CONCOMITANT EFFECTS OF INSULIN ON SURFACE MEMBRANE CONFORMATION AND POLYSOME PROFILES OF SERUM-STARVED BALB/C 3T3 FIBROBLASTS

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ABSTRACT

By scanning and transmission electron microscopy we have shown that insulin rapidly reversed changes in surface membrane conformation and polysome profile induced by the transfer of actively growing Balb/c 3T3 fibroblasts from a serum-containing to a serum-free medium. Morphometric analysis of polysome profiles revealed a 94% aggregation of total ribosomes during logarithmic growth. This figure fell to 78% after 18 h of serum starvation. The number of ribosomes per unit area of cytoplasm also fell. 1 h of insulin treatment restored aggregation to 92% and increased the number of ribosomes per unit area of cytoplasm by 22%.

Scanning electron microscopy of logarithmically growing cells revealed an abundance of surface microvilli, whereas serum starvation promoted a smooth surface with few microvilli. After 1 h of insulin treatment, microvilli reappeared with a distribution and subcellular organization characteristic of exponential growth.

This study shows the combined and rapid effect of insulin on the regulation of polysome formation and the promotion of a specific surface membrane conformation in cultured cells. The observations are consistent with the knowledge that insulin, acting on the surface membrane, can influence such parameters as membrane transport, and the rates of protein and RNA synthesis.

INTRODUCTION

Transferring logarithmically growing Balb/c 3T3 fibroblasts to a serum-free medium modifies rates of various cellular processes including decreases in the rates of protein and RNA synthesis, reduction in the rate of uridine, leucine, and glucose uptake, and an increase in the rate of protein degradation (1, 2). After 18–20 h of serum starvation the addition of insulin or fresh serum to the medium rapidly restores the rates of the above reactions to values associated with the exponential cell growth (1). The integrated manner in which these reactions are regulated by extracellular factors led to the hypothesis that they are coordinated by a common intracellular regulatory mechanism (1). Furthermore, since sepharose-bound insulin elicits responses similar to those brought about by the unconjugated hormone, it was assumed that the growth-promoting re-
sponse results from the interaction of insulin and serum factors with the cell membrane (1).

Current evidence suggests that an elevation of intracellular cyclic adenosine - 3',5' - monophosphate (c-AMP) resulting from serum starvation is a factor in mediating growth inhibition (2). Thus the fall in the level of c-AMP, which occurs shortly after the addition of serum or insulin to serum-starved cells (3), may be responsible for the restoration of cell growth and multiplication. Additional experiments have also implicated cyclic guanosine 3',5'-monophosphate (c-GMP) as an antagonist of c-AMP in the control of cellular growth (4).

We have used the scanning and transmission electron microscope to evaluate structural changes of the Balb/c 3T3 fibroblast cell perimeter and cytoplasm during serum starvation, and after the addition of insulin to serum-starved cells in an attempt to identify possible structural components of the growth-regulating mechanisms. Our morphological findings correlate well with the biochemical changes which occur under these different physiological conditions. A preliminary report of this study has appeared elsewhere (5).

MATERIALS AND METHODS
Balb/c 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (MEM) (6), supplemented with 10% calf serum (Grand Island Biological Co., Grand Island, N.Y.) in a humidified 10% CO₂ incubator. The cells were grown in 60-mm Petri dishes (Falcon Plastics, Oxnard, Calif.), either directly on the plastic (for transmission electron microscopy) or on glass cover slips (for scanning electron microscopy), to a subconfluent density of 2-3 X 10⁵ cells per dish. To induce serum starvation, the cells were washed once with 5 ml MEM and then maintained in 3 ml MEM for 18 h. Fresh medium (MEM) containing insulin (5 μg/ml) was added to these cells and the dishes were subsequently processed for scanning and transmission electron microscopy after 1 and 2 h of insulin treatment. Another series of cultures received unsupplemented MEM and were harvested at corresponding intervals. At the beginning and the end of the experiment, as well as at the time of the medium changes, cultures grown in MEM plus 10% calf serum were processed as controls.

