

# Modulation of Fodrin (Membrane Skeleton) Stability by Cell-Cell Contact in Madin-Darby Canine Kidney Epithelial Cells

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**Abstract.** During growth of Madin-Darby canine kidney (MDCK) epithelial cells, there is a dramatic change in the stability, biophysical properties, and distribution of the membrane skeleton (fodrin) which coincides temporally and spatially with the development of the polarized distribution of the Na<sup>+</sup>, K<sup>+</sup>-ATPase, a marker protein of the basolateral domain of the plasma membrane. These changes occur maximally upon the formation of a continuous monolayer of cells, indicating that extensive cell-cell contact may play an important role in the organization of polarized MDCK cells (Nelson, W. J., and P. J. Veshnock, 1986, *J. Cell Biol.*, 103:1751-1766). To directly analyze the role of cell-cell contact in these events, we have used an assay in which the organization of fodrin and membrane proteins is analyzed in confluent monolayers of MDCK cells in the absence or presence of cell-cell contact by adjusting the concentration Ca<sup>++</sup> in

the growth medium. Our results on the stability and solubility properties of fodrin reported here show directly that there is a positive correlation between cell-cell contact and increased stability and insolubility of fodrin. Furthermore, we show that fodrin can be recruited from an unstable pool of protein to a stable pool during induction of cell-cell contact; significantly, the stabilization of fodrin is not affected by the addition of cyclohexamide, indicating that proteins normally synthesized during the induction of cell-cell contact are not required. Together these results indicate that cell-cell contact may play an important role in the development of polarity in MDCK cells by initiating the formation of a stable, insoluble matrix of fodrin with preexisting (membrane) proteins at the cell periphery. This matrix may function subsequently to trap proteins targeted to the membrane, resulting in the maintenance of membrane domains.

**C**ELLS of transporting epithelia exhibit a striking degree of functional and structural polarity, which is reflected in the asymmetric distribution of enzymes and transport activities between the apical and basolateral domains of the plasma membrane (9, 11, 18, 33, 42, 45). Since membrane proteins are, in general, capable of rapid diffusion in the plane of the lipid bilayer (for review see reference 31), the topogenesis of these different membrane domains requires not only the targeting of proteins to the membrane but also the maintenance of nonrandom distributions of proteins upon arrival at the membrane. Although advances have been made recently in understanding the pathways involved in targeting proteins to specific membrane domains (30, 32, 38, 39; for recent reviews see references 40 and 44), little is known about the mechanism(s) involved in maintaining distinct membrane domains in these cells.

To define potential mechanisms involved in the organization of membrane domains, we initiated a study of the membrane skeleton in Madin-Darby canine kidney (MDCK) cells (35). The rationale for this approach is that the membrane skeleton may play a role in MDCK cells similar to that in erythrocytes, in which the assembly of a stable, insoluble matrix comprised principally of ankyrin, spectrin, and actin on the cytoplasmic surface of the membrane provides tensile

strength to the membrane and results in the immobilization of associated integral membrane proteins (for reviews see references 2, 6, 12). Our recent analysis of one component of the membrane skeleton in MDCK cells, fodrin, the functional and structural homolog of spectrin in nonerythroid cells (2, 3, 7, 15, 16, 27, 34), revealed significant changes in the solubility properties, stability, and subcellular distribution of the protein during development of a continuous monolayer of MDCK cells (35). These changes result in the formation of an insoluble, stable layer of fodrin which appears to be localized to the cell periphery and predominantly in the region of the basolateral domain of the plasma membrane. The formation of this structure coincided temporally and spatially with the development of the polarized distribution of the Na<sup>+</sup>, K<sup>+</sup>-ATPase (35), a marker protein of the basolateral domain of the plasma membrane (8, 21, 22, 25, 29).

Significantly, the full development of cell surface polarity of the Na<sup>+</sup>, K<sup>+</sup>-ATPase and the reorganization of the membrane skeleton (fodrin) occurred in confluent monolayers of MDCK cells and not in single cells or small colonies of cells (35); a similar finding has been reported for other membrane proteins (1, 15) and transport activities (1). These results suggest that, in addition to intracellular mechanisms for establishing and maintaining membrane domains, extensive cell-

cell contact may be an important factor in the development of polarity in MDCK cells. To approach this problem directly, we have modulated the  $\text{Ca}^{++}$  concentration of the growth medium (10) to analyze the interrelationship of cell-cell contact and the organization of fodrin and membrane proteins in confluent monolayers of MDCK cells in the absence of cell division; as a first step, we report here our analysis of the effect of cell-cell contact on the stability and solubility properties of fodrin. The results demonstrate directly that there is a positive correlation between cell-cell contact and increased stability and insolubility of fodrin. These results indicate that cell-cell contact is involved in initiating the formation of a stable, insoluble matrix of fodrin at the cell periphery, which may in turn play a role in trapping, and subsequently maintaining the polarized distribution of proteins targeted to the plasma membrane.

## Materials and Methods

### Cells

The morphology, growth characteristics, and culture conditions of MDCK (clone 8) cells have been described previously (35). For use in these series of experiments, cultures of MDCK cells were maintained at a very low cell density ( $\sim 2\text{--}2.5 \times 10^4$  cells/cm<sup>2</sup>) for 48–60 h. During this period, the cells were subcultured twice by light trypsinization with a solution containing 0.04% (wt/vol) trypsin and 3 mM EDTA, and replating at a density of  $1.8 \times 10^4$  cells/cm<sup>2</sup>; these cultures comprised single cells and a few double cells (data not shown; see reference 35). To produce "instant" confluent monolayers of cells from these cultures, the cells were trypsinized and combined; the trypsin was neutralized in the cell suspension by the addition of an equal volume of low  $\text{Ca}^{++}$  medium (LC medium) containing DME with 5  $\mu\text{M}$   $\text{Ca}^{++}$  (instead of 1.8 mM), supplemented with 10% FBS that had been dialyzed extensively against a solution of 10 mM Tris-HCl, pH 7.5, 120 mM NaCl (Tris-saline). After centrifugation at 569 g for 5 min, the cells were resuspended in LC medium to form a single-cell suspension, and replated at a density of  $2.7\text{--}3.0 \times 10^5$  cells/cm<sup>2</sup> in LC medium on either collagen-coated 35-mm plastic petri dishes (Nunc) or 0.45  $\mu\text{m}$  nitrocellulose filters (Millipore/Continental Water Systems, Bedford, MA); collagen was prepared from rat tails as described previously (37). Cells were grown at 37°C in a 5%  $\text{CO}_2$  in air, humidified atmosphere. As needed (see Results), medium was aspirated and DME containing 1.8 mM  $\text{Ca}^{++}$  and 10% undialyzed FBS (high  $\text{Ca}^{++}$  [HC] medium)<sup>1</sup> was added. In some cases (see Results), the medium was replaced subsequently with LC medium; in both cases, the cell monolayer was rinsed twice with the replacement medium before further incubation. The amount of DNA in the culture was determined by the method described by Labarca and Paigen (23) using a Perkin-Elmer PE-3 fluorimeter;  $\lambda\text{DNA}$  was used as the standard.

