Participation of Plasma Membrane Proteins in the Formation of Tight Junctions by Cultured Epithelial Cells

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ABSTRACT Measurements of the transepithelial electrical resistance correlated with freeze-fracture observations have been used to study the process of tight junction formation under various experimental conditions in monolayers of the canine kidney epithelial cell line MDCK.

Cells derived from previously confluent cultures and plated immediately after trypsin-EDTA dissociation develop a resistance that reaches its maximum value of several hundred ohms-cm^2 after ~24 h and falls to a steady-state value of 80–150 ohms-cm^2 by 48 h. The rise in resistance and the development of tight junctions can be completely and reversibly prevented by the addition of 10 μg/ml cycloheximide at the time of plating, but not when this inhibitor is added more than 10 h after plating. Thus tight junction formation consists of separable synthetic and assembly phases. These two phases can also be dissociated and the requirement for protein synthesis after plating eliminated if, following trypsinization, the cells are maintained in spinner culture for 24 h before plating. The requirement for protein synthesis is restored, however, if cells maintained in spinner culture are treated with trypsin before plating. Actinomycin D prevents development of resistance only in monolayers formed from cells derived from sparse rather than confluent cultures, but new mRNA synthesis is not required if cells obtained from sparse cultures are maintained for 24 h in spinner culture before plating. Once a steady-state resistance has been reached, its maintenance does not require either mRNA or protein synthesis; in fact, inhibition of protein synthesis causes a rise in the resistance over a 30-h period. Following treatments that disrupt the junctions in steady-state monolayers, recovery of resistance also does not require protein synthesis.

These observations suggest that proteins are involved in tight junction formation. Such proteins, which do not turn over rapidly under steady-state conditions, are destroyed by trypsinization and can be resynthesized in the absence of stable cell-cell or cell-substratum contact. Messenger RNA coding for proteins involved in tight junction formation is stable except when cells are sparsely plated, and can also be synthesized without intercellular contacts or cell-substratum attachment.

Cells in transporting epithelia form continuous monolayers which function as selective permeability barriers between compartments. Within such monolayers tight (occluding) junctions located near the apical surface of the cells control the movement of substances across the intercellular spaces (8, 9). Freeze-fracture electron microscopy reveals that tight junctions consist of a complex system of anastomosing intramembranous strands involved in maintaining the close contact of the membranes (2, 10, 19, 38, 39, 42). It has been proposed that these strands contain proteins and perhaps lipids (31, 38, 41), but little direct information is available about the biochemical nature of junctional components and their precise organization into a supramolecular structure.

The cell line MDCK of canine kidney origin provides one of several model systems that have recently been adopted for in vitro studies of the development of epithelia and their functional properties (3–5, 13, 14, 21, 25, 26, 27, 30, 33, 34, 35, 40). Confluent monolayers of these cells exhibit many characteristics of renal tubular epithelia, including the capacity to transport fluid and electrolytes in an apical to basolateral direction (3, 20, 27, 37). At confluence, MDCK monolayers acquire a transepithelial resistance that is correlated with the establish-

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 MARCH 1983 693-702

ment of a complete system of tight junctions detected by thin section and freeze-fracture electron microscopy (3, 4, 27).

Using the development of transepithelial resistance as an index of junction formation we have studied the synthesis of the required proteins and the assembly of tight junctions in cultured MDCK cells. We have assessed the stability of junctional components in established monolayers, as well as the protein and RNA synthetic requirements of cells derived from confluent and sparse cultures for the subsequent development of junctions. We have found that synthesis and assembly of junctional components are temporally separated and that both processes can be studied independently by appropriate experimental manipulations of the culture conditions.

This work suggests that formation of tight junctions requires the participation of specific plasma membrane proteins and that the synthesis of these proteins is a regulated rather than a constitutive process. The growth state of the cells appears to play an important role in controlling the accumulation of mRNA's for proteins required for junction formation, but prolonged intercellular contact or attachment to a substratum does not seem to be required for the synthesis of these proteins.

Results of this work have been presented in preliminary form (6, 12).

MATERIALS AND METHODS

MDCK cells that had been grown or maintained under the various conditions described below were plated onto collagen-coated nylon disks used for electrophysiological measurements (3). All cells were seeded at the same density (10^6 cells/ml, 1 ml/well) onto disks placed in 24-well Falcon dishes (Falcon Labware, Oxnard, CA). Disks were transferred to fresh medium after 1.5 h and the development of an electrical resistance was monitored essentially as previously described (3, 4). In these experiments $t = 0$ is the time of plating. Studies were also carried out with monolayers that had been plated on collagen-coated disks, transferred to fresh medium at 1.5 h, and then maintained in this medium for 48 h before the start of the experiment (steady-state monolayers).

In all experiments six disks were used per time point per variable, and the mean and standard error for each point were calculated. Experiments were repeated several times, but data from single experiments were used to construct the graphs (except as noted), because of variability in the absolute values of control resistances.

The following preparations of cells were used for plating:

1. FRESHLY TRYPSINIZED CELLS: Cells were grown in disposable glass or plastic roller bottles with complete medium consisting of Eagle's Minimal Essential Medium (MEM) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) with 10% fetal calf serum (FCS). Cells from recently confluent cultures (3–7 d after reaching confluence) or sparsely plated cells (sparse cultures) were removed from the roller bottles by incubation at 37°C with 0.25% hog pancreas trypsin (Gibco Laboratories) and 2 mM EDTA and plated onto disks immediately after dissociation. Sparse cultures were obtained from roller bottles in which 5×10^6 cells were plated 24 h before harvesting.

2. SPINNER-MAINTAINED CELLS: Cells from sparsely plated or recently confluent cultures were removed with trypsin-EDTA from roller bottles and maintained for 20–24 h at a density of 5×10^5 cells/ml in spinner flasks containing Joklik-modified Minimum Essential Medium for suspension culture (SMEM) (Gibco Laboratories) with 10% FCS. Under these conditions cell counts were stable for several days, indicating that growth was inhibited, although viability remained excellent for at least 48 h. Cell clumps were removed by filtration through a Nitex (Tetko, Elmsford, NJ) mesh with a pore size of 20 μm. Cells were sedimented by centrifugation at 500 *g* for 15 min and resuspended in complete medium before plating.