Tissue Preparation for Scanning Electron Microscopy (SEM)

Cells processed for scanning electron microscopy were grown on glass cover slips. The cells were fixed in 3% glutaraldehyde in 0.2 M cacodylate buffer for 12 h at 4°C. After this they were washed briefly in buffer. The cells were then rapidly dehydrated in a graded series of concentrations of ethanol which was gradually replaced with amyl acetate. They were then dried at the critical point (7) in CO₂ after the second change of 100% amyl acetate. The specimens were mounted on a holder with silver-conducting paint and coated with gold in an Edwards vacuum evaporator equipped with a rotary stage. The coated samples were viewed in a Kent Cambridge S4 scanning electron microscope and the data recorded on Polaroid S/N film, (Polaroid Corp., Cambridge, Mass.).

Preparative Techniques for Transmission Electron Microscopy

The method employed here was similar to that described for SEM preparation except that the period of glutaraldehyde fixation was 90 min, the cells were postfixied in 0.5% osmium tetroxide in 0.2 M cacodylate buffer, and the cells were en bloc stained for 15 min with 2% uranyl acetate in 70% ethanol. Final dehydration was in absolute ethanol after which the monolayer of cells in each Petri dish was infiltrated with 50% Epon 812 in 100% ethanol for 30 min before final embedding. After polymerization, slices of the resin were mounted to allow the sectioning of cells through horizontal and vertical planes. The thin sections were examined in a Philips EM 300 electron microscope.

Quantitative Evaluation of Polysomes

This method was designed to detect differences in the polysome profiles of logarithmically growing cells, serum-deprived cells, and serum-deprived cells to which insulin had been added. At least two Petri dish populations of each category were sampled. Three to five sample blocks from each Petri dish were

Fig. 1 Balb/c 3T3 fibroblasts grown in medium supplemented with calf serum. This scanning electron micrograph shows a characteristic array of cell shapes and surface projections. × 1,700.

Fig. 2 At higher magnification the distribution of microvilli on the fibroblasts grown in serum-enriched medium can be seen to better advantage. × 14,000.
mounted for sectioning. The sample population was represented by approximately 500 micrographs from each category. 30 electron micrographs from each category were selected for evaluation, using a random numbers table. The evaluation of polysome profiles was undertaken by placing a transparent grid over each micrograph to be evaluated. The grid spaces, or unit areas of cytoplasm, were standardized according to the magnification of the individual micrographs. This magnification varied from 8,100 to 20,500. The grid spaces were numbered and five unit areas per micrograph were chosen by random numbers for evaluation. Single ribosomes were defined as those not making contact with adjacent ribosomes either directly or via a connecting strand. Those which did make contact in this manner were interpreted as being polysome aggregates and were tabulated according to number of ribosomes per aggregate. Membrane-bound ribosomes were not included in our evaluation.

Since polysomes vary in shape (8) and angle of orientation with respect to the plane of section, this method of determining a polysome profile cannot yield true absolute figures. However, considering the large pool size (approximately 23,000 individual ribosomes) and the consistent application of our evaluation technique, relative shifts in polysome profile could be detected using morphologic data.

RESULTS

Cell Surface

The nonadherent or free surface of logarithmically growing Balb/c 3T3 fibroblasts, when viewed by scanning electron microscopy, displayed numerous membrane projections (Fig. 1). Among the general category of surface projections several distinct types could be distinguished. These included uniform microvilli approximately 0.14 μm in diameter, and between 0.28 μm and 1.2 μm in length (Fig. 2), elongated microvillus-like projections between 0.14 μm and 0.28 μm in diameter and up to 20 μm in length (Fig. 1), nondescript blunt and sharp-edged membrane folds of various dimensions (Fig. 1), and pseudopod-like membrane projections (Fig. 1). Only the uniform microvilli seemed to respond to serum starvation and subsequent insulin treatment. These microvilli, appearing individually