### Immunofluorescences

Confluent monolayers of MDCK cells that had been maintained in either LC or HC medium were fixed in 1.75% formaldehyde in PBS, extracted in 0.5% (wt/vol) Triton X-100, and processed with  $\alpha$ -fodrin antiserum (35) for immunofluorescence microscopy as described previously (35), except that instead of using rhodamine-conjugated goat anti-rabbit IgG we used biotinylated goat anti-rabbit IgG (1:150 in PBS for 30 min) and avidin-fluorescein (1:150 in PBS for 30 min) as the second antibody complex. Cells were viewed en face with a 63 $\times$  objective using a Zeiss Universal Microscope equipped with epifluorescence illumination, and photographed on Tri-X pan film.

### Immunoblotting

Proteins were transferred electrophoretically from SDS-polyacrylamide gels to nitrocellulose filters (44) as modified and described previously (35). Filters were incubated with a 1:1,000 dilution of  $\alpha$ -fodrin antiserum and then

1. *Abbreviations used in this paper:* HC and LC medium, high  $\text{Ca}^{++}$  and low  $\text{Ca}^{++}$  medium, respectively.

$\sim 2 \times 10^6$  cpm of <sup>125</sup>I-protein A (10  $\mu\text{Ci}/\mu\text{g}$ ) as described previously (35). The resulting autoradiograms were analyzed with a Beckman DU-7 spectrophotometer equipped with a scanning densitometer; the amount of protein in each sample was within the linear range of detection by immunoblotting (35).

### Metabolic Labeling and Immunoprecipitation

Cultures of MDCK cells were rinsed twice with minimum essential medium (containing 5  $\mu\text{M}$  or 1.8 mM  $\text{Ca}^{++}$ ) without methionine and supplemented with 2.5% dialyzed FBS (LC and HC MEM-Met). The cells were preincubated with the corresponding methionine-free medium for 15 min at 37°C. The medium was discarded, and the cells were incubated at 37°C for 15 min in 500  $\mu\text{l}$  LC or HC MEM-Met containing 120  $\mu\text{Ci}$  [<sup>35</sup>S]methionine (New England Nuclear, Boston, MA;  $\sim 1,200$  Ci/mmol). The radioactive medium was discarded, and the cells were rinsed twice in LC or HC medium containing 2 mM unlabeled methionine (chase medium). The cells were incubated in 3 ml LC or HC chase medium for different times. In some cases (see Results), 4  $\mu\text{g}/\text{ml}$  cyclohexamide was added to the chase medium; experiments showed that this concentration of cyclohexamide inhibited >90% of protein synthesis as determined by total TCA-precipitated cpm (data not shown). At given times, the cells were placed on ice and rinsed twice with ice-cold Tris-saline containing 1 mM PMSF. At 4°C, the cells were scraped from the petri dish in 1 ml Tris-saline and 1 mM PMSF using a rubber policeman, and processed for immunoprecipitation with fodrin antiserum as described in detail previously (35). The resulting immunoprecipitates were applied to an SDS/5% polyacrylamide gel (24), which was processed subsequently for fluorography as described (4). The dried gels were exposed at  $-80^\circ\text{C}$  to XAR-5 x-ray film preflashed to an absorbance of  $\sim 0.15$  at 514 nm (26). Fluorograms were analyzed with a Beckman DU-7 spectrophotometer equipped with a scanning densitometer; the rate of degradation of [<sup>35</sup>S]methionine-labeled fodrin was calculated by regression analysis. Each experiment was performed at least three times, and the rates of turnovers of fodrin varied by < 10%; the data from one experiment is presented.

### Solubility Properties of Fodrin from MDCK Cells

Confluent cultures of MDCK cells in LC or HC medium were extracted in a buffer containing 10 mM Tris-HCl, pH 7.5, 5 mM EGTA, 5 mM EDTA, 1 mM PMSF, 0.5% (vol/vol) Triton X-100, 50 mM KCl, 120 mM NaCl, 0.1 mM DTT as described in detail previously (35). The partitioning of fodrin between the resulting residues and extracts was determined by SDS/5% PAGE and immunoblotting as described previously (35).

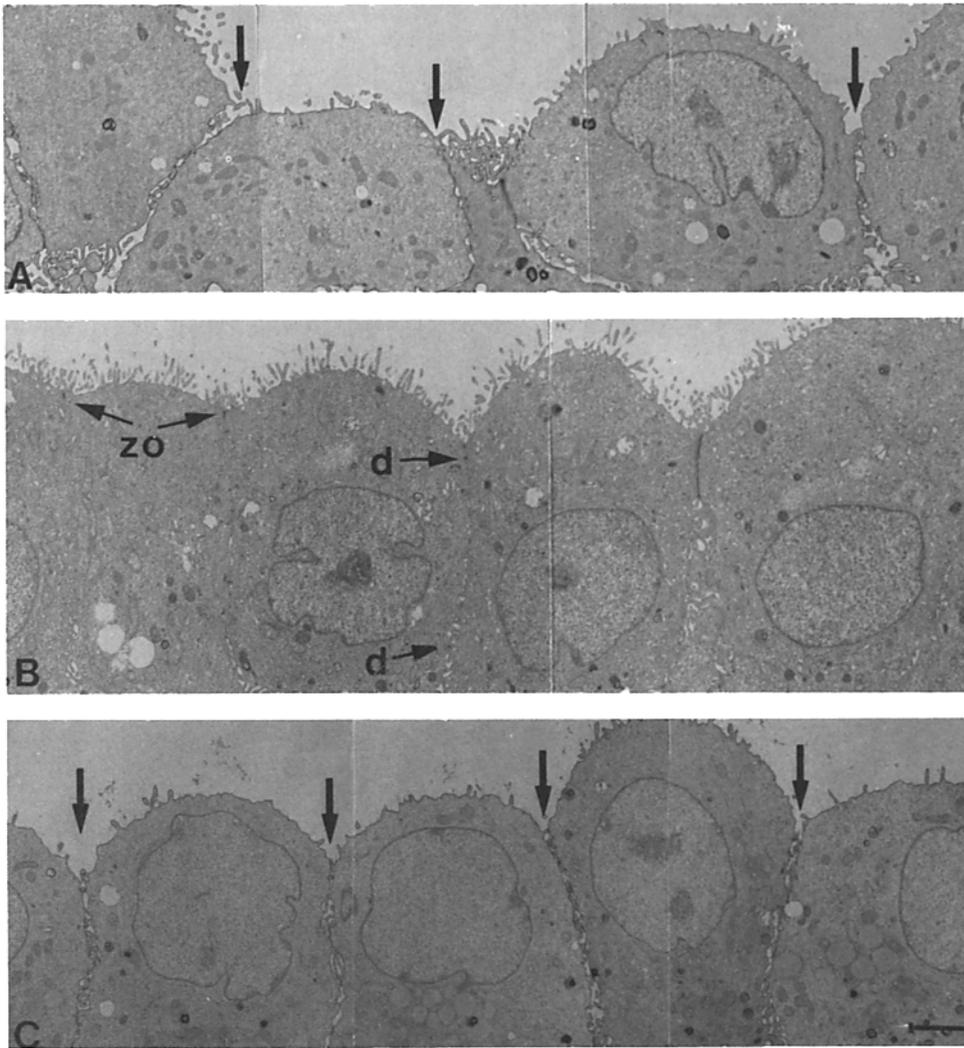
### Electron Microscopy

MDCK cells grown in LC or HC medium were fixed in a solution containing 1.25% glutaraldehyde, 1%  $\text{OSO}_4$  in 50 mM Pipes, pH 7.2, for 5 min at room temperature. The cells were dehydrated and embedded in Epon-Araldite as described previously (35). Sections of cells were cut perpendicular to the substratum, stained in uranyl acetate/lead citrate, and viewed in a Philips 420 transmission electron microscope operated at 80 kV.