3. STEADY-STATE MONOLAYERS: After removal from roller bottles with trypsin-EDTA, cells were suspended in MEM and 10% FCS and immediately plated on collagen disks, but the monolayers were not used until 40–48 h after plating. In these experiments $t = 0$ is the time at which the monolayers were perturbed by the addition of EGTA or Diamide, or by a pH change.

a. EGTA: The medium was removed and replaced with a Ca^{++}, Mg^{++}-free Moscona's solution containing 2 mM EGTA (ethyleneglycol-bis [β-amino-ethyl ether] N-N'-tetra-acetic acid). The kinetics of loss of transepithelial resistance during 1-h incubation at 37°C in this medium and the subsequent recovery of the resistance when monolayers were returned to normal medium were determined.

b. Diamide: Monolayers were treated for different times (15–30 min) with 0.4–0.6 mM Diamide (Sigma Chemical Co., St. Louis, MO) in Dulbecco's phosphate-buffered saline containing Ca^{++}, until the transepithelial resistance dropped to 20 ohms-cm^2. As with EGTA treatment, changes in the resistance during this treatment and after monolayers were returned to normal medium were followed.

c. pH Changes: Disks were incubated in alkaline medium (MEM with serum adjusted to pH 10 with 1 N NaOH) for 1 h. Normal medium was restored and recovery of resistance monitored.

Inhibition of Protein and RNA Synthesis: Media containing 5–10 μg/ml cycloheximide (Sigma Chemical Co.), 2.5–10 μg/ml puromycin (di-HCl, Sigma Chemical Co.), or 1–2 μg/ml actinomycin D, (Aldrich Chemical Co., Milwaukee, WI; prepared from a 2 mg/ml stock solution in ethanol) were added at $t = 0$ and maintained throughout the experiment, unless otherwise indicated.

Rates of protein synthesis were monitored in monolayers developing or established on collagen-coated disks that were incubated with [^{3}H]leucine (10 μCi/ml) for 1 h ([^{3}H]leucine specific activity 5 Ci/mM; New England Nuclear, Boston, MA). Disks were transferred to 10% cold TCA and the precipitated proteins were collected on glass fiber filters that were rinsed three times with 5% cold TCA before counting in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). These measurements demonstrated that cycloheximide and puromycin inhibited protein synthesis by >90% within 10 min of their application.

Freeze-fracture Observations: Cells were plated at the appropriate density onto washed, glutaraldehyde-fixed collagen-coated 100-mm plastic tissue culture dishes each of which also contained three collagen-coated disks. After 1.5 h the dishes were gently rinsed and the medium changed. The disks were removed and their resistances measured before fixation of the monolayers developing directly on the collagen-coated dish surfaces, to correlate the resistance changes with morphological observations. After fixation in situ with cold 2% glutaraldehyde in 0.1 M sucrose and 0.1 M sodium cacodylate buffer for 2 or more hours, the monolayers were scraped off with a Teflon spatula, sedimented into a pellet, rinsed three times in the buffer containing sucrose and impregnated with 20–25% glycerol in 0.1 M sodium cacodylate. The pellets were frozen in liquid Freon 22, stored in liquid N_2 and transferred to a Balzer's model #301 apparatus where they were fractured at −150°C, etched for 0–1 min at −120°C and shadowed with platinum and carbon. The organic material was digested with Clorox and the replicas mounted on grids were examined in a Phillips EM 301 electron microscope. Experimental and control samples from at least two different experiments were examined, with a total of 50–100 cells from each group evaluated.

Scanning Electron Microscopy: Disks whose transepithelial resistance had been measured were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer ± 0.1 M sucrose for at least 1 h. They were impregnated with 1% osmium tetroxide, dehydrated in alcohol, and dried at the critical point in CO_2. Specimens were attached to stubs with double-sided tape, coated with gold-palladium and carbon, and examined in a ETEC or AMR 1000 scanning electron microscope. Experimental and control samples for at least two separate experiments were examined.

RESULTS

Previous observations have shown that when plated at high density on collagen-coated disks, MDCK cells obtained by trypsinization from confluent cultures rapidly adhere to the surface of the disks to form compact monolayers that within a few hours develop a significant transepithelial electrical resistance (3, 4). This rise in electrical resistance is correlated with the formation of an extensive system of tight junctional complexes between the cells (6). Although in monolayers established by this procedure the absolute value of the final resistance and the slope of its initial rise are somewhat variable, in a typical experiment (Fig. 1) the resistance rises to a maximum (~300 ohm-cm^2) usually attained by 20–24 h, and then decreases to steady-state levels (80–150 ohm-cm^2) that are reached by 48 h and have previously been shown to be maintained for weeks thereafter (3, 4; see also Figs. 2 and 3).

Stability of Tight Junctions in Confluent Monolayers

To determine to what extent continuous replacement of proteins involved in tight junctions is necessary for the main-

tenance of the resistance in established monolayers, we examined the effect of cycloheximide added 40–48 h after plating. It was found that inhibition of protein synthesis for 24 h caused no decrease in the electrical resistance (Fig. 2 *a*). Instead, the resistance gradually increased, reaching values up to 30–40% higher than in controls. These observations suggest that junctional proteins critical for the maintenance of the resistance in established monolayers do not turn over rapidly and therefore need not be constantly replaced. Indeed, the rise of the resistance caused by cycloheximide suggests that when protein synthesis is inhibited, previously synthesized proteins necessary for junction formation may be utilized for assembly of new junctions. As expected from these results, inhibition of m-RNA synthesis with actinomycin D did not lower the steady-state value of the resistance (Fig. 2 *b*).

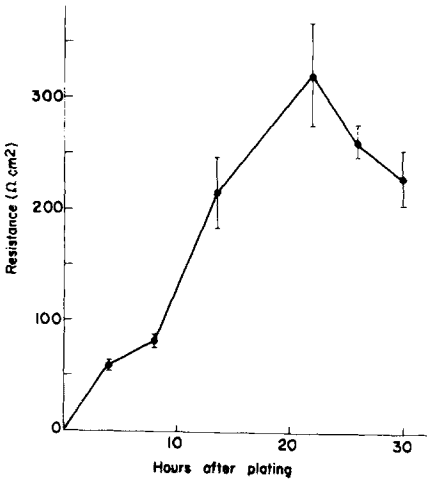

FIGURE 1 Development of resistance in MDCK monolayers formed by plating freshly trypsinized cells obtained from recently confluent cultures. After trypsinization, cells were plated on collagen-coated nylon disks at high density (10^6 cells/ml). After 1.5 h the disks were rinsed and transferred to fresh medium. Measurements of the transepithelial resistance were made at indicated times. Each point is the mean resistance calculated from six different disks.

