specific antibodies from the CSK-soluble pool of proteins. The immunoprecipitates were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose, and biotinylated gp 135/170 was detected with ^{125}I-streptavidin followed by fluorography. The results of a representative experiment from three separate trials are presented.

tact, we chose to analyze the direction of apical protein secretion. Only short times (<5 min) are required to sample the media for the accumulation of secreted proteins in the apical and basal-lateral compartments; for this time period the cell monolayer is sufficient to form a barrier to protein diffusion between compartments in the absence of functional tight junctions (data not shown). We analyzed the direction of secretion of a glycoprotein, termed gp 81. Previous studies have shown that gp 81 is the predominant protein secreted from polarized MDCK cells into the apical medium (Kondor-Koch et al., 1985; Gottlieb et al., 1986b; Urban et al., 1987), and is detected simply by analysis of culture medium by SDS-PAGE without reduction of disulfide bonds (Fig. 7). Confluent monolayers of MDCK cells were pulse-labeled with [35S]methionine/cysteine at the same time that cell–cell contact was induced. Then, at the times indicated, the medium was removed and 1 ml DME was added back to both the apical and basal-lateral compartments for 5 min. 50 μl of the media was collected from the apical and basal-lateral compartments, respectively, for analysis by SDS-PAGE. The results show that 15 min after the induction of cell–cell contact, >85% of gp 81 that is secreted into the medium is found in the apical compartment (Fig. 7). This result strongly indicates that the delivery of newly synthesized gp 81 from the Golgi complex to the apical membrane domain is established at the time of induction of cell–cell contact.

Discussion

The conversion of unpolarized precursor cells to polarized epithelial cells involves the development of restricted distributions of membrane proteins and the formation of structurally and functionally distinct apical and basal-lateral domains of the plasma membrane. These changes are required for specialized functions that are performed by polarized epithelium in the vectorial transport of ions and solutes. Previous studies have reported that induction of cell–cell contact through the Ca^{2+}-dependent cell adhesion protein E-cadherin initiates the development of cell surface polarity during

(A)

Time after cell-cell contact (hrs)	10				12				24			
PM Domain biotinylated	Ap		Bl		Ap		Bl		Ap		Bl	
Triton X-100	s	p	s	p	s	p	s	p	s	p	s	p

gp 135/170 ►

(B)

Percentage at the cell surface

□ Apical
● Basal-lateral

Time after cell-cell contact (hrs)

Figure 6. Delivery of newly synthesized gp 135/170 to the cell surface after induction of cell–cell contact. Confluent monolayers of cells were established on polycarbonate (Transwell) filters in DME/FBS containing 5 μM Ca^{2+} to inhibit cell–cell contact. Cell–cell contact was subsequently induced by raising the Ca^{2+} concentration of the growth medium to 1.8 mM. At different times after induction of cell–cell contact, cells on pairs of filters were pulse-labeled with [^{35}S]methionine/cysteine for 1 h and then biotinylated on either the apical (*Ap*) or basal-lateral (*Bl*) membrane. Cells were extracted with CSK buffer (see Materials and Methods) and the soluble (*s*) and insoluble (*p*) pools of protein were sequentially precipitated with specific antibodies and then avidin–agarose. The immunoprecipitates were processed for SDS-PAGE and fluorography (*A*), and the amount of protein was determined by scanning densitometry (*B*). The results of a representative experiment from four separate trials are presented.

Time after cell-cell contact (min)	15		30		45	
Medium collected	Ap	Bl	Ap	Bl	Ap	Bl
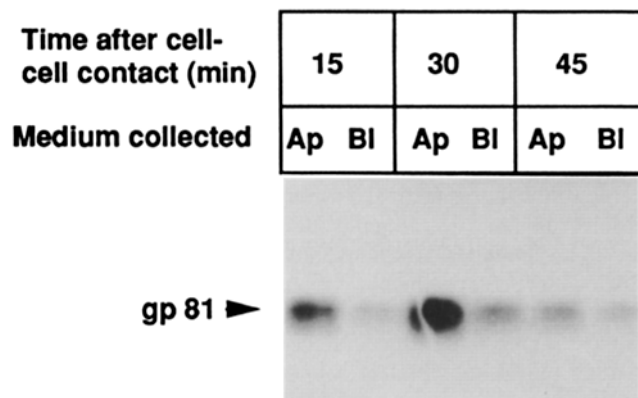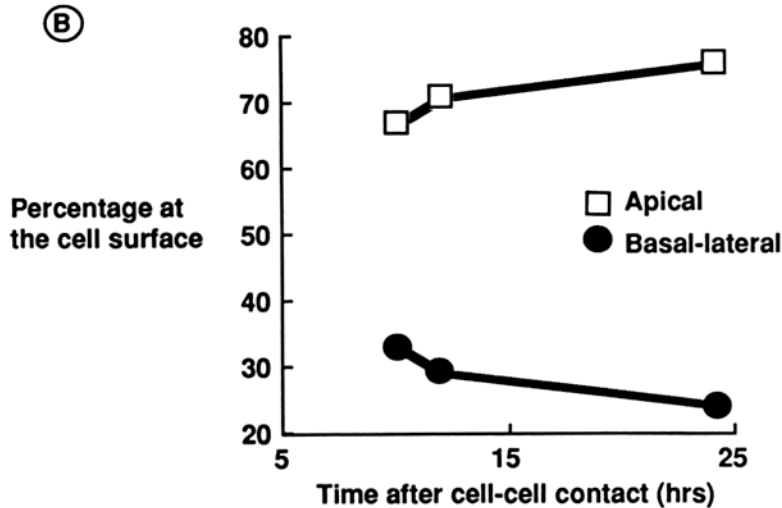
gp 81 ►