## Results

### Growth Properties and Morphology of MDCK Cells in LC Medium

Before establishing confluent monolayers, MDCK cells were maintained at a very low cell density ( $1.8\text{--}2.5 \times 10^4$  cells/cm<sup>2</sup>). Under these conditions, individual MDCK cells have little or no cell-cell contact over a period of up to 60 h in culture. Consequently, when these cells are combined and replated at a high cell density ( $2.7\text{--}3.0 \times 10^5$  cells/cm<sup>2</sup>), we consider them to be cell-cell contact "naive." This precaution was taken so that the effects of the subsequent induction of cell-cell contact in confluent populations of these cells would not be compromised by the participation of residual structures of cell-cell contact that might remain if the cells had been derived from a culture that had exhibited previously a high degree of cell-cell contact (see below).



**Figure 1.** Morphology of MDCK cells in low  $\text{Ca}^{++}$  (LC) and high  $\text{Ca}^{++}$  (HC) medium. MDCK cells were subcultured at low cell density for  $\sim 48$ – $60$  h and then combined at high cell density ( $2.7$ – $1 \times 10^5$  cells/cm $^2$ ). Cells were incubated in LC medium for 8 h (A), followed by HC medium for 1.5 h (B) or 12 h followed by LC medium for 1.5 h (C). For electron microscopy, cells were sectioned perpendicular to the substratum. zo, zonula occludens; d, desmosomes. Bar, 2  $\mu\text{m}$ .

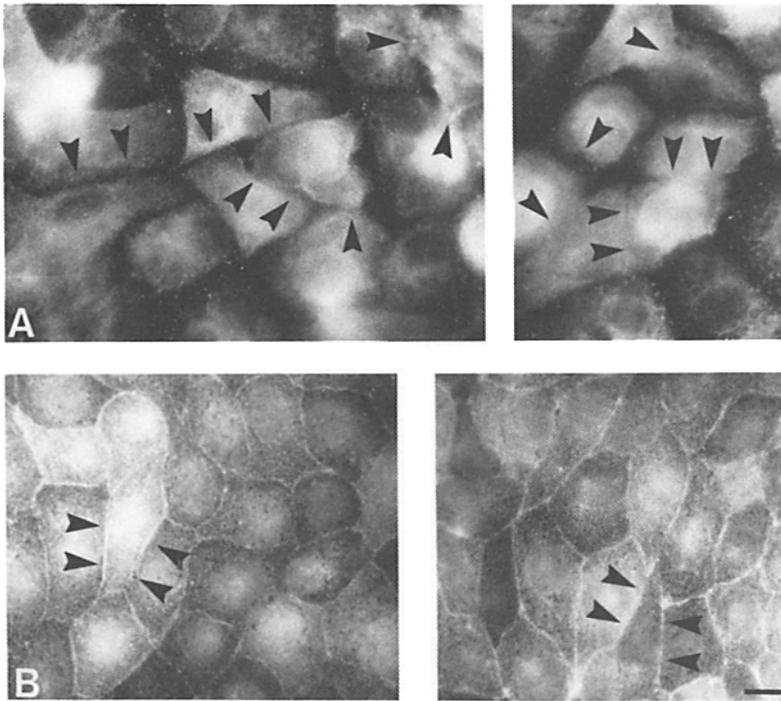
MDCK cells have a high plating efficiency ( $>90\%$ ) in LC medium, which is similar to that of MDCK cells in DME/FBS (35). Cells attach within 2 h of plating, and become packed to form a confluent monolayer within 4–5 h. The packing of cells is due to the high cell density inoculated onto the petri dish and occurs in the absence of cell division (data not shown; see below). The cells are maintained in LC medium for 8–12 h before raising the  $\text{Ca}^{++}$  concentration of the medium. Increasing the  $\text{Ca}^{++}$  concentration of the medium to 1.8 mM does not induce cell division as shown by the maintenance of a constant level of DNA and lack of mitotic figures in the cultures during incubation in either LC or HC medium (data not shown).

Confluent monolayers of MDCK cells established in LC medium appear relatively columnar in lateral view, which is due presumably to the packing of cells inoculated at high cell density. The lateral membranes of adjacent cells exhibit little or no morphological evidence of junctional complex formation (Fig. 1 A); note the absence of electron-dense plaque structures and associated 10-nm filaments characteristic of desmosomes, and the absence of a zonula occludens at the apex of the lateral membranes of adjacent cells. In general, the lateral membranes of adjacent cells are separated by an

intercellular gap of 80–120 nm, compared with an average of  $\sim 20$  nm in MDCK cells grown in the HC medium (35); however, there are rare instances of a close association of adjacent membranes over short distances (see Fig. 1 A) (see also reference 10).

Confluent cultures of MDCK cells that display little or no cell–cell contact in LC medium can be induced rapidly and synchronously to establish cell–cell contact by raising the  $\text{Ca}^{++}$  concentration in the medium to 1.8 mM (HC medium; DME/FBS). Analysis of MDCK cells 1.5 h after raising the  $\text{Ca}^{++}$  concentration reveals that adjacent cells have established extensive cell–cell contact (intercellular gap  $\sim 20$ – $30$  nm) along the whole length of the apposed lateral membranes except in the region of interdigitation (Fig. 1 B), which is a characteristic feature of kidney transporting epithelia (9, 11, 18, 33, 41, 42, 45). In all cases, a zonula occludens is present at the apico-lateral membrane boundary; furthermore, numerous desmosomes are revealed to be in the process of assembly.

The establishment of cell–cell contact and the formation of the junctional complex in these cultures of MDCK cells can also be reversed rapidly by decreasing the  $\text{Ca}^{++}$  concentration of the medium to 5  $\mu\text{M}$  (LC medium) (Fig. 1 C). The



**Figure 2.** Effect of high  $\text{Ca}^{++}$  (HC) and low  $\text{Ca}^{++}$  (LC) medium on the distribution of fodrin in confluent monolayers of MDCK cells. Continuous monolayers of MDCK cells in LC medium (A) or HC medium (B) were fixed in 1.75% formaldehyde, permeabilized in 0.5% (wt/vol) Triton X-100, and processed with  $\alpha$ -fodrin antiserum for immunofluorescence microscopy. Bar, 100  $\mu\text{m}$ .

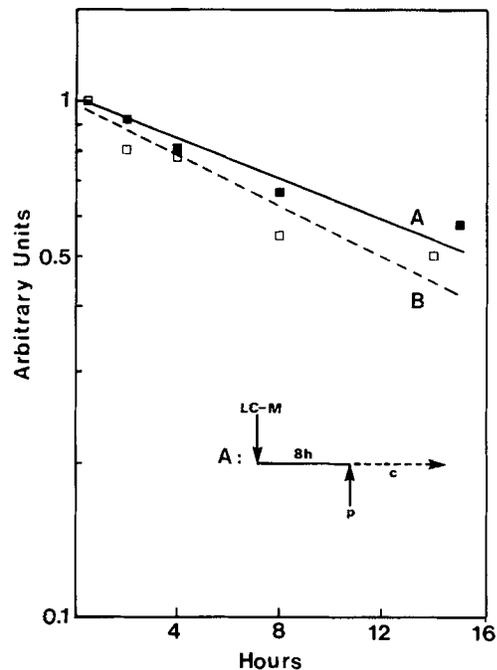
morphology of cells 1.5 h after a decrease in extracellular  $\text{Ca}^{++}$  concentration is similar if not identical to the original monolayer of cells established in LC medium (compare Fig. 1, A and C); there is little or no cell-cell contact and an absence of components of the junctional complex.