Disruption of Established Tight Junctions

Several treatments, such as incubation with media containing EGTA or the weak base diamide or a rise in the pH of the medium, cause a rapid fall in the transepithelial resistance in established monolayers (Fig. 3 *a*, *b*, and *c*). These effects were reversible and the steady-state values of the resistances were restored within 3–5 h after the treated monolayers were transferred to normal medium (Fig. 3). Inclusion of cycloheximide in the medium did not prevent the reappearance of the resistance after removal of the disruptive agent (Fig. 3). Similar results (not shown) were obtained when cycloheximide was added together with EGTA. The observation that protein synthesis is not required for the reassembly of junctions in MDCK monolayers briefly treated with EGTA has been reported by Dolan et al. (6) and Martinez-Palomo et al. (22). It has also been previously shown (22) that the loss in resistance caused by EGTA is correlated with disruption of junctions. Regardless of the specific mechanism by which this disruption occurs, it can be inferred from the results presented in Fig. 3 that proteins necessary for junction formation synthesized before and/or during the disruptive treatments—possibly including some proteins previously involved in the formation of junctional strands—were utilized in the reestablishment of normal levels of resistance.

Formation of Junctions from Newly Synthesized Components by Cells Removed from Confluent Cultures by Trypsinization

The development of electrical resistance that accompanies the formation of new junctions in monolayers made by plating freshly trypsinized cells obtained from confluent cultures is illustrated in Fig. 1. To determine whether under those conditions the synthesis of new proteins is required for the development of tight junctions, we plated cells in the presence of cycloheximide or puromycin (Fig. 4). Although at the concentrations used (10 μg/ml) these inhibitors do not prevent the settling down and spreading of the cells necessary for the establishment of intercellular contacts (Fig. 5), no significant

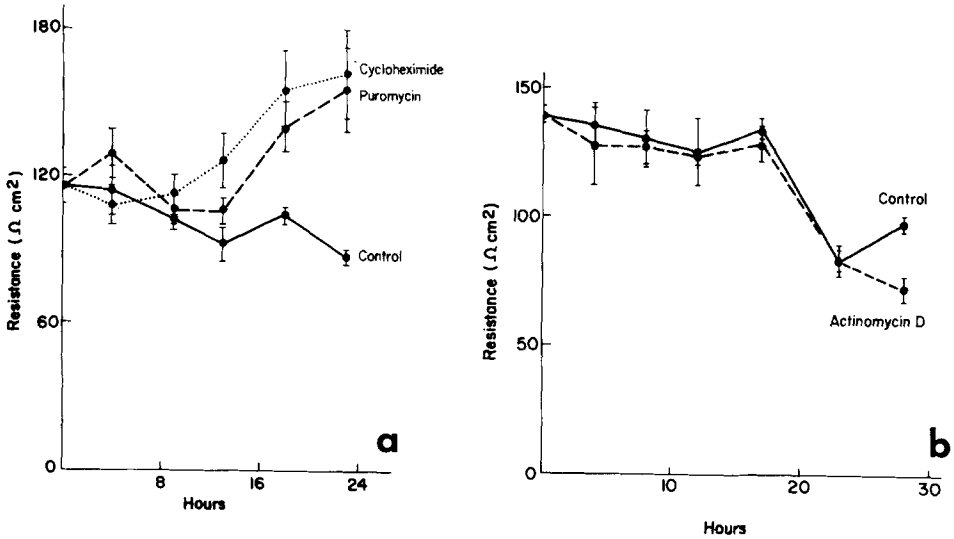

FIGURE 2 Protein and mRNA synthesis are not required for the maintenance of the resistance in established monolayers. (*a*) Effect of inhibition of protein synthesis. Monolayers were prepared by heavy plating of cells as indicated in Fig. 1; 40–48 h after plating, when the resistance had already reached a steady-state value, fresh complete medium containing 5 μg/ml cycloheximide or 2.5 μg/ml puromycin was added ($t = 0$). The presence of cycloheximide resulted in 90% inhibition of protein synthesis, measured by [^{3}H]leucine incorporation. Fresh medium without drugs was added to controls. Data from four experiments have been pooled. (*b*) Effect of inhibition of mRNA synthesis. Steady-state monolayers were prepared as described for Fig. 2 a. 40–48 h after plating fresh medium containing actinomycin D (1 μg/ml) was added ($t = 0$) to the experimental disks and fresh medium without drugs to the controls. Data from three experiments have been pooled.

