

Epidermal Growth Factor Induces Rapid Centrosomal Separation in HeLa and 3T3 Cells

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ABSTRACT Using indirect immunofluorescence, we have found that epidermal growth factor (EGF), at 100 ng/ml, induces centrosomal separation within 20 min in HeLa and 3T3 cells. The effect was evident both in unsynchronized cultures and in HeLa cells blocked in early S phase by hydroxyurea. EGF also induced centrosomal separation in quiescent 3T3 cells blocked in G₀/G₁ by serum deprivation, indicating that DNA replication is not necessary for this effect. The mechanism of this rapid centrosomal separation and its role in the mitogenic effects of EGF remains to be determined.

Epidermal growth factor (EGF) (6,045 mol wt) is probably the most extensively studied mammalian mitogenic peptide (6). EGF was originally isolated from mouse submaxillary glands but it is clearly made by other tissues because submaxillary gland excision does not lower plasma levels of EGF (4). In man, EGF is found in plasma, saliva, and urine but its precise physiological role is unknown (13, 37). Since a wide variety of cells of epithelial (19, 33, 39), fibroblastic (1, 2, 5, 21), and neuronal (45) origin are stimulated to proliferate in vitro in response to EGF, it seems that this peptide is likely to be an important regulator of cell division in vivo.

As with all other peptide hormones studied thus far, the initial event in the action of EGF is binding to specific high-affinity receptors on the external surface of the plasma membrane (1, 5, 6, 21); 15–22 h later DNA synthesis ensues with subsequent cell replication (1, 5, 6, 22). Although it is clear that major changes in the organization and function of the cell must occur in response to EGF before cell division, the key events leading from membrane binding to cell division are unknown.

Centrosomes are perinuclear structures which consist of a pair of centrioles surrounded by an electron-dense cloud of pericentriolar material (34, 40). During interphase many cytoplasmic microtubules originate from this structure (14, 17, 29). During mitosis a centrosome is located at each spindle pole and serves as an anchoring site for microtubules that make up the spindle apparatus. The centriole cycle, which includes duplication, elongation, and polar migration is, in general, believed to be tightly coupled to the cell cycle but the precise relationship between centriole and cell-cycle events remains to be elucidated. For example, while centriole formation can proceed in the absence of DNA synthesis (26, 32) it does require the presence of the nucleus (26). Whether centrosomal behavior influences nuclear events is an open question.

Using a specific antiserum to a high molecular weight microtubule-associated protein (MAP₁), which stains centrosomes

in a variety of cells, we have found that, after addition of EGF, the centrosome splits. The paired daughter centrosomes maintain close contact with the nucleus but migrate away from one another along the nuclear surface. The rapidity of the response and the fact that it precedes S phase by many hours raises the possibility that centrosomal separation may be an important event in the sequence leading to initiation of DNA synthesis in response to EGF.

MATERIALS AND METHODS

Cell Culture and Synchronization

HeLa and BALB c 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal (FCS) and 5% newborn calf serum. HeLa cells (50–70% confluent) were synchronized in early S phase by 48 h of serum deprivation (0.25% newborn) in the presence of 1 mM hydroxyurea (16, 44). <10% of synchronized cells showed nuclear labeling during a 1-h exposure to 1 μ Ci/ml of [³H]thymidine whereas 76% showed incorporation during a 1-h pulse 3 h after serum readdition and removal of hydroxyurea. 3T3 cells (70–80% confluent) were synchronized in G₁ by culturing them for 48 h in DME containing 0.5% FCS (42). <5% of cells showed nuclear labeling during a 2-h exposure to 1 μ Ci/ml of [³H]thymidine. Logarithmically growing 3T3 cells routinely show 50–60% nuclear labeling under these conditions. Cells were processed for autoradiography using Kodak NTB-2 emulsion according to the method of Stein and Yanishevsky (38).