#### **Cell-Cell Contact Coincides with a Redistribution of Fodrin**

Immunofluorescence analysis of confluent monolayers of MDCK cells established and maintained in LC medium for 12–24 h revealed that fodrin has a diffuse distribution (Fig. 2 A). Little or no fodrin was detected in regions of close apposition of adjacent cells in the absence of cell-cell contact. Thus, the distribution of fodrin in confluent monolayers of MDCK cells without cell-cell contact appears similar, if not identical, to that in single cells (see Fig. 7; reference 35). In contrast to these results, confluent monolayers of MDCK cells maintained in HC medium for 36–48 h exhibited a different distribution of fodrin (Fig. 2 B). Fodrin staining was prominent in the regions of contact between adjacent cells. Adjusting the plane of focus between the apical and basal surfaces indicated that fodrin staining was predominantly localized to the basolateral membrane (data not shown), as shown previously (35). These results suggest that cell-cell contact coincides with a redistribution of fodrin from a diffuse pattern to one that is concentrated in regions of cell-cell contact.

#### **The Rate of Turnover of Newly Synthesized Fodrin Is Similar in Confluent Monolayers of MDCK Cells without Cell-Cell Contact and in Single Cells**

The stability of newly synthesized fodrin was compared between confluent monolayers of MDCK cells in LC medium which had little or no cell-cell contact (see Fig. 1 A), and single cells (for example, see Fig. 1 in reference 35). Confluent monolayers of MDCK, which had been established



**Figure 3.** Turnover of newly synthesized fodrin in confluent monolayers of MDCK cells in LC medium (A) and in cultures of single MDCK cells (B). Cells were pulse-labeled (p), with  $[^{35}\text{S}]$ methionine for 15 min and then incubated in chase medium (c) containing >10,000-fold excess of unlabeled methionine. At given time points, cells were scraped from the petri dish and dissolved by boiling in SDS immunoprecipitation buffer. Fodrin was immunoprecipitated with specific antiserum and analyzed by SDS/5% PAGE and fluorography. The relative amount of fodrin remaining was determined from the fluorograms using a DU-7 Beckman spectrophotometer equipped with a scanning densitometer, and plotted as a fraction of the amount present at the first time point.

and grown in LC medium for 8 h, and cultures of single cells were pulse-labeled with [<sup>35</sup>S]methionine for 15 min, and then incubated for up to 14 h in LC medium containing a >10,000-fold excess of unlabeled methionine (chase period). Newly synthesized fodrin was detected by immunoprecipitation and fluorography. The relative amounts of [<sup>35</sup>S]methionine-labeled fodrin, after different periods of chase, were determined by scanning densitometry and the results subjected to regression analysis. Fig. 3 shows that the rate of turnover of newly synthesized fodrin is similar if not identical in confluent monolayers of MDCK cells that do not exhibit cell-cell contact and in single cells ( $t_{1/2} \sim 15$  h). This analysis of fodrin stability was repeated after confluent monolayers of MDCK cells had been maintained in LC medium for 27 h. The results revealed that the rate of turnover of newly synthesized fodrin in MDCK cells observed after 8 h is maintained after 27 h in the absence of cell-cell contact ( $t_{1/2} \sim 15$  h; data not shown). This indicates that the relatively rapid rate of turnover of newly synthesized fodrin is not a transient effect caused by establishing a confluent monolayer of MDCK cells in LC medium, but a long-term effect that coincides with the continued absence of cell-cell contact (see below). Furthermore, that fodrin synthesized in single cells is relatively unstable ( $t_{1/2} \sim 15$  h) in either LC or HC medium (see reference 35) supports the notion that LC medium does not, by itself, affect fodrin stability.

#### The Rate of Turnover of Newly Synthesized Fodrin Is Decreased after Induction of Cell-Cell Contact in Confluent Monolayers of MDCK Cells

To determine whether cell-cell contact affects the stability of

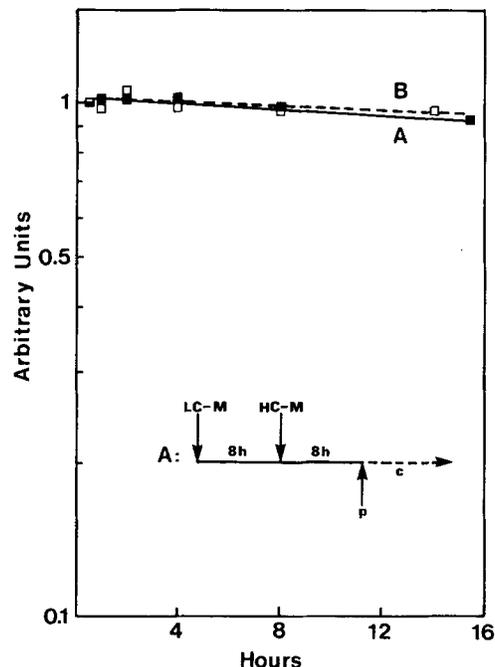


Figure 4. Turnover of newly synthesized fodrin in confluent monolayers of MDCK established in LC medium and maintained subsequently in HC medium (A), or grown from single cell cultures in HC medium (B). The rate of turnover of fodrin was determined using the pulse (p)-chase (c), immunoprecipitation, and fluorography protocol described in the legend to Fig. 3.

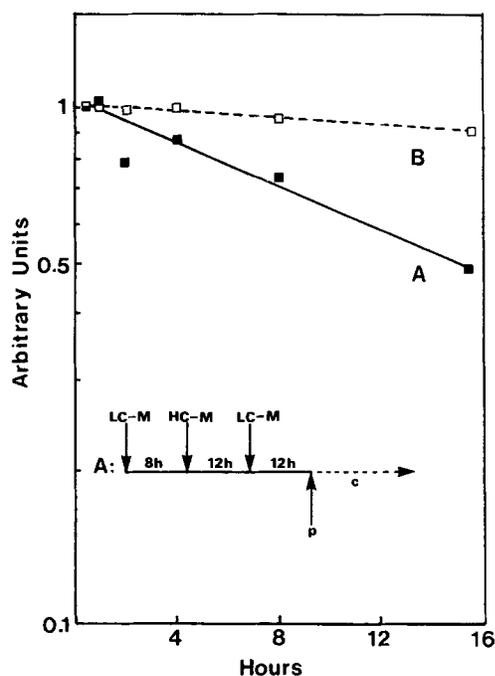


Figure 5. Turnover of newly synthesized fodrin in confluent monolayers of MDCK cells maintained in HC medium (B) and incubated subsequently in LC medium (A). The rate of turnover of fodrin was determined by pulse (p)-chase (c) labeling with [<sup>35</sup>S]methionine, immunoprecipitation, and fluorography (for details, see text).

newly synthesized fodrin, confluent monolayers of MDCK cells were established without cell-cell contact in LC medium for 8 h. Subsequently, the medium was changed to HC medium and, after 12 h, the cells were pulse-labeled with [<sup>35</sup>S]methionine and incubated for a period of chase in HC medium containing unlabeled methionine. Newly synthesized fodrin was detected by immunoprecipitation, fluorography, and scanning densitometry as described above. Fig. 4 shows that the rate of turnover of newly synthesized fodrin was decreased dramatically after synchronous induction of cell-cell contact in confluent monolayers of MDCK cells ( $t_{1/2} > 70$  h; compare with Fig. 3). Significantly, the rate of turnover of fodrin in these cells is similar, if not identical to that in MDCK cells that had gradually formed confluent monolayers from single cells in HC medium ( $t_{1/2} > 70$  h; see also reference 35).

