Gap Junction Assembly and Endocytosis Correlated with Patterns of Growth in a Cultured Adrenocortical Tumor Cell (SW-13)

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ABSTRACT

After seeding at subconfluent densities, human adrenocortical adenocarcinoma cells (SW-13) proliferate to form a typical epithelial monolayer upon the culture vessel substratum. Single cells of the confluent monolayer are spontaneously released into the medium, however, where they may remain isolated or reaggregate with other floating cells. In this study, we examined the growth patterns of these cells in culture and analyzed alterations of their most prominent intercellular contact specialization, the gap junction, as cellular relationships changed during growth and development of the cell culture. We report here details of gap junction assembly during cellular aggregation and correlate the apparent endocytosis of gap junctions with the spontaneous release of single cells from the monolayer into the medium.

INTRODUCTION

Many cell lines grow 2-dimensionally in culture forming a confluent monolayer upon the vessel substratum (1, 4, 7, 12, 15). Even though cells in subconfluent cultures may round and even detach from the bottom of the flask or dish prior to division, they generally return and flatten again, ultimately interacting with other flattened cells at their lateral borders. As confluence approaches, however, such rounding and division, most frequent at the advancing edge of the expanding tissue sheet (7, 11, 16), may be reduced or may cease altogether (7, 48, 49). Several other cell lines, including several malignant cells of epithelial and fibroblastic origin, however, often continue to round, detach, and divide even after cells have completely covered the substratum and may do so generally in any region of the culture dish. Usually, these cells are viable and continue to proliferate within the medium as single cells or may reaggregate with others, "piling up" above the monolayer (4, 6, 19, 42).

We have undertaken studies of a human adrenocortical adenocarcinoma (SW-13) which "piles up" in cell culture, and because an earlier investigation indicated that cells of the monolayer were capable of forming extensive gap junctions (35), we wondered how these specialized cell-to-cell connections might be altered as cells spontaneously aggregated and disaggregated during their growth in culture. Earlier studies have demonstrated that gap junctions may be rapidly assembled at the cell surface in both cultured (27) and intact (2, 3, 10, 14) cells. Other reports have suggested that gap junctions may be removed from the cell surface either through an endocytic mechanism (2, 33, 34) or by dispersal within the plane of the membrane (29, 57). Although previous studies have published details of cellular behavior in culture or have examined specific aspects of gap junction assembly or removal processes, the present report attempts to analyze possible relationships between the spontaneous behavior of a cultured cell and the dynamic behavior of its junctional specializations.

MATERIALS AND METHODS

Cells. Cultured SW-13 cells were obtained from Leibovitz (Scott and White Clinic, Temple, Texas) in their 149th passage. These cells have been described as adenocarcinoma of the adrenal cortex (35) and have been demonstrated to produce tumors when injected into nude mice (18).

Since it has been suggested that peptide hormones may influence junctional behavior (10, 13, 31), the ability of SW-13 cells to produce cAMP in response to ACTH and CT was tested by measuring cAMP and steroids with procedures described by Decker et al. (13). Both Y-1 (BS-1) and Y-1 (mutant) adrenal cortical tumor cells were assayed for unstimulated and CT- and ACTH-mediated cAMP and steroid synthesis along with the SW-13 cells as controls for the assay procedures, and their culture is described by Decker et al. (13).

Cell Culture. The cells used in the growth and ultrastructural studies were cultured in L-15 nutrient medium (Grand Island Biological Co., Grand Island, N. Y.) with cortisone (10 mg/ml), insulin (0.02 unit/ml), penicillin (0.06 mg/ml), streptomycin (0.1 mg/ml), and Fungizone (0.01 mg/ml), all from Grand Island Biological Co., buffered with L-arginine at pH 7.1 to 7.2. All cells were grown in 35-mm Falcon Petri plates or 25-ss sq mm Falcon flasks (Falcon Plastics, Oxnard, Calif.) at 37° in a 5% CO2 atmosphere. Direct cell counting after trypan blue and the counting of cells in time lapse films revealed a confluent density of at least 106 cells/cm2 for this cell line. The viability of cells in various phases of culture growth was determined by dye exclusion studies (trypan blue), by replating, and by an analysis of mitotic index.