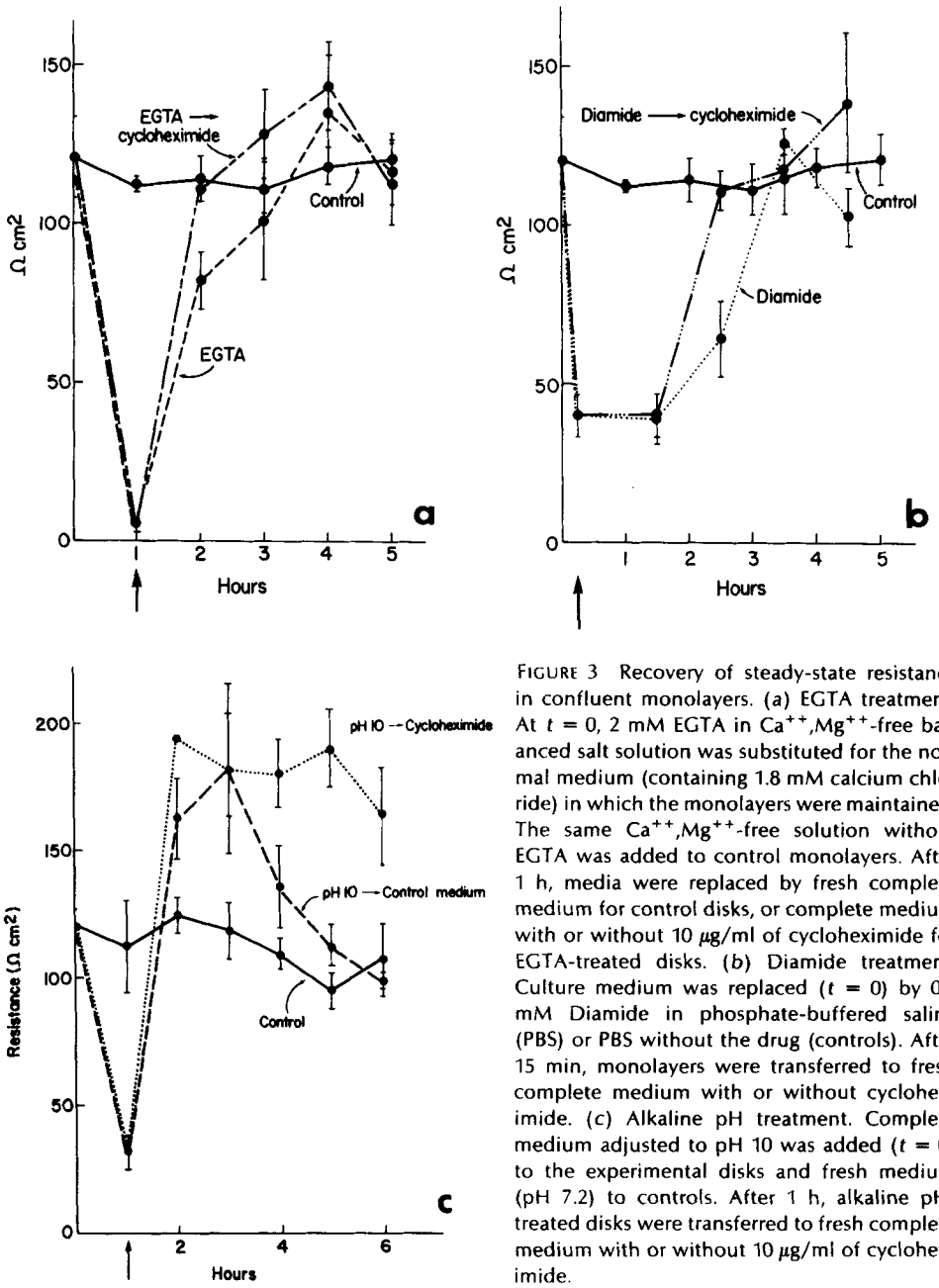

FIGURE 3 Recovery of steady-state resistance in confluent monolayers. (*a*) EGTA treatment. At $t = 0$, 2 mM EGTA in Ca^{++},Mg^{++}-free balanced salt solution was substituted for the normal medium (containing 1.8 mM calcium chloride) in which the monolayers were maintained. The same Ca^{++},Mg^{++}-free solution without EGTA was added to control monolayers. After 1 h, media were replaced by fresh complete medium for control disks, or complete medium with or without 10 μg/ml of cycloheximide for EGTA-treated disks. (*b*) Diamide treatment. Culture medium was replaced ($t = 0$) by 0.4 mM Diamide in phosphate-buffered saline (PBS) or PBS without the drug (controls). After 15 min, monolayers were transferred to fresh complete medium with or without cycloheximide. (*c*) Alkaline pH treatment. Complete medium adjusted to pH 10 was added ($t = 0$) to the experimental disks and fresh medium (pH 7.2) to controls. After 1 h, alkaline pH-treated disks were transferred to fresh complete medium with or without 10 μg/ml of cycloheximide.

resistance developed in the monolayers as long as the inhibitors were present (Fig. 4*a*). Correspondingly, freeze-fracture observations revealed that while extensive junctional complexes were formed in control monolayers during the first 8 h after plating (Fig. 6*a*), the formation of tight junctional strands was suppressed during incubation with cycloheximide for the same period: in cycloheximide-treated monolayers at 8 h, very few complete junctions were found, and patches of junctional fragments not observed in control cultures were occasionally seen (Fig. 6*b*). The inhibition caused by cycloheximide appears to be fully reversible, since upon removal of the drug even 8 h after plating a resistance begins to develop almost immediately with nearly normal kinetics (Fig. 4*b*). The requirement for protein synthesis suggests that trypsin causes irreparable damage to proteins necessary for junction formation.

Experiments in which cycloheximide was added at different times after plating (Fig. 4*c*) show that the synthesis of proteins required for the formation of junctions is completed during the first 10 h after plating. At this time the transepithelial resistance is not yet fully developed, but addition of cycloheximide does not prevent the continuing rise of the resistance, which attains its normal maximum 10–12 h later. These observations suggest that the synthesis of junctional components and their assembly into mature tight junctions are stages that may be temporally separated by several hours.

It was shown in a preceding section (Fig. 2*a*) that the maintenance of tight junctions in established monolayers does not require the continuous synthesis of junctional components. Having shown that establishment of a normal transepithelial resistance in monolayers of freshly trypsinized cells derived from confluent cultures requires protein synthesis, we therefore considered it of interest to determine whether mRNA's coding for proteins necessary for junction formation were present in freshly trypsinized cells obtained from confluent cultures. It