#### Loss of Cell-Cell Contact in Confluent Monolayers of MDCK Cells Results in an Increased Rate of Turnover of Newly Synthesized Fodrin

The preceding results demonstrate clearly that the induction of cell-cell contact in confluent monolayers of MDCK cells correlates with a dramatic decrease in the rate of turnover of newly synthesized fodrin. If this effect is correlated with cell-cell contact we would predict that the rate of turnover of newly synthesized fodrin would increase if cell-cell contact is disrupted in a confluent monolayer of MDCK cells. To test this prediction, confluent monolayers of MDCK cells were established in LC medium, then induced to form cell-cell contact for 12 h in HC medium, and finally returned to LC medium for 12 h (Fig. 5); electron microscopy of these

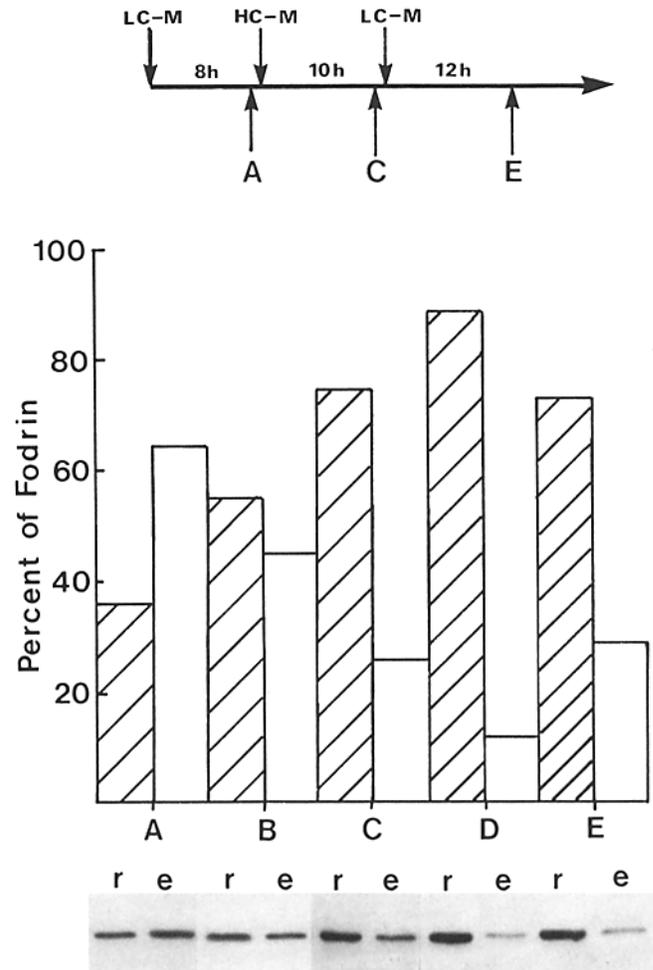
cells showed a clear disruption of cell-cell contacts and absence of components of the junctional complex (Fig. 1 C). Analysis of the rate of turnover of newly synthesized fodrin using the pulse-chase and immunoprecipitation protocol described above demonstrated that there is a dramatic increase in the rate of turnover of newly synthesized fodrin 12 h after disruption of cell-cell contact ( $t_{1/2} \sim 15$  h; Fig. 5). In contrast, fodrin exhibited a half-life of  $>70$  h in confluent monolayers of cells maintained in HC medium throughout the same period of time (Fig. 5). Together these results demonstrate clearly a positive correlation between the induction of cell-cell contact and an increased stability of newly synthesized fodrin.

### Induction of Cell-Cell Contact in Confluent Monolayers of MDCK Cells Results in an Increase in the Insolubility of Fodrin

Our earlier study indicated that the degree of solubility of fodrin decreased during development of a continuous monolayer of MDCK cells (35). To determine directly whether the solubility of fodrin is affected by cell-cell contact, confluent monolayers of MDCK cells were established without cell-cell contact in LC medium for 8 h. The cells were incubated subsequently in HC medium to induce cell-cell contact. Representative cell monolayers in LC or HC medium were extracted in a solution containing 0.5% (vol/vol) Triton X-100 and 150 mM salt (NaCl + KCl). It should be noted that LC medium by itself does not appear to solubilize fodrin/spectrin from the plasma membrane of erythrocytes and lens plasma membranes (data not shown). The resulting extracts and residues from equivalent amounts of starting material were analyzed by SDS/5% PAGE and immunoblotting (for details, see Materials and Methods). The partitioning of fodrin between the soluble (*e*) and insoluble (*r*) fractions (Fig. 6) was quantitated by densitometric scans of the resulting autoradiogram.

The results demonstrate that in the absence of cell-cell contact fodrin is relatively soluble ( $\sim 65\%$ ) in buffers containing Triton X-100 and 150 mM salt (Fig. 6 A) or 6 M urea (data not shown); this degree of solubility is similar to that of fodrin in cultures of single MDCK cells ( $\sim 45\text{--}55\%$ , Fig. 6 B; see also reference 35). Significantly, fodrin remained relatively soluble in confluent monolayers of MDCK cells in LC medium throughout the time period examined (27 h; data not shown), suggesting that this is also a long-term effect that coincides with the absence of cell-cell contact (see above).

A significant difference in the degree of solubility of fodrin was found after induction of cell-cell contact with HC medium in confluent monolayers that had been established and maintained previously for 8 h in LC medium (Fig. 6 C). Fodrin became relatively insoluble ( $>75\%$  in residue) in a buffer containing Triton X-100 and 150 mM salt (Fig. 6 C). These solubility properties are similar, if not identical, to those of fodrin in confluent monolayers of cells that had developed over 5–7 d from a culture of single cells (Fig. 6 D). Taken together these results indicate that the decrease in the degree of solubility of fodrin is correlated with the formation of cell-cell contact between adjacent MDCK cells. Significantly, fodrin present in confluent monolayers with cell-cell contact is resistant to extraction following loss of cell contact in LC medium, at least in the short term (Fig. 6 E; for further discussion, see below).



**Figure 6.** Analysis of solubility properties of fodrin from MDCK cells in the presence or absence of cell-cell contact. Confluent monolayers of MDCK cells were established in LC medium (A), followed by HC medium (C), followed by LC medium (E); alternatively, single cell cultures (B) and confluent monolayers (D) of MDCK cells were established and maintained in HC medium. Monolayers were extracted on the petri dish with a solution containing 0.5% (wt/vol) Triton X-100 and 150 mM salt, and the partitioning of fodrin in the residue (*r*) and extract (*e*) determined by SDS/5% PAGE and immunoblotting.