Time Lapse Photography. Cells used for time lapse studies were established as monolayers on the surface of acid-cleaned sterile glass coverslips (25 mm; No. 1) in the bottom of a 35-mm Petri dish. Dishes were seeded at superconfluent densities, and 24 to 72 hr after a confluent monolayer was established, all cells above the monolayer were washed away, and the coverslip was mounted in a Sykes-Moore chamber (Bellco Glass, Inc., Vineland, N. J.) filled with L-15 medium equilibrated to a 5% CO2 atmosphere. These preparations were then mounted on the stage of a Leitz Orthoplan microscope equipped with a ×16 objective lens. A Sage air curtain (Sage Instruments, White Plains, N. Y.) was used to maintain culture temperature at 37°. The cells were filmed with a Bolex H16M camera (Saitue Croix, Switzerland) through Nomaski optics of the microscope at a total magnification of ×16.7 on the 16-mm plus-X Pan reversal film 7276. One 2-sec exposure was made each min and controlled by a Sage series 500 cinematographic apparatus. Periods of 1 to 3 days of cell growth were continuously monitored with this technique. Time lapse films were...
analyzed with a Magnasynch Moviola 16-mm viewer and frame counter (North Hollywood, Calif.). The total magnification of cell cultures on the Moviola screen was \( \times 210 \). The positions and times of single-cell rounding were recorded with the aid of a calibrated plastic grid attached to the viewing screen.

**Thin-Section and Freeze-Fracture Studies.** Cells were fixed for 10 to 15 min at room temperature in 2.5% glutaraldehyde and 2% sucrose buffered to pH 7.2 with 0.05 M cacodylate buffer or in 2.5% glutaraldehyde with 2% tannic acid neutralized with NaOH according to the procedure of Van Deurs (52). Tissue for thin sectioning was rinsed in 0.05 M cacodylate buffer containing 10% sucrose and fixed in 1% osmium tetroxide buffered to pH 7.2 with 0.05 M cacodylate. After a buffer wash, the tissue was dehydrated in ethanol and embedded in Araldite (R. P. Cargille Labs, Inc., Cedar Grove, N. J.). Tissue used in freeze-fracture studies was infiltrated with 30% glycerol for 3 hr after 10 min of fixation in 2.3% glutaraldehyde and 2% sucrose in 0.05 M cacodylate buffer at pH 7.2. The tissue was then frozen in Freon and fractured with a mirror image device in a Balzers BAE 121 freeze-fracture apparatus (Balzers, Hudson, N. H.). Complimentary platinum-carbon replicas were cleaned and mounted on uncoated 300-mesh grids. Micrographs were taken with a Philips 300 electron microscope. Cells floating in the monolayer were simply poured with the tissue culture medium into an equal volume of double-strength fixative while cells clustered above the monolayer were freed from the monolayer prior to fixation by vigorous pipetting. Normal-strength fixative was flooded onto the intact monolayer after medium was removed, and these cells were sectioned in the dish or were scraped free from the dish with a rubber policeman and inserted into sample holders prior to freeze fracture.

**Electron Microscopy.** Cells were prepared for scanning electron microscopy by fixation in their flasks in 2.5% glutaraldehyde in 0.05 M cacodylate and 2% sucrose at 37\(^\circ\)C for 1 hr. The cultures were washed in cacodylate buffer (pH 7.2) for 30 min at room temperature, fixed in 1% cacodylate-buffered osmium, dehydrated in alcohol, critical point dried in carbon dioxide, and sputter coated with gold-platinum alloy. The intact cells were then scanned in a Cambridge S4 or a JEOL-JSM 35c scanning electron microscope.