![Fig. 3 Scanning electron micrograph of Balb/c 3T3 fibroblasts after 18 h of serum starvation. Note that the number of microvilli are greatly reduced, particularly on the flattened cells. X 1,000.](image-url)
as well as in clusters, were widely distributed over the free surface of logarithmically growing cells and, in most cells, disappeared from large regions after 18 h of serum starvation (Figs. 3, 4). Microvilli subsequently reappeared after 1 h of insulin treatment (Fig. 5). The microvilli which appeared after insulin treatment had a diameter of 0.14 μm but were on the average shorter than...
Fig. 5 Serum-deprived Balb/c 3T3 fibroblasts exposed to insulin for 1 h. The distribution of microvilli now closely resembles that observed in the fibroblast grown in the calf serum supplemented medium. Note the adhesion pads. × 2,400.

Fig. 6 Higher magnification of an insulin-treated cell surface. The distribution of microvilli closely resembles that observed in cells grown in calf serum-supplemented medium. Note, however, that the length of these microvilli is often shorter, with a few appearing to be like small buds. × 18,000.
those of actively growing cells (Fig. 6). Small surface elevations also appeared and were interpreted as developing microvilli (Fig. 6). The average length of microvilli was slightly greater after 2 h of insulin treatment. Elongated surface projections up to 20 µm in length and 0.28 µm in diameter originated along the free surface margin and were much less, if at all, influenced by serum starvation (Fig. 3). These projections had a rigid straight outline similar to microvilli, but unlike microvilli of the free surface they were in contact with the glass substratum or the surface of an adjacent cell. We were unable to determine by transmission electron microscopy if the core of these structures resembled that of microvilli.

By light microscopy the tapered cell bodies which lend a typical bipolar shape to serum-starved fibroblasts appeared to become shorter after 1 h of insulin treatment. This was interpreted as evidence of peripheral retraction since adhesion pads were frequently seen by scanning electron microscopy along the edge of the insulin-treated cells.

Ectoplasm

The ectoplasm of (3T3) fibroblasts, a differentiated zone of cytoplasm immediately adjacent to the surface membrane, contains microtubules and populations of 70- and 100-Å filaments as observed by McNutt et al. (9). We were interested in the possible role of microtubules and filaments in relation to the surface projections described above. Microvilli of the free surface were seen by transmission electron microscopy to occur both as single structures and in clusters (Fig. 7). The core of microvilli invariably contained aggregates of 70-Å filaments, and 100-Å filaments occasionally were seen at the base of microvilli (Fig. 7). The ectoplasm underlying single microvilli of logarithmically growing, serum-starved, and insulin-treated cells occasionally contained bundles of mixed 70- and 100-Å filaments organized parallel to the cell surface. It appeared that only the 70-Å filaments branched from these bundles into the core of microvilli. The ectoplasm underlying clusters of microvilli was expanded in comparison to zones underlying single microvilli and nonprojecting regions of the cell surface. Both 70-Å and 100-Å filaments were found within these expanded regions. The alignment of 100-Å filaments in these regions seemed random, whereas 70-Å filaments were polarized in aggregates extending into the core of microvillus projections. These patterns of organization were seen after the addition of insulin to serum-starved cells (Figs. 7a, b, c) and similar patterns were observed in logarithmically growing cells. Ectoplasmic microtubules were often seen running parallel to the cell surface but were not directly associated with surface membrane folds or projections. The pattern of microtubule organization was similar beneath both morphologically quiet regions of the cell surface and regions of membrane plasticity.