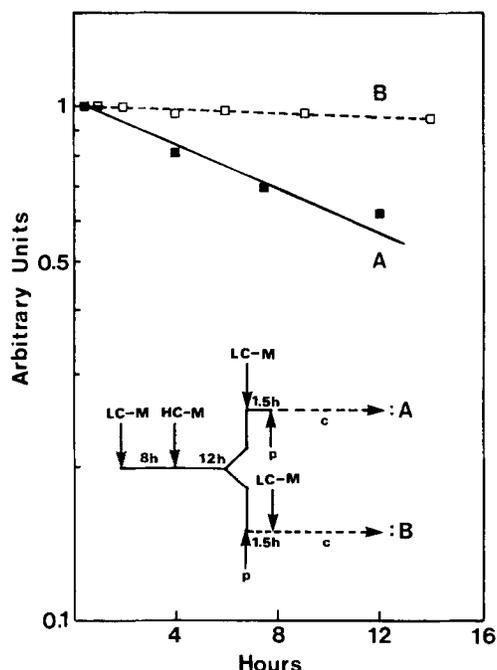
### Fodrin Synthesized in Confluent Monolayers of MDCK Cells 1.5 Hours after Loss of Cell-Cell Contact Is Not Stabilized

To determine how closely coordinated cell-cell contact and the induction of fodrin stability are, the rate of turnover of newly synthesized fodrin was determined in confluent monolayers of MDCK cells that had lost cell-cell contact 1.5 h before initiating the period of [ $^{35}$ S]methionine labeling (see Fig. 7). Confluent monolayers of MDCK cells were established in LC medium, and then induced to form cell-cell contact in HC medium. After 10 h, the medium was replaced with LC medium, and after a further 1.5 h the cells were pulse-labeled with [ $^{35}$ S]methionine and incubated for different periods of chase to determine the rate of turnover of newly synthesized fodrin. The results show clearly that newly synthesized fodrin is degraded relatively rapidly with a half-life of  $\sim 15$  h (Fig. 7 A) which is similar to that of fodrin

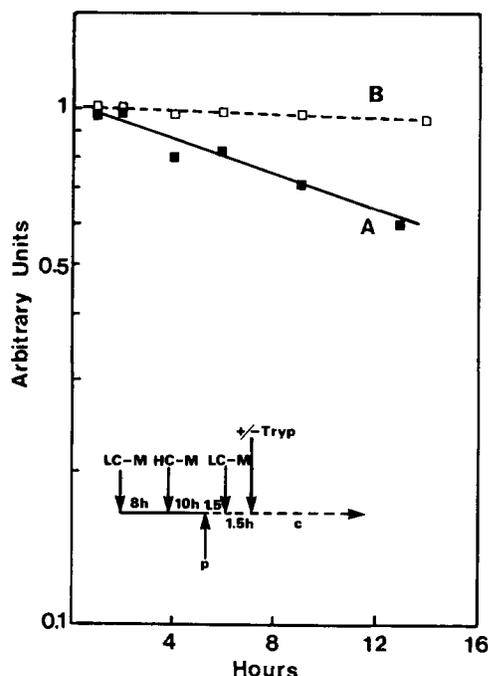
synthesized in confluent monolayers of MDCK cells that had been established and maintained in LC medium (compare Figs. 7 and 3). That fodrin synthesized 1.5 h after loss of cell-cell contact is degraded relatively rapidly indicates that cell-cell contact and fodrin stabilization are closely coordinated events in time.

**Stability of Fodrin Synthesized in Confluent Monolayers of MDCK Cells Is Not Altered by the Subsequent Loss of Cell-Cell Contact in LC Medium**

The results described above clearly establish a correlation between cell-cell contact and decreased solubility and increased stability of newly synthesized fodrin in confluent monolayers of MDCK cells. In the next series of experiments, we sought to determine whether fodrin that had been stabilized in the presence of cell-cell contact could be destabilized through the subsequent loss of cell-cell contact. Confluent monolayers of MDCK cells were established and maintained in LC medium for 8 h, and cell-cell contact was induced by the substitution of HC medium. After 10 h, the cells were pulse-labeled with [<sup>35</sup>S]methionine for 15 min and then incubated in HC medium with unlabeled methionine for 1.5 h, at which time the medium was replaced with LC medium to disrupt cell-cell contact (Fig. 7). At given times throughout the chase period in LC medium the relative amount of newly synthesized fodrin remaining was deter-



**Figure 7.** Turnover of fodrin synthesized in confluent monolayers of MDCK cells 1.5 h before (B) or after (A) loss of cell-cell contact. Duplicate sets of MDCK cells cultures were established at high cell density in LC medium (8h) and maintained subsequently in HC medium (12h). One set of cultures (B) was pulse-labeled for 15 min with [<sup>35</sup>S]methionine (p), incubated in chase medium (HC medium) for 1.5 h and then in LC medium for the remainder of the period of chase (c). The other set of cultures (A) was incubated in LC medium for 1.5 h before the pulse-label with [<sup>35</sup>S]methionine and subsequent period of chase in LC medium. The rate of turnover of fodrin was determined by immunoprecipitation and fluorography (for details, see text).



**Figure 8.** Turnover of newly synthesized fodrin in confluent monolayers of MDCK cells after loss of cell-cell contact (B) and light trypsinization of the cell surface (A). Confluent monolayers of MDCK cells were established in LC medium (8h) and maintained in HC medium (10h). The cells were pulse-labeled (p) with [<sup>35</sup>S]methionine and then incubated in HC medium for a further 1.5 h (c). Subsequently, the cells were incubated in LC medium for 1.5 h followed by light trypsinization of the cell surface for 5 min. The cells were incubated in LC medium containing 4 μg/ml cyclohexamide for the remainder of the period of chase (c). The rate of turnover of fodrin was determined by immunoprecipitation and fluorography (for details, see text).

mined by immunoprecipitation and fluorography as described above. The results of this analysis show that fodrin synthesized in MDCK cells that have cell-cell contact is relatively stable ( $t_{1/2} > 50$  h) after loss of cell-cell contact in LC medium (Fig. 7 B). This indicates that in MDCK cells that have extensive cell-cell contact, newly synthesized fodrin is sequestered in a stable pool of protein that is resistant to destabilization by loss of cell-cell contact through a decrease in the concentration of extracellular  $Ca^{++}$ .

That fodrin is sequestered in a stable compartment in MDCK cells that exhibit cell-cell contact is reflected also in the resistance of fodrin in these cells to extraction after loss of cell-cell contact (Fig. 6). Extraction of confluent monolayers of MDCK cells that had been allowed to establish cell-cell contact for 12 h and were then disrupted in LC medium for 8 h revealed that at steady state fodrin remained relatively insoluble (>75% in the residue) in a buffer containing 0.5% (vol/vol) Triton X-100 and 150 mM salt (Fig. 6 E).