**Stereological Studies and Statistical Analysis.** The fractional areas and mean areas of formation plaques and gap junctional aggregates were determined by measuring individual formation plaques, gap junctional aggregates, and nonjunctional membrane with an Imaging Quantimat 720 Automatic Image Analyzing Computer (Imanco-Metals Research, Monsey, N. Y.) from tracings of electron micrographs. Nonjunctional membrane areas were traced directly from negatives taken at 4800 magnification. The junction and formation plaque areas were calculated on a per cell basis by determining the total number of equivalent cell surfaces scrutinized. The total number of cell surfaces scrutinized was determined by dividing the total membrane area measured by the estimated average surface area of a single cell which was calculated from measurements of cell outlines from scanning electron micrographs printed at \( \times 440 \). It was assumed that upper and lower surfaces of the monolayered cells were identical, and on this basis we calculated an average surface area of 161 monolayer cells as 2469.7 ± 446.8 (S.E.) sq \( \mu \text{m} \). In actuality, the area of apical membrane in flattened cells of the monolayer is slightly greater than the area of basal membrane because of cell thickness. Calculations based on the thickest cell measured would increase, at most, the total membrane per cell by about 10%, and since any increase would result in an increase in the average amount of junctional membrane per cell which in the 24-hr monolayer is already 8 to 400 times greater than junctional areas in 48- and 24-hr floating reaggregated cells, respectively, compensations in our calculations for this increased membrane area would simply tend to increase these large differences. The average surface area of clustered cells collected from the medium was determined by measuring the diameters of rounded cells and calculating the average surface area on the assumption that these cells were spherical. The average area of 96 clustered cells was 400.7 ± 13.3 sq \( \mu \text{m} \). It was assumed that average cell surface areas of 2- and 24-hr monolayers were identical and that the average surface areas of 24- and 48-hr clustered cells were identical. In actuality, the floating cells are not perfect spheres. Approximately one-third of these cells possess extensive surface blebs which may increase the average cell surface area significantly. These blebs, however, are readily identifiable in freeze-fracture preparations, and in all replicas scrutinized, only one junctional plaque was demonstrated on these surface membrane specializations. These membranes, therefore, were not included in measurements of junctional or nonjunctional membrane, and so their presence does not significantly alter estimates of junctional area or frequency on a per cell basis.

Eighty to 90 negatives taken at random of SW-13 cell surface membrane from 2 to 4 separate 25-sq cm culture flasks of each experimental group analyzed were randomly organized into 6 different groups of about 13 negatives each. A similar amount of membrane from each individual flask was analyzed in each respective culture type, and the total amount of membrane in each group of 13 negatives was approximately equal. Each of these groups was then treated as a single sample which was then analyzed with respect to the variables listed in Table 1. The differences in means of most pertinent concern in our studies were compared by one-way analysis of variance. Pairwise differences between means were determined by Duncan’s multiple comparison procedure (47).

**Localization of Acid Phosphatase Activity.** Cultured cells were fixed in 2.5% glutaraldehyde and 2% sucrose buffered with 0.05 M cacodylate, pH 7.2. After 30 min of fixation at room temperature, the cells were washed in buffer (2 X 15 min) and then treated in one of the following ways. Cells were incubated in 15 ml of a solution consisting of 40% 0.2 M Tris Maleic acid buffer (pH 5.0), 40% 0.125% \( \beta \)-glycerophosphate (filtered), approximately 6% of 1% PbNO\(_3\), 4% of water, and with or without approximately 10% of 0.1 M MgCl\(_2\). Some cells were incubated in complete medium minus \( \beta \)-glycerophosphate and others were incubated in complete medium also containing 1% NaF. All cells were incubated for 30 min at 37\(^\circ\)C in a gyratory water bath, washed twice in cacodylate buffer, fixed in osmium, dehydrated, and embedded in Araldite. This technique is Trout’s modification of the method of Wachstein and Meisel (54).

**RESULTS**

In order to investigate any possible correlation between gap junctional distribution and SW-13 cell growth, we found it necessary first to undertake a more detailed study of the behavior of SW-13 cells in culture. In the following sections, we will first describe morphological and functional characteristics of SW-13 cells, their patterns of growth under various culture conditions, and finally, the frequency and distribution of gap junctions in SW-13 cells in different phases of growth.