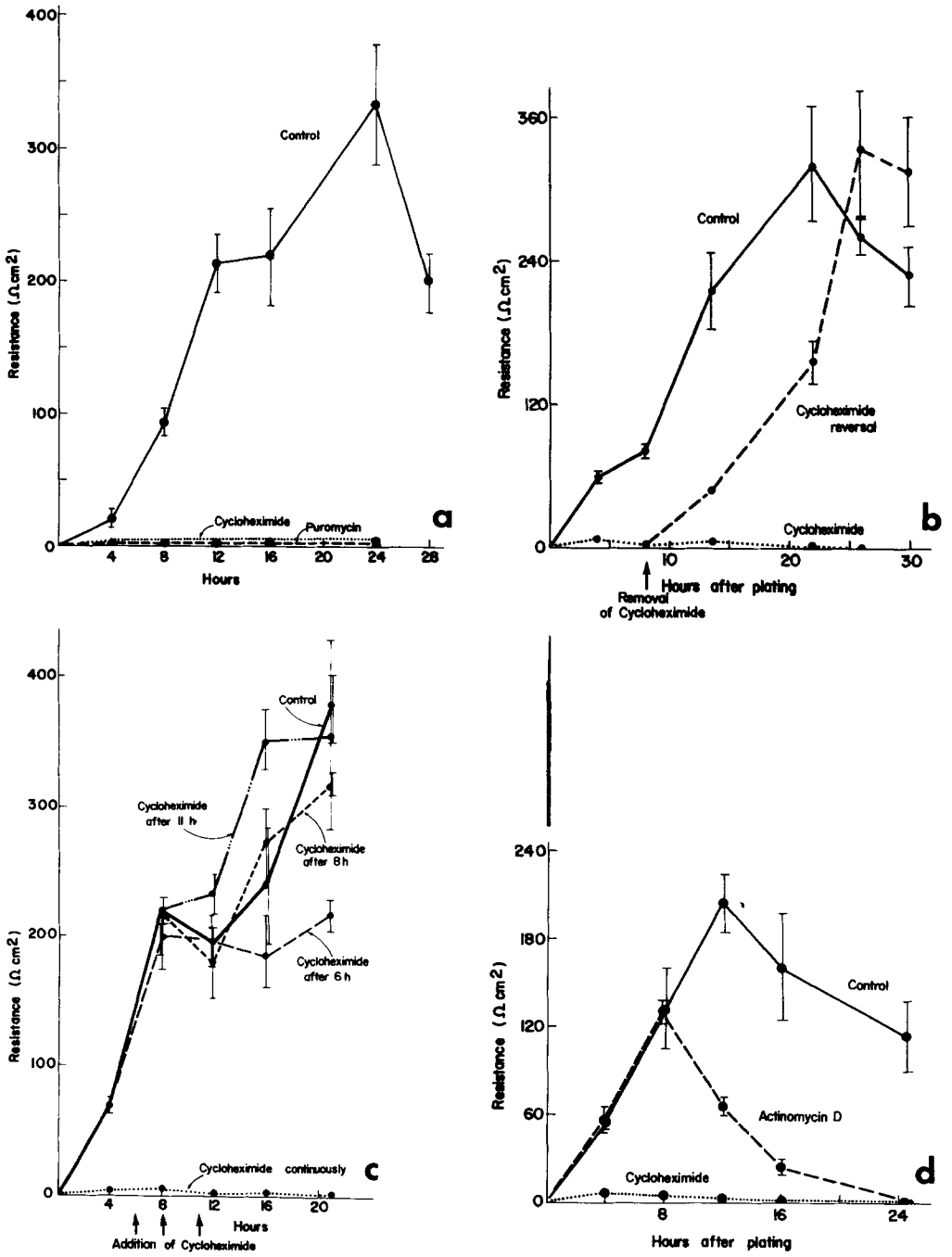

FIGURE 4 Effect of inhibitors of protein and mRNA synthesis on the development of resistance in monolayers formed by freshly trypsinized cells. Cells were plated and measurements were made as described in Fig. 1. (*a*) Development of resistance is prevented by protein synthesis inhibitors. Cycloheximide (10 μg/ml) or puromycin (10 μg/ml) was added at the time of plating and was present continuously. (*b*) A transepithelial resistance develops after removal of cycloheximide. Cells were placed in cycloheximide-containing medium at the time of plating; after 8 h, the disks were rinsed five times and placed into fresh complete medium free of cycloheximide. (*c*) Cycloheximide does not prevent development of the resistance when added more than 8 h after plating. Cycloheximide was added to disks (as indicated by the arrows) at 6, 8, and 11 h. (*d*) Actinomycin D (2 μg/ml) added at the time of plating did not prevent the initial rise of the resistance.

was found (Fig. 4*d*) that transcription of new mRNA's was not required for the formation of new tight junctions in such cells. Addition of actinomycin D at the time of plating does not prevent the rise of the resistance that occurs during the first 8 h after plating: only after this time does the drug cause a fall in the rising transepithelial resistance, probably as a result of its toxic effects.

Formation of Junctions from Newly Synthesized Components by Cells Obtained from Sparse Rather than Confluent Cultures

The preceding observations led us to study the development of the transepithelial resistance in monolayers of MDCK cells obtained by trypsinization from sparsely plated cultures.

Freeze-fracture electron microscopic observations show that complete junctional complexes are absent from sparse MDCK cells, which only occasionally contain some junctional strands in regions of intercellular contacts. A direct comparison was made of the kinetics with which resistances developed in monolayers formed by cells that had either been previously confluent for 2–5 d or had been sparsely plated 24 h before harvesting. Monolayers made with the latter cells showed significantly lower initial rates of development of resistance (Fig. 7*a*). Most importantly, and in dramatic contrast to the observations made with previously confluent cells (Fig. 4*d*), actinomycin D added at the time of plating completely prevented the development of a transepithelial resistance (Fig. 7*b*). These observations suggest that mRNA molecules that direct the synthesis of proteins necessary for the establishment of tight junctions are not present in sparse cells, at least at levels adequate to support the formation of complete junctional complexes in all cells, and that in these cells transcription of new mRNA must precede the rise in resistance.

FIGURE 5 Monolayers plated and maintained in the presence of cycloheximide for 8 h. The apical surface of a monolayer treated with cycloheximide (10 μg/ml, added at the time of plating) looks similar to that of control monolayers (not shown). Cells have spread and appear to be in contact, although occasionally some areas of retraction are seen. Bars, 10 μm. × 1,180.

Formation of Junctions from Newly Synthesized Components by Cells Obtained from Long-confluent Cultures

When monolayers are formed by cells obtained by trypsinization from cultures maintained at confluence for 2–3 wk before dissociation, the transepithelial resistance rises at a rate lower than it does in monolayers formed by cells derived from recently confluent cultures (Fig. 8*a*). Although this low rate was comparable to that observed when cells from sparse cultures were plated, in the case of long-confluent cultures actinomycin D did not inhibit the development of resistance (Fig. 8*b*). This suggests that mRNA for proteins required for junction assembly is present and turns over slowly in long-confluent cells, in which junctional proteins also turn over at a very low rate. The slow development of resistance is probably a consequence of a marked decrease in overall protein synthesis, documented by reduced incorporation of [^{3}H]leucine in these cells as compared with controls from recently confluent cultures (Table I).

Dissociation of the Synthesis and Assembly of Junctional Components

A complete dissociation of the synthesis of junctional components from their assembly into junctions was accomplished when, before plating on disks, freshly trypsinized cells derived