Antibodies and Immunofluorescence

To prepare antibody to MAP₁ we used twice-cycled rat brain microtubule protein (36) which was electrophoresed on 7.5% SDS polyacrylamide gels. The MAP₁ band was cut from the stained gel, homogenized with Freund's adjuvant (Gibco, Grand Island Biological Co., Grand Island, NY), and the suspension, containing ~30 μ g of MAP₁, was injected into a rabbit. After 1 mo the rabbit was boosted and then bled 10 d later. Rabbit serum was used at a dilution of from 1:20 to 1:30 to stain centrosomes. At lower dilutions, microtubules were also stained and centrosomes were less easily identified. Fluorescein-conjugated goat anti-rabbit immunoglobulin (Miles-Yeda Laboratories, Elkhart, IN) was used at a dilution of 1:40. Guinea pig antitubulin antiserum (a kind gift of S. Blose, Cold

Spring Harbor Laboratory, Cold Spring Harbor, NY) was used at a dilution of 1:20 and rhodamine-conjugated goat anti-guinea pig immunoglobulin (U. S. Biochemical Corp., Cleveland, OH) was used at a 1:30 dilution. To stain centrosomes, cover slips were rinsed once in phosphate-buffered saline (PBS) at room temperature and then immersed in absolute methanol at -20°C for 5 min. After drying, the first antibody was applied for 30 min at 37°C in a humidified chamber. The cover slips were then rinsed three times in PBS, overlaid with fluorochrome-labeled goat anti-rabbit antibody, and incubated for 30 min at 37°C . They were then rinsed three times in PBS, mounted on slides in PBS containing 50% glycerol, and the edges were sealed with nail polish. Slides could be stored in the dark for up to 2 mo at 4°C without evident fading of fluorescence.

Cells were viewed with a Zeiss Photoscope III equipped for epifluorescence and photographed on Kodak Tri-X 35 mm film. If the distance between centrosomes was greater than their diameter, they were scored as separated. Mitotic cells or those in which separation was equivocal were not counted. At least 100 cells were scored per cover slip. Slides were coded and the observer was not aware of the treatment group.

To measure the degrees of arc between centrosomes, randomly selected fields containing 8–15 cells were photographed and the angular distance was measured directly from projections with a protractor.

EGF (Ultrapure) was purchased from Laref SA (Cadempino-T1, Switzerland).