**Characterization of SW-13 Cells.** SW-13 cells obtained directly from Leibovitz demonstrated extensive gap junctions between cells of the monolayer as originally reported (35). The cytoplasm of all cells contained abundant smooth endoplasmic reticulum, elongate mitochondria with lamellar cristae, annulate lamellae, and lipid inclusions. The number of chromosomes of SW-13 cells obtained from Leibovitz and cultured for 165 successive passages was somewhat altered from that reported previously by Leibovitz et al. (35), demonstrating a range of 67 to 76 with 93% hyperdiploid and 7% hypodiploid. None of the SW-13 cultures tested were capable of secreting detectable levels of cAMP into the medium in response to ACTH or CT or were capable of producing fluorogenic steroids in response to...
and clusters above the monolayer had been rinsed. Cell clusters were allowed to form for 24 or 48 hr after 24-hr-old confluent monolayers had been completely rinsed of all cells above the monolayer and then fixed and freeze fractured. Based upon one-way analysis of variance and Duncan’s multiple comparison procedure (47), the amount of gap junctional membrane per cell in the 24-hr monolayer is significantly greater than the amount of gap junction per cell in any other culture. The amount of formation plaque per cell in the 24-hr monolayer is significantly greater than that in the 24- or 48-hr cell clusters but not significantly greater than that in the 2-hr monolayer. The number of gap junctions per cell is significantly greater in the 24-hr monolayer than in the 2-hr monolayer and in the 24- and 48-hr clusters. In all cases, differences were significant at $p \leq 0.05$.

Table 1

<table>
<thead>
<tr>
<th>Total areas of SW-13 cell membrane devoted to gap junctions and formation plaques, mean areas of gap junctions and formation plaques per cell in monolayered cells and cell clusters above the monolayer after varying periods of culture</th>
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<tbody>
<tr>
<td>Mean ± S.E. of 2 flasks.</td>
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<tr>
<td>Mean ± S.E. of 3 plates.</td>
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<tr>
<td>Mean ± S.E. of 2 flasks.</td>
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<tr>
<td>24-hr clusters</td>
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<tr>
<td>Mean ± S.E. of 2 flasks.</td>
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<tr>
<td>Mean ± S.E. of 3 plates.</td>
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<tr>
<td>Mean ± S.E. of 2 flasks.</td>
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<tr>
<td>2-hr monolayer</td>
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<tr>
<td>24-hr monolayer</td>
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<tr>
<td>48-hr monolayer</td>
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<tr>
<td>Formation plaque/cell</td>
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<tr>
<td>Formation plaque size range (sq µm)</td>
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<tr>
<td>Total monolayered cells and cell clusters above the monolayer after varying periods of culture</td>
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<td>Total formation plaque/ cell (sq µm)</td>
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<tr>
<td>No. of gap junctions/cell</td>
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</table>

*Mean ± S.E. of 2 flasks.

ACTH in contrast to Y-1 tumor cells tested at the same time (Table 2).

Pattern of SW-13 Cell Growth. When SW-13 cells were seeded into 35-mm Falcon Petri plates at a subconfluent density of approximately 5 x 10^4 cells/sq cm, almost all of the cells flattened and attached to the substratum within 2 hr. This was true of cells obtained just prior to plating by trypsinization of mature monolayers or from suspension after spontaneous detachment of cells from the monolayer. Within 24 hr, small colonies of flattened cells were present (Fig. 1) and cell rounding and division above these colonies or at the colony edges were apparent. Confluence of the typical epithelial monolayer (Fig. 2) occurred within approximately 4 to 5 days, but SW-13 cells in culture did not cease to round and divide once confluence had been attained. Time lapse cinematography of cells grown in Sykes-Moore chambers revealed that almost all cells in culture did not divide in the absence of immediately preceding cell contact. After an arrest period of 3.5 hr (using 0.06 mg colchicine per ml), we calculated a mean mitotic index of 11.7 ± 1.1 % for 5 samples of a floating cell population grown for 71 to 74.5 hr in spinner culture.

As noted, single cells rising above the monolayer could rapidly aggregate with other single cells and cell clusters, and within 24 hr, the majority of cells above the monolayer were aggregated into clusters (Fig. 4). Continued rounding and division resulted in continuing increases in cell number until the monolayer was almost completely obscured by aggregated cells (Fig. 5). In such advanced cultures, time lapse films demonstrated that floating cells also originated directly from clusters.