Cytoplasmic Polysome Profile

Polysome profiles of nonmembrane-bound cytoplasmic ribosomes (f ribosomes) were evaluated by morphometric techniques and are summarized in Figure 8. A change in the extent of polysome formation is reflected by a change in the percentage of total f ribosomes found in polysomes. In logarithmically growing fibroblasts, on the average, 94% of the total f ribosomes were found in polysomes, whereas after 18 h of serum starvation only 78% of the f ribosomes were in polysomes. The profile of polysome size distribution was also altered by serum starvation and the total number of f ribosomes per unit area of cytoplasm fell by 46%. The shift of a large percentage of cells to an elongated gross morphology which occurs during serum starvation might have affected the number of total f ribosomes per unit area of cytoplasm if it were accompanied by an increase in cell size. Evaluating the volume of cells in monolayer is exceedingly difficult; however, light microscopy failed to indicate an increase in cell area. The addition of insulin to serum-starved cells increased polysome formation. After 1 h of insulin treatment the total number of f ribosomes per unit area rose from 56% to 71% (Table I) of that found in logarithmically growing (3T3) fibroblasts. Furthermore, the polysome size distribution was restored to that characteristic of logarithmically growing cells (Figs. 8, 9).
Intracellular Membranes

The endoplasmic reticulum and Golgi apparatus of actively growing (3T3) fibroblasts were moderately well developed. Neither 18 h of serum starvation nor the readdition of insulin had an obvious effect on these membranes. Serum starvation for a period of 18 h did markedly increase autolysosomal activity (Fig. 9a). Whole segments of the cytoplasm became enclosed by intracellular membranes, and the contents of autophagic vacuoles revealed several stages of lysozomal digestion of effete organelles. Residual bodies were often observed at the cell surface, presumably to be eliminated by exocytosis. Up to 2 h of insulin treatment did not reduce the number of autolysosomal vacuoles and residual bodies in serum-starved cells. Finally, small surface membrane vacuoles often formed at the base of microvilli clusters.

DISCUSSION

The transfer of logarithmically growing fibroblasts to a serum-free medium gives rise to several structural modifications described in this study. After 18 h of serum starvation the percentage of cells with an elongated, typically bipolar morphology was increased, surface microvilli disappeared from large regions of the free cell sur-

Fig. 7. Transmission electron micrographs of serum-deprived Balb/c 3T3 fibroblasts exposed to insulin for 1 h. A variety of surface microvilli arrangements are seen in this series of micrographs. The ectoplasm is expanded beneath the clusters of microvilli and often contains many 70-Å filaments. 100-Å filaments (indicated by the arrows) are also present but loosely organized. Mixed bundles of 100- and 70-Å filaments sometimes aggregate in the ectoplasm underlying single microvilli. Only 70-Å filaments are observed in the microvillus core. (a) × 37,000; (b) × 37,000; (c) × 39,000; (d) × 44,000.
TABLE I

Average Number of Ribosomes per Micrograph

|                  | Serum-Enriched (S+) | Serum-Starved (S--) | Insulin-Treated (I+) |
|------------------|---------------------|---------------------|---------------------|
| **Mean**         | 334 ± 111           | 186 ± 64            | 237 ± 101           |
| **SD**           | 21.03               | 10.39               | 20.00               |
| N                | 28                  | 38                  | 26                  |

*Five unit areas of cytoplasm were evaluated per micrograph. N = number of micrographs. See Materials and Methods.

face, the percentage of ribosomes incorporated into polysomes as well as the total number of ribosomes per unit area of cytoplasm were reduced and nonspecific autolysozomal activity was increased. This multifaceted response to serum starvation was rapidly reversed by exposure of these cells to insulin.