**Stability of Fodrin Synthesized in Confluent Monolayers of MDCK Cells Is Decreased by Subsequent Disruption of Cell-Cell Contact in LC Medium and Light Trypsinization of the Cell Surface**

Although loss of cell-cell contact in LC medium does not alter the stability or degree of solubility of fodrin, we sought

to determine whether loss of cell-cell contact together with degradation of externally exposed membrane proteins would affect fodrin stability. Confluent monolayers of MDCK cells were established in LC medium for 8 h, and then allowed to form cell-cell contacts in HC medium for 12 h. Cells were labeled metabolically with [<sup>35</sup>S]methionine for 15 min and then incubated in chase medium. After 1.5 h, the medium was changed to LC medium. After a further 1.5 h, by which time cell-cell contact is lost (see Fig. 1 C), the cells were lightly trypsinized with a solution containing 0.04% (wt/vol) trypsin in LC medium for ~5 min (Fig. 8); under these conditions the cells do not detach from the substratum (data not shown). The medium was discarded and the cell monolayer incubated in LC medium containing 4 μg/ml cyclohexamide for a further 20 h. At given time points, the amount of [<sup>35</sup>S]methionine-labeled fodrin remaining was determined by immunoprecipitation and fluorography. The results demonstrate clearly that light trypsinization of cells following loss of cell-cell contact results in a dramatic decrease in the stability of fodrin synthesized previously in the presence of cell-cell contact ( $t_{1/2}$  ~20 h; see Fig. 8). This result indicates that while loss of cell-cell contact per se does not alter the stability of preexisting fodrin, degradation of externally exposed membrane proteins results in the destabilization of the same population of fodrin.

### Protein Synthesis Is Not Required upon Cell-Cell Contact to Stabilize Newly Synthesized Fodrin

The results described above indicate that membrane proteins may be involved in the association and stabilization of fodrin with the membrane. To determine if proteins synthesized during induction of cell-cell contact are involved in this process, the rate of turnover of newly synthesized fodrin was determined after induction of cell-cell contact in the presence of 4 μg/ml cyclohexamide. Confluent monolayers of MDCK cells were established in LC medium. After 8 h, the cells were pulse-labeled with [<sup>35</sup>S]methionine and incubated for 1.5 h in LC medium with unlabeled methionine. At this time the medium was exchanged for HC medium with or without cyclohexamide (Fig. 9). Analysis of the levels of [<sup>35</sup>S]methionine-labeled fodrin revealed that the rates of turnover of newly synthesized fodrin in cells induced subsequently to establish cell-cell contact was identical either in the presence or absence of protein synthesis at the time of cell-cell contact ( $t_{1/2}$  >50 h; see Fig. 9). Two important conclusions can be drawn from these results. First, proteins present constitutively at the time of induction of cell-cell contact appear to play a critical role in the stabilization of newly synthesized fodrin; it should be noted that MDCK cells are able to establish cell-cell contact and form components of the junctional complex in the presence of cyclohexamide (our unpublished results; see also reference 18). Second, the fact that newly synthesized fodrin can be stabilized subsequently upon cell-cell contact indicates that fodrin can be recruited from an unstable pool of protein to a stable pool during induction of cell-cell contact.

### Discussion

Studies of endogenous protein organization in MDCK cells have demonstrated that the polarized distribution of many membrane proteins and transport activities, and components

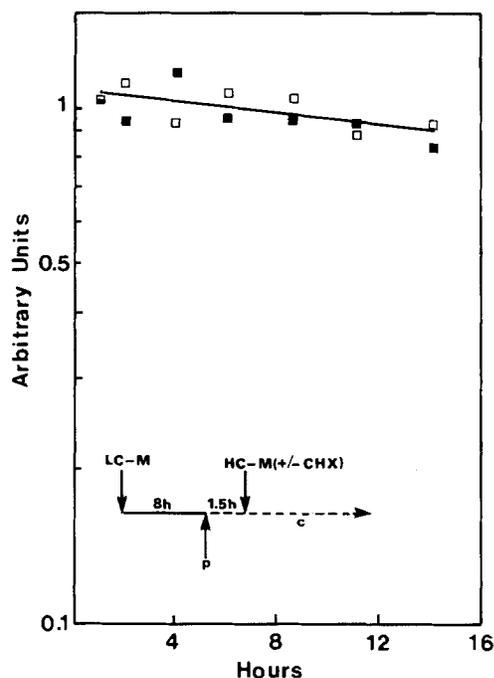


Figure 9. Turnover of newly synthesized fodrin in confluent monolayers of MDCK cells after induction of cell-cell contact in the absence or presence of protein synthesis. Confluent monolayers of MDCK cells were established in LC medium (8h), pulse-labeled with [<sup>35</sup>S]methionine (p), and the cells were incubated in LC medium for 1.5 h (c). The medium was changed to HC medium containing either 4 μg/ml cyclohexamide (■) or the equivalent volume of ethanol (2.5 μl) (□) and the period of chase was continued. The rate of turnover of fodrin was determined by immunoprecipitation and fluorography.

of the membrane skeleton (fodrin) is fully established and maintained only upon the formation of a continuous monolayer of cells (1, 15, 19, 35). The coincidental timing of these events suggests that, in addition to intracellular mechanisms for establishing and maintaining membrane domains, extensive cell-cell contact may be an important factor in the development of MDCK cell polarity. To address directly the interrelationship of these three processes, we have used a controlled assay in which the organization of the membrane skeleton (fodrin) and membrane proteins is analyzed in confluent monolayers of MDCK cells in the presence or absence of cell-cell contact by adjusting the Ca<sup>++</sup> concentration of the growth medium (10).

This protocol fulfills three important criteria for this analysis (for details, see Materials and Methods and Results). First, confluent monolayers of MDCK can be established with a high plating efficiency, and maintained subsequently in the absence of cell-cell contact. Second, the cells used to form these "instant" confluent monolayers are cell-cell contact "naive" to ensure that induction of cell-cell contact produces a de novo response, rather than the possible use of (protein) structures remaining from cells maintained previously at high cell densities. Third, cell-cell contact can be induced rapidly and synchronously throughout the cell monolayer, a process which can also be reversed rapidly. Together, these characteristics indicate that this assay is a meaningful approach to analyzing the establishment and maintenance of membrane domains and the role of cell-cell contact in these

processes. In the study presented here, we have addressed the first question raised in our previous study, which concerns the effect of cell-cell contact on the stability and insolubility of fodrin. Subsequently, we plan to analyze in detail the effect of cell-cell contact on the spatial organization of the membrane skeleton and membrane proteins.

### **Regulation of Fodrin Stability and Solubility by Cell-Cell Contact**

The initial part of this study sought to determine whether there was a correlation between the establishment of cell-cell contact and increased fodrin stability and insolubility. The results showed clearly that fodrin was relatively unstable ( $t_{1/2} \sim 15$  h) and soluble in confluent monolayers of MDCK cells in the absence of cell-cell contact. However, upon establishment of cell-cell contact, these properties of fodrin changed dramatically. Newly synthesized fodrin was relatively stable ( $t_{1/2} > 70$  h) and insoluble. Significantly, when cell-cell contact was lost subsequently by reducing the concentration of the extracellular  $\text{Ca}^{++}$ , and the stability of newly synthesized fodrin was analyzed 12 h later, the rate of turnover had increased ( $t_{1/2} \sim 15$  h) to a level similar to that in confluent monolayers of MDCK cells that had been established and maintained continuously in the absence of cell-cell contact. Previously, our analysis of different populations of single-cell and confluent monolayers of MDCK cells demonstrated a similar change in these properties of fodrin, which we interpreted as an effect of extensive cell-cell contact (35). Using the assay developed in the present study, our results directly demonstrate a positive correlation between the establishment of cell-cell contact and an increase in the stability and insolubility of fodrin. In addition, our immunofluorescence analysis of fodrin in MDCK maintained in LC medium or HC medium revealed that cell-cell contact coincides with a dramatic change in the distribution of fodrin and results in the predominant localization of fodrin at the basolateral domain of the plasma membrane as indicated previously (35).