Cells removed from any location within the culture, whether washed gently from suspension, rinsed forcefully from clusters above the monolayer, or trypsinized free from the culture dish substratum, were usually 90 to 98% viable (dye exclusion) and were all capable of establishing new cultures, identical in growth pattern to those just described. In summary, it appears that SW-13 cells cultured in 35-mm Falcon Petri dishes or in Sykes-Moore Chambers may (a) spontaneously relocate from monolayer to cluster or to suspension or (b) from clusters to suspension or (c) from regions above the monolayer back to the monolayer, and (d) may reestablish cultures producing cells in all phases of growth after seeding into clean culture vessels. A diagrammatic representation of these transformations is provided in Fig. 6.

Membrane Junctions of SW-13 Cell Monolayers. Only 2 junctional types were identified with thin-section or freeze-fracture procedures in SW-13 cells in culture. These are the intermediate junction and the gap junction. Intermediate junc-
cells in the monolayer (Fig. 13). Rarely, annular profiles of gap junction membrane were observed within the cytoplasm of SW-13 cells at the cell surface (Fig. 12). Neither the total amount of gap junction formation plaque per cell nor the mean area of individual formation plaques, however, increased significantly between 2 and 24 hr. Occasionally, in thin sections, large involutions of gap junction membrane were also observed between cells of the monolayer, and in several cases it was apparent that these structures were connected to the cell surface (Fig. 12). Rarely, annular profiles of gap junction membrane were observed within the cytoplasm of SW-13 cells in the monolayer (Fig. 13).

### Table 3

Summary of behavior of 70 cells observed on time lapse film

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Total no.</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>Rounding cells that divide</td>
<td>56</td>
<td>88</td>
</tr>
<tr>
<td>Rounding cells not dividing</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>Cells rounding and spreading repeatedly</td>
<td>3</td>
<td>4.8</td>
</tr>
<tr>
<td>Rounding cells dividing twice</td>
<td>4</td>
<td>6.3</td>
</tr>
</tbody>
</table>

* Time between rounding and division, 2.3 ± 0.2 hr (range, 1.1 min to 5.6 hr).
* Time between first and second division, 11.3 ± 2.8 hr (range, 2 to 23 hr).

**Gap Junctions of Single Cells Floating in Suspension.** Single floating cells, obtained by gentle agitation and rinsing of the medium directly into fixative (Fig. 14) within 3 hr after all previously produced floating cells had been removed, infrequently demonstrated evidence of gap junctions at the cell surface. Those observed were characteristically small and always occurred between single intact cell bodies and small cell fragments apparently broken away from another cell. Annular junctional profiles, however, were frequently observed within the cytoplasm of single floating cells and were located both near the small surface junctions described above, as well as deep within the cytoplasm (Figs. 15 to 17). A single annular junctional profile was occasionally observed within another (Figs. 16 to 17) and others were frequently clustered together in small groups (Fig. 16). In preparations incubated in medium containing NaF or lacking the substrate β-glycerophosphate. Additional evidence for an interaction of these internalized gap junctional vesicles with the digestive apparatus of the cell was the rare observation of continuity between the membrane of a cytoplasmic junctional profile and an apparent lysosome (Fig. 21). In some cases, the annular junctional profile appeared to be undergoing degradation since inner and outer membranes were separated in some areas (Fig. 22).

**Gap Junctions of Cells Reaggregated above the Monolayer.** Cells localized in aggregates above the monolayer were also rinsed into fixative after vigorous pipeting and examined for cell junctions with thin-section and freeze-fracture techniques. Here, relatively large numbers of gap junction formation plaques, as well as more mature gap junctional forms, were documented (Figs. 23 and 24). The frequency and size of gap junctions and formation plaques in cell clusters produced over 24- and 48-hr periods are presented in Table 1. Thin-section electron microscopy also revealed the presence of intermediate junctions and annular cytoplasmic gap junctional profiles in these cells (Fig. 25).
DISCUSSION

The results of the correlated ultrastructural, time lapse, and growth studies just described support the hypothesis that gap junctions in cultured SW-13 cells are formed rapidly as cells come into contact and are removed from the cell surface through an endocytotic mechanism prior to or during spontaneous cell detachment. The following discussion will review current studies relevant to these conclusions and then will explore the possible functional significance of these relationships.