Figure 7. Direction of secretion of newly synthesized gp 81 after induction of cell–cell contacts. Confluent monolayers of cells were established on polycarbonate (Transwell) filters. Cells were pulse-labeled with [^{35}S]methionine/cysteine for 15 min and then incubated for different periods of chase in the absence of labeled amino acids. At the time of the initiation of the chase period, cell–cell contact was induced by raising the Ca^{2+} concentration of the growth medium to 1.8 mM. At different times (15, 30, and 45 min) the medium was replaced with fresh medium and a 50-μl aliquot was removed after 5 min from either the apical (*Ap*) or basal-lateral (*Bl*) compartment of the filter. The medium was subjected to SDS-PAGE in the absence of reducing agents, and then to fluorography. Under these conditions, gp 81 is the major protein

mesenchyme to epithelium conversion in vivo (Ekblom et al., 1986) and the differentiation of MDCK cells in vitro (Balcarova-Ständler et al., 1984; Vega-Salas et al., 1987). This study is the first to investigate how protein distributions are remodeled during early stages in the formation of the apical and basal-lateral membrane domains in MDCK cells.

Development of Restricted Protein Distributions after Induction of Cell–Cell Contact

Our results showed that restricted distributions of two basal-lateral membrane proteins, Dg-1 and E-cadherin, were established within 8 h of the induction of cell–cell contact (see Figs. 1 and 2). At that time, however, newly synthesized Dg-1 and E-cadherin were delivered predominantly to the forming apical membrane and not to the basal-lateral membrane (see Fig. 3). The residence time of these proteins in the apical membrane was very short ($t_{1/2}$ 30–60 and 120 min, respectively), compared with that in the basal-lateral membrane ($t_{1/2}$ 24 and 8 h, respectively). Thus, development of the cell surface polarity of these proteins appears to be regulated by

detected in the gel (Kondor-Koch et al., 1985; Gottlieb et al., 1986b; Urban et al., 1987). The results of a representative experiment from three separate trials are presented.

the differential removal of these proteins from the apical membrane and their retention in the basal-lateral membrane, rather than by direct delivery of newly synthesized proteins to the forming basal-lateral membrane.

Dg-1 internalized from the forming apical membrane appeared to be relocated to the basal-lateral membrane. Fig. 4 showed a threefold increase in the detectable amount of newly synthesized Dg-1 present on the basal-lateral membrane between 2 and 4 h of chase after a 15-min period of labeling with [^{35}S]methionine/cysteine, in spite of the fact that all newly synthesized Dg-1 is delivered to the plasma membrane within 90 min of synthesis (Pasdar and Nelson, 1989). Significantly, the increase in the amount of Dg-1 on the basal-lateral membrane coincided with a threefold decrease in the amount of Dg-1 in the apical membrane. Since functional tight junctions had formed, it is unlikely that Dg-1 moved from one membrane domain to the other by diffusion in the plane of the membrane. Rather, the result indicates that Dg-1 was internalized from the apical membrane and delivered by transcytosis to the basal-lateral membrane. Pathways for transcytosis exist in MDCK cells as shown directly for the receptor-mediated transcytosis of the Poly-IgA (Mostov and Deitcher, 1986) and Fc receptors (Hunziker and Mellman, 1989) in these cells (see also Matlin et al., 1983 and Brändli et al., 1990). At present we do not know whether the internalization of Dg-1 requires a specific signal, as has been shown for receptor-mediated endocytosis.