These experiments addressed the fate of fodrin that was synthesized in confluent monolayers of MDCK cells in which cell-cell contact had been present or absent for a substantial period of time, 8–12 h. Having demonstrated clearly a correlation between cell-cell contact and the stability of fodrin, we sought to gain an insight into the nature of these two events by analyzing the fate of fodrin synthesized in a narrow window of time during the induction or loss of cell-cell contact. A window of 1.5 h was chosen since maximum incorporation of [ $^{35}\text{S}$ ]methionine into newly synthesized fodrin, following a pulse label of 15 min, was obtained within 30–60 min (our unpublished results); and second, electron microscopy revealed that full cell-cell contact was established or lost, depending upon the concentration of extracellular  $\text{Ca}^{++}$ , within this time period (see Fig. 1).

It was noted above that fodrin synthesized in the absence of cell-cell contact was relatively unstable ( $t_{1/2} \sim 15$  h); however, when cell-cell contact was induced 1.5 h after synthesis, the same population of fodrin became stabilized ( $t_{1/2} > 70$  h). The time required to stabilize fodrin after cell-cell contact was very rapid since we were unable to detect a biphasic rate of degradation of fodrin (Fig. 7). This result demonstrates that a relatively unstable pool of fodrin can be rapidly recruited into a stable, insoluble pool of protein upon

cell-cell contact. In this context, it may be significant that our preliminary results indicate that the population of relatively unstable and soluble fodrin in these MDCK cells is in the form of heterodimers ( $\alpha\gamma$ ) which theoretically could be rapidly recruited to the membrane given an appropriate signal (Nelson, W. J., manuscript in preparation).

How is this pool of newly synthesized fodrin recruited and stabilized? A simple explanation is that proteins synthesized during the induction of cell-cell contact are involved. Significantly, however, the recruitment and stabilization of fodrin occurred in cells in which protein synthesis was inhibited by cyclohexamide during and after induction of cell-cell contact (Fig. 9). These results indicate that while cell-cell contact and the stabilization of fodrin are closely coordinated events in time, proteins synthesized during and after cell-cell contact are not required for fodrin stabilization.

That membrane proteins are involved in maintaining the stability of fodrin is suggested by the results of an experiment in which newly synthesized fodrin, that had become stabilized ( $t_{1/2} > 70$  h; Fig. 8 B) in cells with cell-cell contact, was shown to be destabilized ( $t_{1/2} \sim 20$  h; Fig. 8 A) after loss of cell-cell contact and light trypsinization of the cell surface. We interpret these results to indicate that destabilization of membrane proteins through the degradation of their extracellular domains by trypsin results sequentially in the destabilization of the associated membrane skeleton (fodrin) on the cytoplasmic surface of the membrane. Although we do not yet know the molecular linkage of fodrin to the membrane in MDCK cells (see below), these results indicate that proteins present constitutively in MDCK cells, in the presence or absence of cell-cell contact, may be involved in the stabilization of fodrin. However, this possibility must be qualified to take account of the fact that fodrin synthesized in the absence of cell-cell contact or 1.5 h after loss of cell-cell contact is relatively unstable ( $t_{1/2} \sim 15$  h) and soluble. Taken together, these results indicate that the stabilization and increased insolubility of fodrin in MDCK cells upon cell-cell contact is not simply due to the presence of preexisting proteins. In addition, cell-cell contact must result in changes in the conformation and/or affinity of proteins that cause the stabilization of fodrin; we are at present investigating these possibilities, together with the role of an ankyrin-like protein in MDCK cells.

The effect of cell-cell contact on fodrin stability and insolubility in MDCK cells appears similar to the effect of external ligands on the properties and distribution of fodrin in lymphocytes (5, 27, 28, 29, 36) and spectrin in erythrocytes (13, 14, 20, 43). Upon addition of cell surface ligands, fodrin/spectrin has been shown to become cross-linked and insoluble on the membrane, and in lymphocytes to become redistributed rapidly in patches and then a cap in a complex with the ligand and receptors (5). Significantly, in lymphocytes the association of fodrin with one surface glycoprotein receptor, the T-lymphoma-specific glycoprotein T-200, appears to be induced upon addition of the ligand and the onset of patching (5). The results of the present study demonstrated that perturbation of MDCK cell membranes by cell-cell contact also induces a rapid increase in the insolubility of fodrin, as well as increased stability. Furthermore, we showed here that the formation of extensive cell-cell contacts coincides with a dramatic change in the distribution of fodrin with a predominant localization in the regions of cell-cell contact.

These results indicate that cell-cell contact in MDCK cells and the binding of ligands to receptors on lymphocytes may induce a similar transmembrane signal(s) that initiates the association of fodrin with the membrane and a coordinate change in the spatial organization of membrane proteins.

Although cell-cell contact results in the stabilization and increased insolubility of fodrin in MDCK cells, our results indicate that, at least in the short term ( $\sim 12$  h), continued cell-cell contact is not required to maintain these new properties of fodrin. This was demonstrated through the analysis of the stability and degree of insolubility of fodrin synthesized in MDCK cells in the presence of cell-cell contact 1.5 h before loss of cell-cell contact by a decrease in the extracellular  $\text{Ca}^{++}$  concentrations. Under these conditions, fodrin remained relatively stable ( $t_{1/2} > 70$  h) and insoluble, despite the fact that fodrin synthesized after the loss of cell-cell contact was unstable ( $t_{1/2} \sim 15$  h; see above). These results indicate that the effect of cell-cell contact is to initiate the formation of a stable, insoluble fodrin matrix on the cytoplasmic surface of the plasma membrane. However, once established cell-cell contact per se is not required to maintain these properties of the fodrin matrix, at least in the short term.

### Implications for the Formation of Membrane Domains

Our initial analysis of the membrane skeleton (fodrin) in MDCK cells revealed that during development of a continuous monolayer of cells there are dramatic changes in the solubility properties, stability, and distribution of fodrin, which appeared to coincide temporally and spatially with the development of the polarized distribution of the  $\text{Na}^+, \text{K}^+$ -ATPase, a marker protein of the basolateral domain of the plasma membrane (35).

How might the effect of cell-cell contact on the stability and solubility of fodrin reported in this study play a role in the organization of fodrin and membrane domains? We suggest that the organization and distribution of fodrin in MDCK cells is regulated by a process of differential stabilization and accumulation that is initiated upon cell-cell contact. Since fodrin is stabilized and rendered insoluble by cell-cell contact, we suggest that fodrin will gradually accumulate at these regions of the membrane; on the other hand, since fodrin is relatively unstable and soluble in the absence of cell-cell contact, we suggest that fodrin will not accumulate to the same extent in regions of the membrane that are not involved in cell-cell contact (the apical membrane). This process would result in the gradual development of an asymmetric distribution of fodrin in MDCK cells that had extensive cell-cell contact, as indicated in our previous study (35). Thus, the gradual development of an asymmetric distribution of fodrin by a process of differential stabilization and accumulation, as hypothesized above, could occur without the need for fodrin to be targeted directly to the basolateral domain of the plasma membrane. Finally, we suggest that a consequence of the formation of this fodrin matrix at the cell periphery may be that membrane proteins targeted directly to the basolateral domain of the plasma membrane (e.g., the  $\text{Na}^+, \text{K}^+$ -ATPase [8]) become trapped in this matrix and hence constrained from diffusion in the plane of the lipid bilayer.

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