**Junctional Assembly in SW-13 Cells.** The freeze-fracture studies described in this report suggest that gap junction "formation plaques" may form rapidly when SW-13 cells are forced into contact at the bottom of the Petri dish after seeding at superconfluent density or when single cells spontaneously aggregate after their rounding and detachment from well-established confluent monolayers. These membrane specializations are similar to structures postulated to represent formative stages of gap junction development in reaggregated Novikoff hepatoma cells (27), differentiating amphibian ependymogial cells (14), developing granulosa and lutein cells (2, 3), and ovarian interstitial cells (10). Since the single floating cells used to seed the cultures in our studies were devoid of formation plaques and possessed only rare gap junctional plaques connected to small cell fragments, those junctional structures observed in newly established monolayers must have been assembled just subsequent to seeding. Our quantitative studies indicate an approximately 24-fold increase in gap junction membrane between 2 and 24 hr of culture which is consistent with data from earlier studies of reaggregated Novikoff hepatoma cells (27), suggesting that formation plaques and gap junctions may begin to form even within min after single cells have been brought into contact. It seems likely that the gap junctions and formation plaques present in cells spontaneously reaggregating for 24 and 48 hr above more mature confluent monolayers were also newly assembled as these cells made contact, since these cell clusters are initially formed by the spontaneous aggregation of single cells which rise and round at different times from the monolayer. The evidence that formation plaques occupy larger areas of membrane than more mature gap junctional aggregates after relatively short periods of cellular contact and less after longer periods in monolayers and in clusters provides additional support for the hypothesis that these formation plaques represent gap junctional precursors.

**SW-13 Cell Gap Junctional Endocytosis.** Many earlier studies have documented the presence of annular gap junctional profiles within the cytoplasm of several normal and malignant cell types (30), and on the basis of lanthanum tracer and serial sectioning studies (17, 20, 39), it has been suggested that some of these annular profiles are completely isolated from the cell surface. The present study which demonstrates the presence of annular gap junction profiles in single floating SW-13 cells also supports this conclusion since the inner junctional membrane of these profiles was most probably derived originally from a formerly adjacent cell. No evidence for dispersal of gap junction particles within the plane of the membrane, as reported in 2 other cell systems (29, 57), was observed in SW-13 cells.

Ultrastructural and cytochemical studies of cytoplasmic gap junction vesicles in SW-13 cells have also provided evidence for the degradation of these structures after internalization. Although annular gap junction profiles in SW-13 cells have not been observed within autophagolysosomes as reported in the developing chick otocyst (24), we have documented the apparent fusion of gap junction vesicles with lysosomes; the structural disorganization of bimembranous gap junction vesicles; and the presence of reaction product, indicative of hydrolytic enzyme activity, within the junctional vesicle matrix. This evidence is in agreement with earlier observations of the cytoplasmic gap junction vesicles of rabbit granulosa cells (33). Whether or not cytoplasmic gap junction vesicles or their remnants in SW-13 or rabbit granulosa cells are ultimately incorporated within autophagolysosomes remains to be determined.

A previous study also provides evidence for the presence of a basketwork of 4 to 7-nm-thick filaments enveloping some of the SW-13 cytoplasmic gap junctional vesicles as has also been reported in granulosa cells and cultured B16 melanoma cells (34). In the granulosa cell, S-1 decoration experiments have positively identified the membranes as actin containing, but additional studies directed to the presence and organization of other contractile proteins, such as myosin and a-actinin, will be required to determine whether or not an actin-based contractile apparatus plays a central role in gap junctional endocytosis in any of these cell systems.

Although it is possible that junctional interiorization may be predominantly unidirectional, since annular junction profiles appeared to be much more frequent in single floating cells than in cells of the monolayer, the relative frequencies of internalized gap junctions in these 2 populations may also depend upon other uncontrolled factors including (a) the relative rates of cytoplasmic gap junction vesicle degradation in monolayer and floating cells and (b) the selection of cells containing interiorized gap junction vesicles when recently detached floating cells are collected for ultrastructural analysis, in contrast to the more random sampling of the monolayer which may include large numbers of cells not involved in cell detachment and therefore demonstrating little or no evidence of junctional endocytosis.