A similar analysis of the distribution of newly synthesized E-cadherin showed that ~75% of newly synthesized protein was delivered directly to the apical membrane (Fig. 3), but that within 2 h 70% of newly synthesized E-cadherin that had been delivered to the cell surface was restricted to the basal-lateral membrane (Fig. 4). However, we did not detect a concomitant increase in the detectable amount of newly synthesized E-cadherin in the basal-lateral membrane during the time that protein was removed from the apical membrane (Fig. 4), indicating that the internalized E-cadherin is degraded.

The significant differences between the rates of removal of these proteins from the apical and basal-lateral membrane domains indicates that endocytosis plays an important role in the rapid development of cell surface polarity. The rates of removal of both Dg-1 and E-cadherin from the apical membrane ($t_{1/2}$ 30–60 and 120 min, respectively) correspond well with a previous study that reported that <2 h were required for constitutive internalization of surface markers from the apical and basal-lateral plasma membrane domains of MDCK cells (Balcarova-Ständer et al., 1984). This rate is significantly faster than the rate of removal of Dg-1 and E-cadherin from the basal-lateral membrane ($t_{1/2}$ 24 and 8 h, respectively). A rapid constitutive rate of removal of cell surface proteins would act as a counter-force to the accumulation of proteins being delivered to that region of the membrane, whereas a decrease in the rate of removal would result in protein accumulation in the membrane.

A decrease in the rate of endocytosis of proteins could result from the formation of complexes with other membrane and cytoplasmic proteins. Previously, we (Pasdar and Nelson, 1988, 1989) and others (Penn et al., 1987) showed that induction of cell–cell contact results in the assembly of Dg-1 into a large protein complex containing other components of the membrane core domain of the desmosome, cytoplasmic

plaque proteins (predominantly, desmoplakin I/II), and the cytokeratin intermediate filament network. Assembly of the desmosome results in an increase in the insolubility of the component proteins in buffers containing Triton X-100 and an increase in their metabolic stability, which are consistent with the formation of a large protein complex (Figs. 1 and 4; see also Penn et al., 1987 and Pasdar and Nelson, 1988, 1989). We suggest that a consequence of protein complex formation is a decrease in the rate of protein removal from the cell surface that would normally occur if the component proteins remained in an unassembled state. Significantly, in the absence of cell–cell contact unassembled Dg-1 is rapidly removed from the cell surface ($t_{1/2}$ <2 h; see Penn et al., 1987 and Pasdar and Nelson, 1989); this coincides also with the rapid rate of loss of Dg-1 from the apical membrane where there is no cell–cell contact and hence no induction of desmosome assembly (this study). It is also noteworthy that loss of cell–cell contact results in the rapid internalization and degradation of the desmosomal proteins and other cell adhesion proteins (Kartenbeck et al., 1991).

Induction of cell–cell contact also results in the formation of protein complexes between the cell adhesion protein E-cadherin and components of the actin-based cytoskeleton (Hirano et al., 1987; Ozawa et al., 1989; Nelson et al., 1990). These interactions appear to occur specifically on the membrane at sites of cell–cell adhesion and not elsewhere on the membrane (Hirano et al., 1987; McNeill et al., 1990). Thus, specific linkage of E-cadherin to the actin cytoskeleton may also reduce the rate of removal of this protein from sites of cell–cell contact. On the other hand, we found that E-cadherin is rapidly removed from the apical membrane where there is no cell–cell contact and, hence, where complex formation with the actin cytoskeleton would not be induced.

Recent studies indicate that selective retention of proteins in the membrane through complex formation with other membrane and cytoplasmic proteins may not be restricted to components of the junctional complex. Na$^+$, K$^+$-ATPase, a basal-lateral membrane protein in MDCK cells (Caplan et al., 1986; Morrow et al., 1989), is selectively retained in the basal-lateral membrane and rapidly removed from the apical membrane; the rate of removal of Na$^+$, K$^+$-ATPase from the apical membrane is similar to that of Dg-1 and E-cadherin (Hammerton et al., 1991). Significantly, Na$^+$, K$^+$-ATPase is directly linked to the membrane-associated cytoskeleton through binding to ankyrin and fodrin (Koob et al., 1987; Nelson and Veshnock, 1987b; Morrow et al., 1989). Assembly of this complex occurs at sites of E-cadherin-induced cell–cell contacts (McNeill et al., 1990). Assembly of Na$^+$,K$^+$-ATPase into the membrane-cytoskeleton may result in a decrease in the rate of internalization of Na$^+$, K$^+$-ATPase from the membrane and hence its selective retention and accumulation in that region of the plasma membrane (Hammerton et al., 1991).