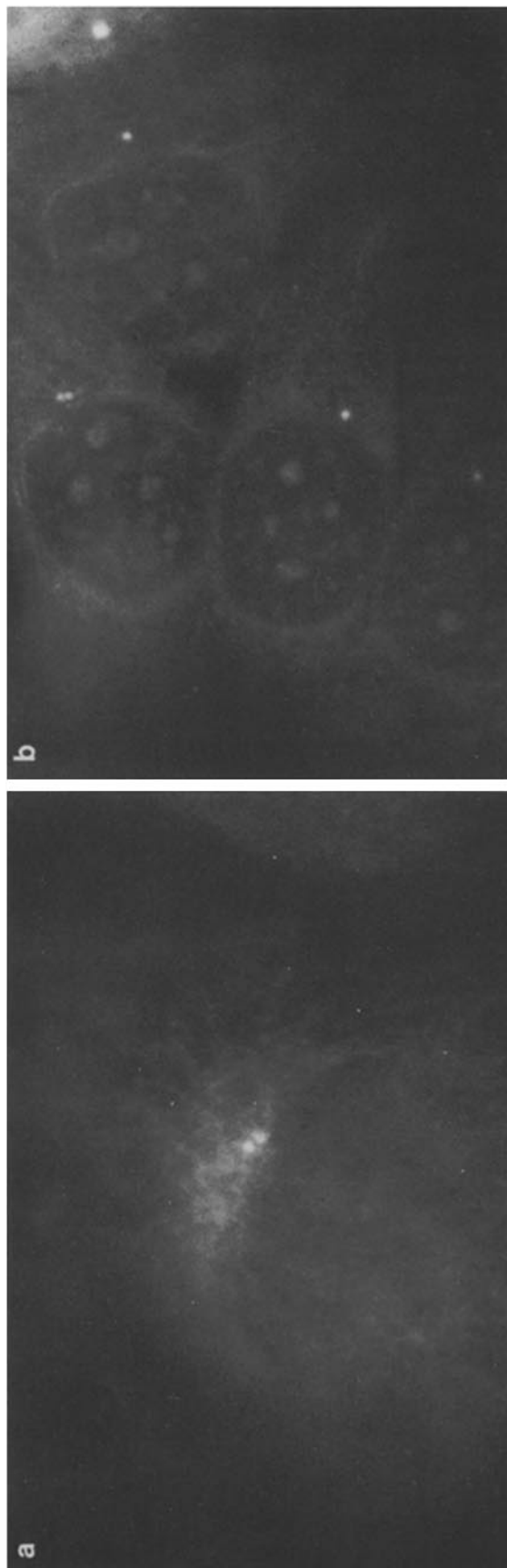
RESULTS

Unsynchronized HeLa and 3T3 cells (80–90% confluence) showed intensely stained centrosomes (Fig. 1 *a, b*) or spindle poles (Fig. 1 *e, f*). No centrosomal staining was observed with preimmune serum (Fig. 1 *c, d*). In over 95% of nonmitotic HeLa and 3T3 cells, centrosomes were either single or within 5° of one another. Within 20 min after addition of EGF (100 ng/ml) to cultures, a substantial proportion of cells (21% in 3T3, 14% in HeLa) showed centrosomes separated by $>5^{\circ}$. Representative cells are shown in Fig. 2. After 45 min, 32% of 3T3 and 28% of HeLa cells had separated centrosomes, at 90 min 34% of 3T3 and 35% of HeLa cells showed separation and the average distance between them had increased. No further increase was seen with longer incubation (240 min: 35% in 3T3; 31% in HeLa).

Our initial thought was that EGF was stimulating centrosomal separation in the subpopulation of cells in G_2 before prophase. To test this, we arrested HeLa cells in early S phase with 1 mM hydroxyurea for 48 h (16, 44). Within 20 min after addition of EGF (100 ng/ml) many cells with widely separated centrosomes were observed (Control 6%, EGF [20 min] –19%, EGF [45 min] –28%, EGF [90 min] –35%, EGF [4 h] –92%).

To further confirm the somewhat surprising finding that EGF induced rapid centrosomal separation at the onset of or prior to DNA replication, we synchronized 3T3 cells in G_0/G_1 by 48 h of serum deprivation (0.5%) after which we added EGF (100 ng/ml) and, after various intervals, fixed and stained the cover slips. In serum-arrested 3T3 cells, $<3\%$ of cells had separated centrosomes, but within 20 min after exposure to EGF 22% of the cells showed separation, by 45 min 32% showed separation, and by 4 h 91% of centrosomes were separated. These results suggested that EGF was capable of inducing centrosomal separation in previously quiescent cells arrested in G_0/G_1 and that DNA replication was not required for this event since in these cells there is a 15- to 20-h lag period before the onset of DNA synthesis in response to EGF (5, 6). In parallel cover slips pulsed with [^3H]thymidine at the time of addition of EGF, labeled nuclei were not seen by autoradiography until 20 h later (data not shown).

The frequency distribution of angular arc separation between centrosomes in hydroxyurea-arrested HeLa cells is shown for four time points in Fig. 3. As is evident, the average separation increases with time after EGF exposure; however, even at 240 min, $<5\%$ of cells show separation $>120^{\circ}$.