**Gap Junction Behavior and SW-13 Growth Patterns.** Since newly formed SW-13 monolayers and cell clusters forming above the monolayer become established through the aggregation of single cells, it is not surprising that gap junction formation plaques and junctional aggregates are formed as cells make initial contact. The absolute area of these structures increases on a per cell basis, however, during early periods of culture, and so it also seems likely that these new cellular associations are sufficiently stable to allow the net addition of junctional elements to the membrane. A significant increase in the mean area of gap junctional membrane per cell, as previously mentioned, has been documented in the monolayer between 2 and 24 hr of culture. On the other hand, however, time lapse cinematography suggests that certain individual cells of the monolayer are capable of dynamic mobility, moving several cell diameters from their initial location or spontaneously disaggregating and detaching from the monolayer altogether to float into the medium. The possibility that gap junctions are removed from the cell surface by endocytosis, before or during the period when a cell of the monolayer begins to round and detach, is supported by the observation of numerous cytoplasmic gap junction vesicles in newly detached cells. Even when
spontaneously detaching single cells have been allowed to reaggregate with other floating cells for 24 hr, the area of gap junction membrane per cell at the cell surface is approximately 400-fold less than in 24-hr-old monolayers. Even when the area of nonjunctional membrane in monolayer cells and clustered cells is not taken into account, the difference in fractional gap junction area is still about 65-fold. Equally dramatic, the mean number of gap junctions per cell in 24-hr-old monolayers is about 20 whereas only about one in 3.5 cells in 24-hr clusters possesses a gap junction on average. Even after an additional 24 hr of clustering, the average junctional membrane area per cell is still about 8 times less than that measured in 24-hr-old monolayers. Since earlier studies documenting the ripping out of intact gap junctions during mechanical, enzymatic, or ionic cell disaggregation procedures (21, 41, 46) suggest strong cohesive membrane-to-membrane bonding at gap junctions, it is not surprising to find evidence of a mechanism of junctional removal in SW-13 cells after spontaneous disaggregation. This evidence, all taken together then, suggests that gap junctional endocytosis is necessary for the spontaneous disaggregation of cultured SW-13 cells.

Our analysis of time lapse films also demonstrated that a majority of cells which rose above the monolayer divided within the next 5.6 hr, thus raising the question of a functional relationship between cell detachment and cell division in cultured SW-13 cells. Earlier work suggests that mitotic cells are less adhesive to their immediate neighbors, as well as to the culture vessel substratum (44, 50, 51), and other studies have demonstrated that cells which are free of cell contact may divide at higher rates than aggregated cells within the colony (12, 16, 23, 25, 28, 53). In contrast, however, other investigators have not been able to observe a correlation between division behavior and gap junctional frequency or ionic coupling (26, 40, 43), and so the question of the gap junctions role in cell division remains uncertain.

In addition, several previous studies demonstrating a reduction or complete absence of gap junctions in certain tumors have implicated gap junctions in the malignant transformation of several cell types (5, 22, 32, 37, 38). Alternative explanations for these findings have also been suggested (55), however, in addition, other studies have demonstrated a high frequency of gap junctions in a variety of transformed or malignant cells (4, 27, 31, 35, 36, 45). A possible explanation for these contradictory findings may be provided by results of the present study which suggests that junctional frequency in some cell types may vary significantly with the stage of cell growth.

These studies have correlated the spontaneous assembly of gap junctional plaques with SW-13 cell aggregation in the monolayer and in clusters above the monolayer and suggest that these structures are removed from the cell surface by endocytosis prior to or during the spontaneous detachment of cells from the monolayer. Although our evidence also supports the hypothesis that net junctional endocytosis is a necessary condition for spontaneous cell disaggregation in SW-13 cells, more certain conclusions regarding this hypothesis await studies directed to the experimental manipulation of gap junctional assembly and endocytosis in a cell system sensitive to pertinent physiological agents. Given the complex nature and variety of possible mechanisms which contribute to cell cohesion (8, 56), such studies are required before the relative contribution of the gap junction to cell-cell cohesion and their influence upon the initiation, stability, and dissolution of intercellular interactions can be more accurately ascertained.