The appearance of microvilli within 1 h after insulin treatment is coincident with a previously reported increase of uridine, leucine, and glucose uptake (1, 2). This correlation seems to agree with the general associations of microvilli with cell surfaces specialized for active transmembrane uptake and exchange (10). Regardless of the function of microvilli as a differentiated structure, the mechanisms which might couple rapid conformational changes of the surface membrane, such as the formation of microvilli with an increase in the rate of uptake, are unknown. Since serum starvation decreases the transport of amino acids, glucose, uridine, and other small molecules (1, 2, 11), it might be argued that the existence of microvilli depends on the intracellular concentration of one or more of these substances. It is unlikely that the decrease in amino acid uptake is responsible for the morphological surface changes since preliminary observations have shown that amino acid starvation does not result in a disappearance of microvilli (R. B. Evans and V. Mohnhenn, unpublished observations).

Intracellular levels of c-AMP are elevated during serum starvation and fall during the restoration of growth by insulin (2, 3). The regulation of cell surface conformation may therefore be related to intracellular levels of cyclic nucleotides. In accord with our findings others have noted that analogues of c-AMP can bring about cell elongation in malignant and virally transformed cell lines (12–14) and a loss of projecting structures from the surface of transformed and untransformed cells (15). Furthermore, transformed cells which contain decreased levels of cyclic nucleotide (16) also display more surface microvilli than do their untransformed counterparts (17).

The Balb/c 3T3 cells studied here were growing logarithmically at a subconfluent density and, unlike their SV 40-transformed counterparts, showed biochemical sensitivity to serum starvation.

If there is a relationship between cyclic nucleotides and surface changes, the mechanism is unknown. Our observations indicate that microfilaments are the structural elements most directly involved with the apparent regeneration of microvilli; however, since microtubule inhibitors enhance membrane transport during serum starvation without changing the level of c-AMP, as well as reverse the prostaglandin-induced lowering of transport, perhaps microtubules are somehow involved as well (4).

Generally speaking, cell contact at subconfluent density did not interfere with the cell response to serum starvation. It should be noted, however, that serum-starved cells in contact displayed a variety of cell shapes (Fig. 3). Those cells with a flattened morphology lacked surface microvilli, whereas the surface of elongated or spindle-shaped cells was less influenced by serum starvation. Variations in cell shape have been reported to reflect different phases of the cell cycle (18), and perhaps the variety seen in Fig. 3 indicates that the serum-starved cells are in different phases of the cycle. Elongated cells existing free had, in comparison to elongated cells in contact, fewer surface microvilli in response to serum starvation. Cell contact is known to in-
Figure 9 a-c (a) shows the polysome configurations typical of Balb/c 3T3 fibroblasts grown under serum-starved conditions. 24% of the ribosomes were seen in thin sections as single ribosomes. Note also the number of autophagic vacuoles in this preparation. (b) and (c) show portions of cytoplasm from two serum-starved cells exposed to insulin for 1 h. In these preparations, 8% of the ribosomes appear as single ribosomes. Arrow heads point to microtubules (b), and arrows to 100-Å filaments. (a) X 24,000; (b) X 30,000; (c) X 38,000.
fluence the surface conformation of untransformed Chinese hamster ovary cells (19).

Cell surface changes are not the only effects of serum starvation which are rapidly reversed by insulin. The level of polysome aggregation observed in these experiments parallels the rate of protein synthesis determined in biochemical studies (1), and confirms the results of Wool and Cavicchi (20) that insulin can stimulate polysome formation. Cyclic nucleotides may also influence polysome formation since the elevation of c-AMP by prostaglandin E₁ or serum starvation is associated with a reduction in the rate of protein synthesis (4). Insulin is known to lower intracellular c-AMP (2) and therefore may influence polysome aggregation via this nucleotide. Our observations are consistent with regulation at the initiation step in protein synthesis.

The structural changes described in this study coincide with the earliest biochemical events of growth restoration. The fact that diverse structural substrates and a correlative array of biochemical functions become altered over a short period of time, if not simultaneously, suggests a pervasive activity for any cytoplasmic mediator of growth. Whether these structural changes represent the direct activity of such a mediator and whether any of these morphological manifestations of growth restoration are essential for regulation to occur remains to be clarified.

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