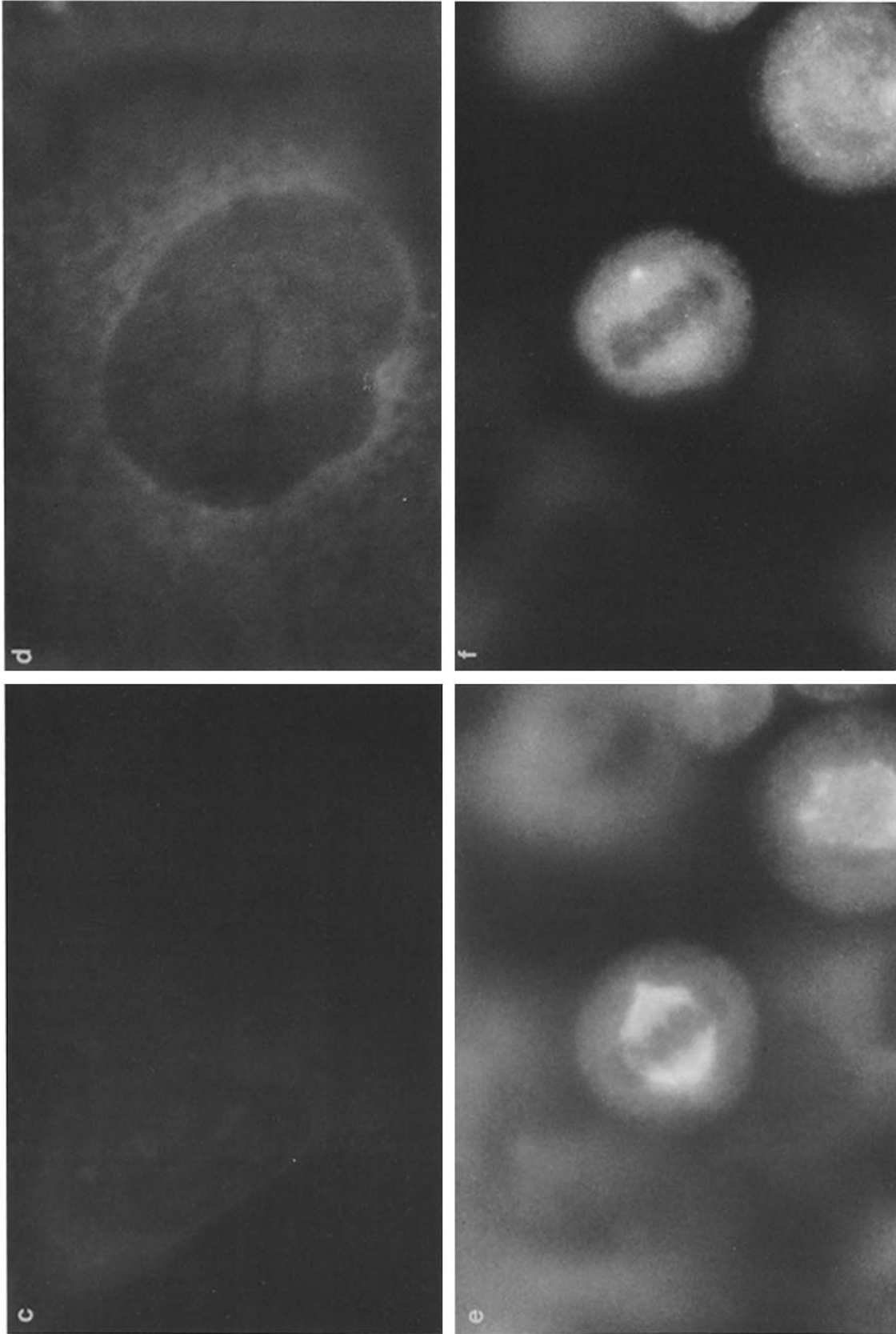


FIGURE 1 Unsynchronized 3T3 and HeLa cells were fixed with absolute methanol at -20°C for 5 min and processed for indirect immunofluorescence. A rabbit antiserum directed against MAP, was used as the first antibody and a fluorescein-conjugated goat anti-rabbit IgG as the second antibody. (a) 3T3 cell demonstrates a centrosome at one o'clock in the perinuclear area. ($\times 2,000$). (b) HeLa cells with one or adjacent centrosomes in the perinuclear region. ($\times 1,000$). (c) 3T3 cell processed for indirect immunofluorescence with the preimmune serum as first antibody. No centrosomes are visible. ($\times 1,000$). (d) HeLa cells stained with preimmune serum. No centrosomes are visible. ($\times 2,000$). (e) HeLa cell in metaphase stained with guinea pig antitubulin antiserum and rhodamine-conjugated goat anti-guinea pig second antibody. ($\times 2,000$). (f) Same HeLa cell as in e stained with antacentrosome antiserum. Mitotic spindle pole fluoresces strongly; the opposite one is out of the plane of focus. ($\times 2,000$).