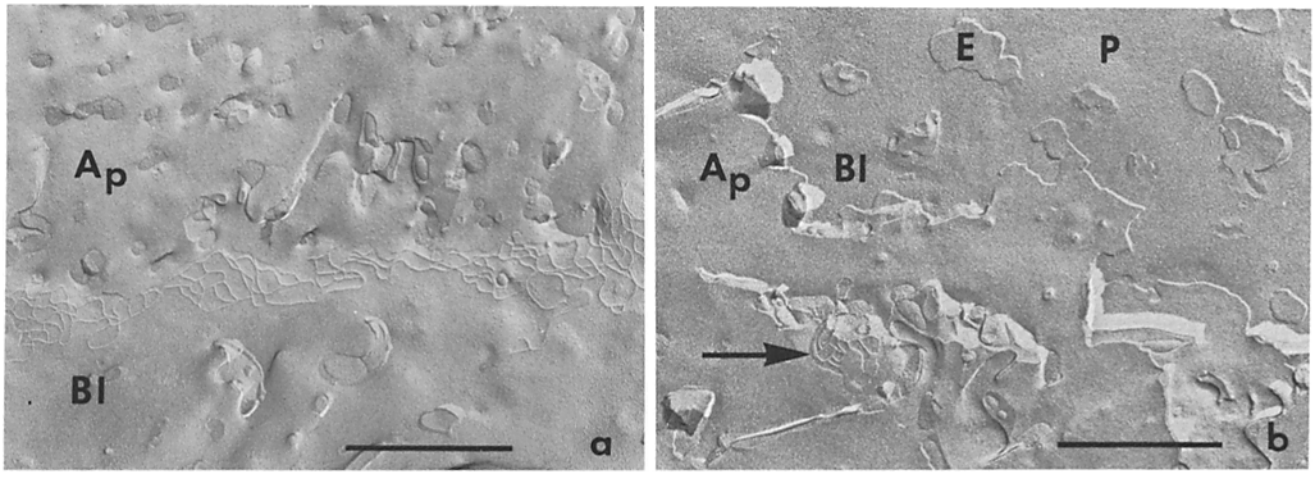

FIGURE 6 Cycloheximide inhibition of the development of tight junctions. 8 h after plating, control (*a*) or cycloheximide-treated monolayers (*b*) were fixed in glutaraldehyde, scraped, and processed for freeze fracture. (*a*) In control monolayers a network of tight junctional strands marks the boundary between apical (*Ap*) and basolateral (*Bl*) plasma membrane domains. Occasionally, immature junctions with as few as one strand are also seen (not illustrated). Bar, 1 μm. × 23,000. (*b*) In monolayers treated with cycloheximide (10 μg/ml, added at the time of plating) complete tight junctions are seen very rarely, although occasionally a few junctional strands are present (arrow) between apical (*Ap*) and basolateral (*Bl*) surfaces. That extensive contact between neighboring cells has taken place, however, is indicated by frequent patches of the membrane (*E* face) of one cell which remain attached to the membrane of the adjacent cell (*P* face). Bar, 1 μm. × 23,000.

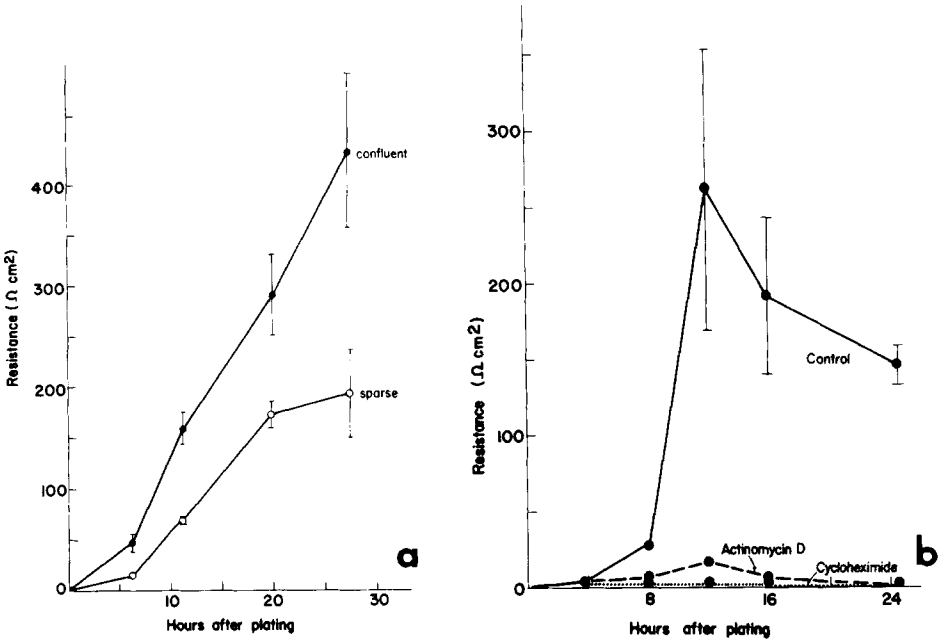

FIGURE 7 Development of transepithelial resistance in monolayers formed by cells derived by trypsinization from sparse compared with confluent cultures. (*a*) The rate of rise of the resistance is lower when cells obtained from sparse cultures (5×10^6 cells/roller bottle) rather than recently confluent cultures ($0.5\text{–}1 \times 10^8$ cells/roller bottle) were seeded on discs at the same density (10^6 cells/ml). (*b*) Actinomycin D (2 μg/ml) as well as cycloheximide (10 μg/ml) added at the time of plating prevent the development of resistance in monolayers formed by plating freshly trypsinized cells obtained from sparse cultures.

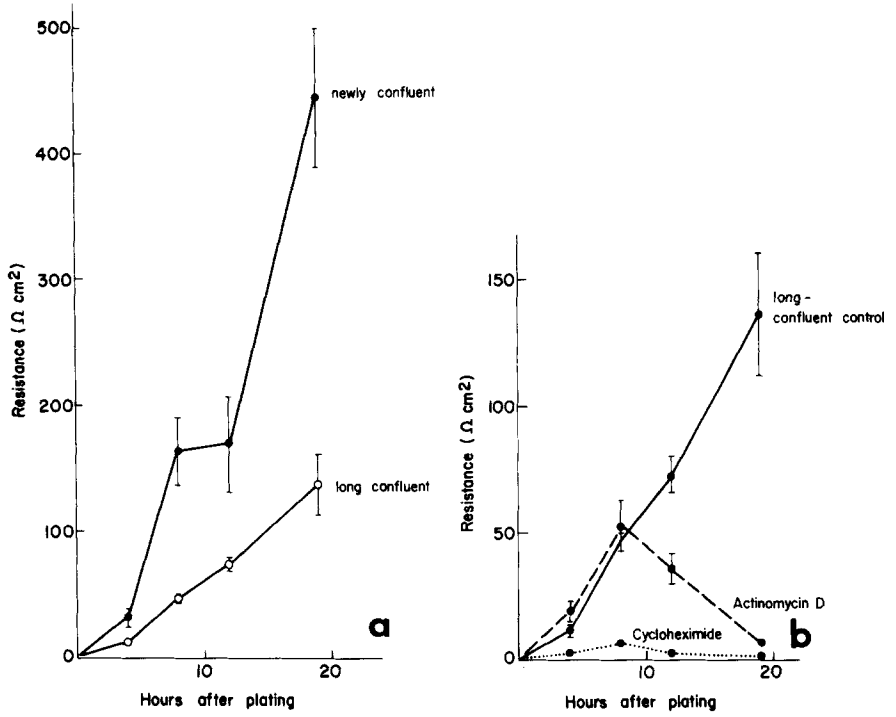

FIGURE 8 Development of transepithelial resistance in monolayers formed by freshly trypsinized cells derived from cultures maintained at confluency for varying periods. Cells obtained from cultures confluent for 2 d (newly confluent) or for 15 d (long-confluent) were plated at high density (10^6 cells/ml) and measurements were made as described in Fig. 1. (*a*) The resistance rises at a higher rate when cells obtained from newly confluent cultures are plated. (*b*) Actinomycin D does not prevent the initial rise of the resistance in monolayers formed with cells derived from long-confluent cultures. Actinomycin D (2 μg/ml) and cycloheximide (10 μg/ml) were added at the time of plating.

from confluent cultures were incubated for 24 h as suspension cultures in spinner flasks. Monolayers formed by these cells (Fig. 9 *a*) develop an electrical resistance more rapidly than those made from freshly trypsinized cells and achieve earlier a peak resistance that is generally of a higher value. Most strikingly, treatment of spinner cells with cycloheximide continuously from the time of plating does not prevent the development of a resistance (Fig. 9 *b*), which, however, often does not reach peak values as high as those in controls. These results clearly show that the synthesis of proteins necessary for the formation of junctions can proceed in suspension cultures, in which cells do not make stable contacts with other cells or with a solid substratum. Such proteins appear to have been transferred to the cell surface, since treatment of spinner-maintained cells with trypsin-EDTA immediately before plating renders them incapable of developing a resistance in the absence of protein synthesis (Fig. 9 *c*).