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Fig. 3. Individual frames from time lapse films demonstrating rounding, division and detachment behavior of SW-13 cells. a to f, no colchicine. × 170. g to i, with 0.06 mg colchicine per ml. × 170. In a, a cell rises and rounds at 0 time (arrow). In b, the same cell has migrated above the monolayer and divides 4.1 hr after rounding (arrow). In c, the daughter cells separate (arrow) at 0 + 4.8 hr. In d, one of the daughter cells (arrow) detaches and floats away from the site of original attachment to the monolayer at 0 + 4.9 hr. In e, the detached cell (arrow) has floated 2 to 3 cell diameters by 0 + 5.1 hr and an additional 4 or 5 cell diameters by 0 + 5.3 hr (f). In colchicine-treated culture, a cell (arrow) rises at 0 time (g), without dividing, detaches (arrow) at 0 + 0.9 hr (h), and floats 9 or 10 cell diameters away by 0 + 0.96 hr (i).

Fig. 4. Cell clustering above the monolayer within 24 hr after rinsing a confluent monolayer of single cells or cell clusters, numerous clusters have again formed (arrows). Scanning electron micrograph. × 250.

Fig. 5. By 1 week, the monolayer is almost entirely obscured by aggregated cells. Phase contrast, × 165.

Fig. 6. Possible movements of cultured SW-13 cells. Cells may spontaneously move in the direction of any of the arrows. c, cytokinesis.
Figs. 7 to 13. Intermediate and gap junctions in monolayered SW-13 cells.

Fig. 7. Stained thin section of intermediate junction characterized by ~12-nm intercellular gap and dense mats of cytoplasmic filaments (arrows). × 42,000.

Fig. 8. Thin-section profile of gap junction. Total width of the profile is ~20 nm. These profiles are characterized by periodic intercellular densities and dense cytoplasmic laminae (arrows). × 140,000.

Fig. 9. Freeze-fractured SW-13 gap junction demonstrating P face particles and E face pits. Particles are organized in columns separated by particle-free aisles. × 63,000. P, P fracture face; E, E fracture face.

Fig. 10. Large gap junctional aggregate demonstrating P and E fracture faces. This aggregate measures ~2.5 × 4 μm.

Fig. 11. Formation plaque regions in the membrane of a cell in a confluent monolayer. Note the clusters of 11-nm particles (arrows). × 48,000.

Fig. 12. Gap junctional invaginations connected to nonjunctional membrane (arrow) in monolayered cells. × 24,000.

Fig. 13. Small annular junctional profile in SW-13 monolayer cell (arrow). × 39,000.
Fig. 14. Single cells washed gently from the medium above a mature SW-13 cell monolayer, fixed, and centrifuged onto a Millipore filter. Scanning electron micrograph. x 680.
Figs. 15 to 22. Interiorized gap junctional vesicles in single floating SW-13 cells gently washed from the medium into fixative.

Fig. 15. An annular junctional profile deep within the cell interior. × 46,000.

Fig. 16. Several smaller annular gap junctional profiles grouped within the cytoplasm. A very small annular junctional profile can be observed within the matrix of the largest one. × 45,000.

Fig. 17. One annular junctional profile within another. × 44,000.

Fig. 18. Acid phosphatase reaction product within Golgi saccules (arrows). Unstained. nuc, nucleus. × 24,000.

Fig. 19. Acid phosphatase within annular profile composed of apparent double membrane (arrow). × 37,000.

Fig. 20. Apparent lysosome with associated acid phosphatase reaction product (solid arrow) within annular junctional profile (open arrow). × 66,000.

Fig. 21. Apparent fusion of gap junctional vesicle with lysosome; note continuity of membranes (arrows). × 81,000.

Fig. 22. Separation and revesiculation of junctional membrane of cytoplasmic gap junction vesicle. Note reformation of junctional specializations where vesicles make contact (arrows). × 91,000.
Figs. 23 to 25. Junctional structures in clustered cells above the monolayer.

Fig. 23. Freeze-fractured plasma membrane with large formation plaque (arrow). nuc, nucleus. × 9,400.

Fig. 24. Enlargement of formation plaque in Fig. 23. Note numerous dispersed 11-nm particles (open arrows) and small developing gap junctional aggregates (solid arrows). × 49,000.

Fig. 25. Small annular gap junctional profile deep within the cytoplasm near the nucleus (arrow). nuc, nucleus. × 108,000.
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