Development of Cell Surface Polarity: Role of Vectorial Delivery of Proteins from the Golgi Complex

Previous studies have shown that in fully polarized MDCK cells newly synthesized proteins are efficiently delivered to either the apical or basal-lateral membrane domains (reviewed in Simons and Fuller, 1985, and Rodriguez-Boulan and Nelson, 1989). The question arises when direct delivery

of proteins from the Golgi complex to the apical or basal-lateral membrane is established in these cells. We sought to determine whether protein sorting occurred at the time of induction of cell–cell contact by analyzing the direction of secretion of gp 81, the major apically secreted protein in polarized MDCK cells. We showed that >85% of newly synthesized gp 81 is secreted directly into the apical medium within 15 min of the induction of cell–cell contact (see Fig. 7). This result indicates that shortly after cell–cell contact intracellular sorting of gp 81 occurs before delivery to the cell surface. However, at the earliest time that delivery of membrane proteins to the cell surface could be analyzed (2 h after cell–cell contact), we detected delivery of >70% of both apical and basal-lateral membrane proteins to the apical membrane. There are at least two possible explanations of this result: Either there is little or no sorting of apical and basal-lateral proteins in the Golgi complex at this time and >70% of all vesicles are delivered directly to the forming apical membrane; this implies that time is required to establish the putative sorting machinery in the Golgi complex. Or sorting of apical and basal-lateral membrane proteins in the Golgi complex occurs constitutively in these cells regardless of the state of cell surface polarity, but initially delivery of both populations of vesicles is directed to the apical membrane; this implies that vesicle delivery to the apical surface is regulated at this time. At present we are not able to distinguish unequivocally between these possibilities, although the finding that gp 81 is delivered directly to the apical membrane indicates that sorting of apical and basal-lateral proteins may occur before the development of cell surface polarity.

That the majority of newly synthesized Dg-1, E-cadherin, and gp 135/170 are initially delivered to the apical membrane indicates the presence of a facilitated pathway of delivery of vesicles from the Golgi complex to the apical membrane at this time. Previous studies have shown that microtubules facilitate the delivery of vesicles to the apical membrane in fully polarized MDCK cells (Achler et al., 1989; Eilers et al., 1989; Paraczyk et al., 1989; Hunziker et al., 1990; Matter et al., 1990b; van Zeijl and Matlin, 1990; Gilbert et al., 1991, see also Bacallao et al., 1989). Depolymerization of microtubules with drugs causes a decrease in the transport of proteins to the apical membrane and some missorting of proteins to the basal-lateral membrane (Achler et al., 1989; Eilers et al., 1989; Paraczyk et al., 1989; Hunziker et al., 1990; Matter et al., 1990b; van Zeijl and Matlin, 1990; Gilbert et al., 1991). The presence of a facilitated pathway of vesicle delivery from the Golgi complex to the apical cell surface in single MDCK cells and in cells shortly after the induction of cell–cell contact may provide an explanation for the polarity of apical proteins in these cells. It will be important to determine whether the disruption of microtubules affects the delivery of vesicles to the apical surface during the development of cell surface polarity following the induction of cell–cell contact in MDCK cells.

Our studies indicate that direct delivery of proteins to the basal-lateral membrane domain does not occur until >48 h after the induction of cell–cell contact. This may reflect the time required to reorganize part of the cytoskeleton that allows delivery of vesicles to the basal-lateral membrane, or the time required for these vesicles to achieve the capacity to recognize and fuse with the basal-lateral plasma membrane. Docking proteins on the basal-lateral membrane may be involved as a recognition system necessary for basal-lateral vesicle fusion with that plasma membrane domain and may not be in position in sufficient quantity before this time to accept vesicles being delivered from the Golgi complex.

Similarity in Mechanisms of Sorting Cell Surface Proteins in Different Polarized Epithelia

Previous studies of protein sorting pathways in polarized epithelial cells have indicated a fundamental difference between MDCK cells and two other epithelial cell types, Caco-2 (intestinal epithelial cells) and hepatocytes (Simons and Wandinger-Ness, 1990). Caco-2 cells directly insert basal-lateral and some apical proteins into the basal-lateral membrane, where the apical proteins are then sorted from resident basal-lateral proteins, internalized, and redirected to the apical cell surface (Matter et al., 1990a; Le Bivic et al., 1991). In the hepatocyte, all membrane proteins are delivered to the basal-lateral membrane (sinusoidal surface), from where apical proteins are selectively removed and delivered to the apical membrane (bile canaliculus) (Bartles et al., 1987). Our results show that the formation of membrane domains in MDCK cells is accomplished by the sorting out of mixtures of proteins on the cell surface by different rates of protein internalization, rather than the direct delivery of proteins to the appropriate membrane. Thus, MDCK cells, Caco-2, and hepatocytes may share a similar mechanism of protein retrieval from the cell surface, which may underlie a common mechanism for protein sorting in these different epithelial cell types that is used at different stages during the establishment and maintenance of cell polarity.

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