Transcription of mRNA's required for the synthesis of junctional proteins also takes place when cells derived from sparse cultures are maintained in spinner conditions. Thus, while monolayers made of freshly trypsinized sparse cells do not develop a transepithelial resistance when incubated with either actinomycin D or cycloheximide (Fig. 7 *b*), these inhibitors do not abolish the rise of the resistance, but only partially reduce it, when the same cells are plated after having been maintained for 24 h in spinner culture (Fig. 9 *d*).

TABLE I

Rates of Protein Synthesis in Monolayers Formed by Plating Freshly Trypsinized Cells from Newly Confluent or Long Confluent Cultures

[^{3}H]Leucine incorporation during 1 h (cpm × 10^3)		
Time	Newly confluent	Long confluent
h		
3	12.7 ± 5.0	9.5 ± 0.6
7	23.1 ± 1.2	8.6 ± 3.2
11	20.3 ± 0.8	12.7 ± 1.2

Cells were obtained from cultures confluent for 2 d (newly confluent) and 15 d (long confluent) and plated at high density as described for the measurement of resistance. [^{3}H]leucine (10 μCi/ml) was added to disks for 1 h pulses at 3, 7, and 11 h after plating. Total proteins on each disk were precipitated with 10% TCA, rinsed with 5% cold TCA and counted in a liquid scintillation counter. Each value is the mean of 4 disks ± standard error.

DISCUSSION

The experiments described in this paper provide insights into the nature and regulatory features of the biosynthetic processes necessary for the assembly of functional tight junctions in epithelial cells. The previously reported finding (4, 6), analyzed in more detail in this paper, that MDCK cells require protein synthesis to reestablish a transepithelial resistance after trypsin dissociation most likely indicates that proteins that are exposed on the cell surface participate in the process of junction formation. Our observations also suggest that confluent MDCK cells do not contain a large intracellular pool (i.e., inaccessible to trypsin) of proteins that can be utilized in junction formation in the absence of protein synthesis. A similar conclusion has been reached from freeze-fracture observations, which indicate that junction formation does not take place when cycloheximide is added to developing monolayers formed by cells dissociated from subconfluent cultures with EGTA (15).

Synthesis of proteins necessary for junction formation appears to be completed within 8 h after trypsinized cells are plated at high density. After this time, assembly of the junctions, as manifested by an increase in transepithelial resistance, is unaffected by the addition of cycloheximide and proceeds even when the drug is present for an additional 12–14 h. A complete temporal dissociation between the assembly of tight junctions and synthesis of the required proteins can be achieved under several experimental conditions. Cells from previously confluent cultures are capable of reestablishing junctions in the absence of protein synthesis, if after trypsinization they are maintained in a spinner culture for 24 h before plating. As expected, the kinetics of junction formation by these cells are faster than with cells not incubated in spinner culture but plated immediately after trypsinization. Proteins required for junction formation are probably incorporated into the cell surface during maintenance of the cells in spinner culture, since trypsinization immediately before plating abolishes the capacity of such cells to form junctions in the presence of cycloheximide.

Assembly of junctions in the absence of protein synthesis can also be achieved in monolayers briefly treated with various agents that rapidly and reversibly lead to a loss of transepithelial resistance. Although it is likely that these agents only lead to junction disruption secondarily, most likely as a result of primary effects on cell shape (1, 7, 11, 24, 25, 32), the rapid recovery of the resistance in the presence of cycloheximide after removal of the perturbing agents indicates that the capacity of preexisting proteins to participate in junction formation is not affected. Using freeze-fracture electron microscopy, Meldolesi et al. (23) drew similar conclusions after examining the effects of cycloheximide on guinea pig pancreatic acinar cells whose junctions were disrupted by Ca^{++} chelation.

It is known that the plasma membrane is a dynamic structure in which protein components may turn over with independent rates (16, 18, 29, 36). The possibility that junctional structures may undergo remodeling is suggested by the observation that peak levels of resistance, usually attained 24 h after plating of trypsinized cells, decrease during the next 24 h to steady-state levels, which are often >50% lower (3). The extent to which synthesis of new proteins is required for the maintenance of established tight junctions was examined in mature monolayers, in which protein synthesis was inhibited during long periods of incubation with cycloheximide. The finding that prolonged treatment with cycloheximide does not lead to a decay in resistance suggests that, if proteins are involved in maintaining the junctional complexes, such proteins do not normally turn over with a high rate.

The unexpected observation that cycloheximide treatment of mature monolayers leads to a 30–40% increase in resistance within 24 h may reflect a reorganization of junctional elements that takes place during this period and renders the junctions more impermeable to ions. This could possibly involve the recruitment of a pool of plasma membrane components that for some reason had not previously been integrated into functional junctions. It has been observed that different treatments can induce a proliferation of tight junction strands in natural epithelia (23, 28), and it has recently been shown that a massive increase in the total length of tight junction strands between prostatic epithelial cells takes place during incubation of the tissue at 37°C (17). This effect was not prevented by cycloheximide and it was therefore suggested that it reflected the assembly of preexisting components into new junctions (31).

An increase in resistance during incubation with cycloheximide is also consistent with more speculative interpretations in which the suppression of the synthesis of a labile inhibitor of junction formation leads to a decrease in transepithelial permeability. If such a feedback mechanism to maintain tight junction permeability exists, it might also explain why transepithelial resistance reaches a peak before assuming steady-state values (Fig. 1) and also why, after disruption of established monolayers, the resistance initially rebounds to higher than steady-state values (Fig. 3).

In the experiments presented in this paper, cells were always plated on collagen disks at a sufficiently high density that formation of a confluent monolayer and development of tight junctions could proceed without cell division. This allowed us to compare the readiness of cells previously found in different growth states to form junctions. Cells derived from cultures maintained at confluence for 2–5 d or even longer contained a level of mRNA adequate for the synthesis of proteins necessary for junction formation, which therefore proceeded even in the presence of actinomycin D. On the other hand, cells that had not yet reached confluence at the time of trypsinization because they had been sparsely plated and cultured for only 24 h did not contain sufficient levels of mRNA to synthesize the proteins necessary for junction formation in the presence of actinomycin D. If one discounts the possibility of greater toxicity of actinomycin D on sparse than on confluent cultures, these results suggest that expression of genes involved in tight junction formation may be regulated by the growth state of the cells so

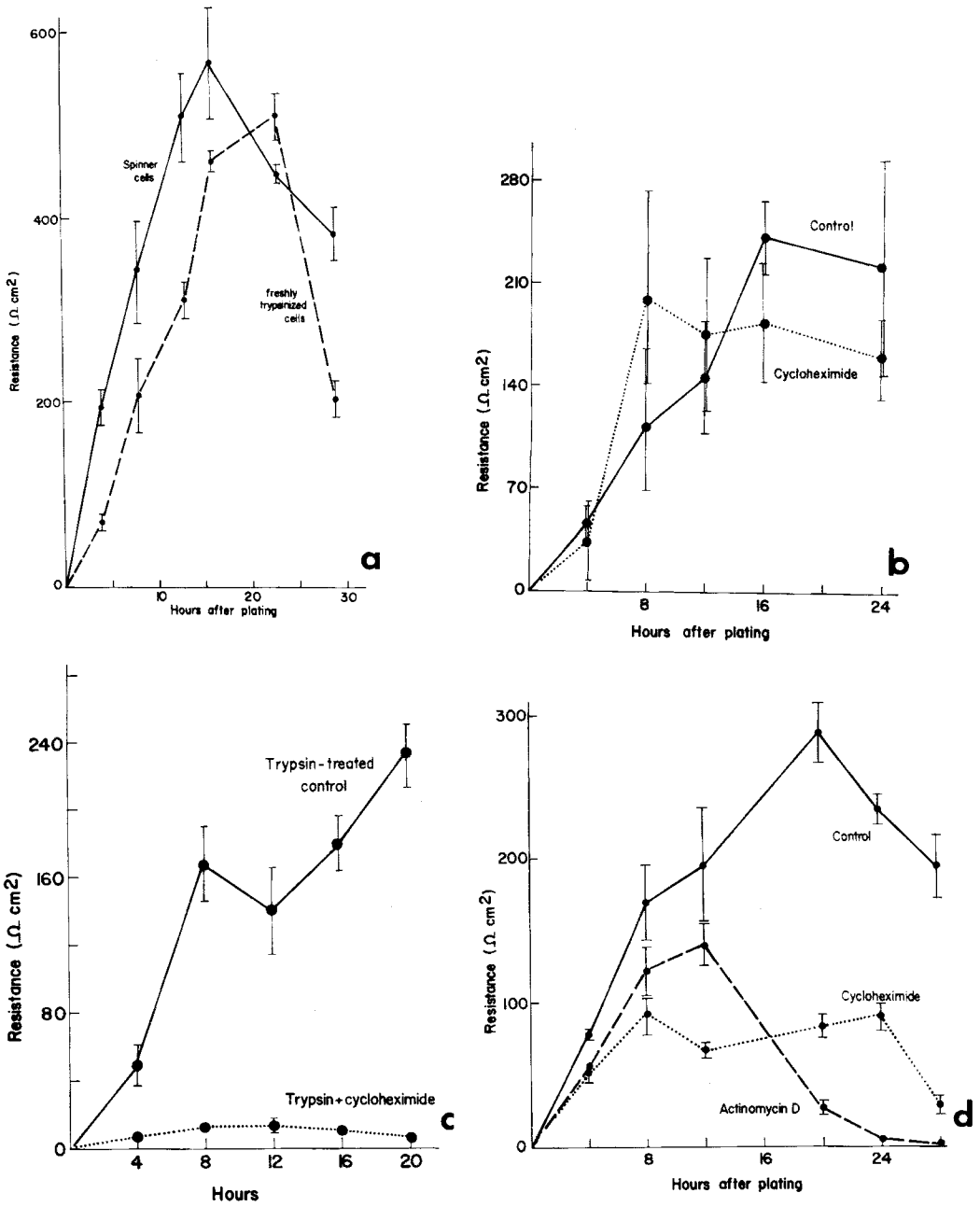

FIGURE 9 Development of the transepithelial resistance in monolayers formed by cells that after trypsinization are maintained in spinner culture for 20–24 h before plating. Cells obtained by trypsinization from confluent (*a*, *b*, and *c*) or sparse (*d*) cultures were maintained for 20–24 h in spinner culture before plating on disks at high density. (*a*) The resistance develops faster when cells were maintained in spinner medium following trypsinization. (*b*) Cycloheximide (10 μg/ml) added at the time of plating does not prevent the development of a resistance when cells previously maintained in spinner culture were plated. (*c*) Treatment with trypsin before plating restores the requirement for protein synthesis when cells previously maintained in spinner culture were plated. Cells were treated with trypsin-EDTA in a Ca^{++}, Mg^{++}-free salt solution for 15 min before plating on disks. Cycloheximide (10 μg/ml) was added at the time of plating. (*d*) Maintenance in spinner cultures allows the transcription of messengers and the synthesis of proteins required for the development of a transepithelial resistance in cells obtained from sparse cultures. Actinomycin D (2 μg/ml) and cycloheximide (10 μg/ml) were added at the time of plating.

that adequate levels of the corresponding mRNA's normally accumulate only after confluence. However, the finding that, in cells that were obtained from sparse cultures by trypsinization and maintained in suspension for 24 h before plating, the necessary mRNA's also accumulate in levels sufficient to support junction formation indicates that neither attachment to a substratum nor extensive intercellular contacts are required for messenger accumulation. Readiness to establish junctions may follow the cessation of growth that occurs after confluence in monolayer cultures or during maintenance in suspension.

The experiments presented in this paper emphasize the role of some plasma membrane proteins in the process of junction formation. They do not, however, demonstrate that these proteins are structural components of the junction. An understanding of the ability of cells to regulate the synthesis of required components and their assembly into tight junctions may facil-

itate the identification of those proteins that play a specific role in the process of junction formation.

We acknowledge with gratitude the expert technical assistance of Harriet Snitkin, Susan Malamet, and Heide Plesken. We wish to thank Joyce Mixon, Sonia Martinez, and Myrna Chung for their help with the typing of the manuscript, and Jody Culkin and Brian Zietlow for photographic work.

E. B. Griepp was supported by National Institutes of Health (NIH) postdoctoral fellowship HD 05076 and subsequently by an Investigatorship from the New York Heart Association. This work was supported by NIH grant AG 00378.

Received for publication 28 September 1982, and in revised form 7 December 1982.

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