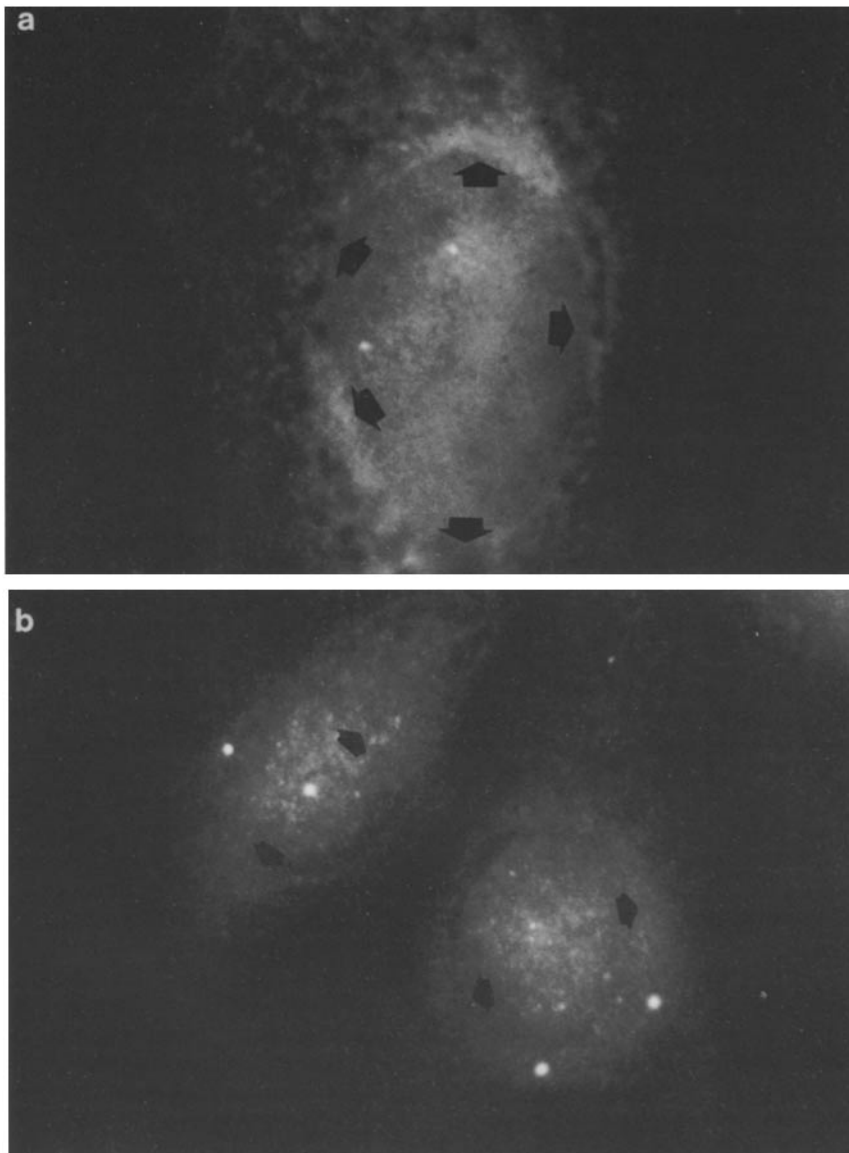


FIGURE 2 Unsynchronized near-confluent 3T3 and HeLa cells were incubated in culture medium containing EGF (100 ng/ml) for 45 min and then processed for indirect immunofluorescence. Arrows indicate nuclear borders. (a) 3T3 cell, demonstrating centrosomal separation. ($\times 2,000$). (b) A pair of HeLa cells demonstrating centrosomal separation. ($\times 2,000$).

DISCUSSION

With the use of a specific immunofluorescent marker we have found that EGF causes rapid centrosomal separation in HeLa and 3T3 cells. Other rapid morphological changes have also been described in response to EGF, notably stimulation of fluid-phase pinocytosis (20) and membrane ruffling (9) in A-431 cells. In calcium-deprived A-431 cells, EGF induces an energy-dependent shape change from a flattened epithelioid configuration to a spherical form within 20 min of exposure (10). However, it is unclear how these changes might relate to the mitogenic effect of the hormone. Similarly, rapid biochemical alterations have been described in cells in response to EGF, including an increase in α aminoisobutyric acid uptake in human fibroblasts (22), an increase in 2-deoxyglucose uptake and glycolysis in 3T3 cells (3, 15), and an increase in $^{86}\text{Rb}^+$ influx (measure of K^+ transport) in 3T3 cells (35). Again, however, we do not know how these early biochemical responses to EGF relate to DNA replication and cell division which occur many hours later.

The effect of EGF to stimulate a membrane-associated, tyrosine-specific protein kinase (7, 8, 43) may be the key event which triggers both the early biochemical and morphological

responses and ultimately cell proliferation. Protein tyrosine phosphorylation appears to be necessary for transformation and consequent cell proliferation induced by Rous sarcoma (RSV) and other retrovirus (11, 23, 27). Furthermore, EGF has recently been shown to induce tyrosine phosphorylation of at least two proteins in intact A-431 cells within 1 min of exposure (24). The further identification of the *in vivo* target proteins phosphorylated by the src and EGF kinase(s) would seem then to be essential for understanding how RSV and EGF alter cell morphology and trigger proliferation. It will then be necessary to determine how the function of these proteins is altered by tyrosine phosphorylation.

Microtubules, or a protein which regulates microtubule assembly, may be an important target for EGF or the EGF-stimulated kinase. We know that microtubule depolymerization can initiate DNA synthesis in quiescent fibroblasts in the absence of added growth factors (12, 28). Furthermore, a number of studies have now shown that the mitogenic effect of growth factors including EGF is substantially augmented by antimicrotubule drugs (18, 25, 30, 31, 41). Thus, there is reason to suspect that microtubule reorganization is necessary for initiation of DNA synthesis by EGF and other growth factors.

Since centrosomes are microtubule organizing centers, the

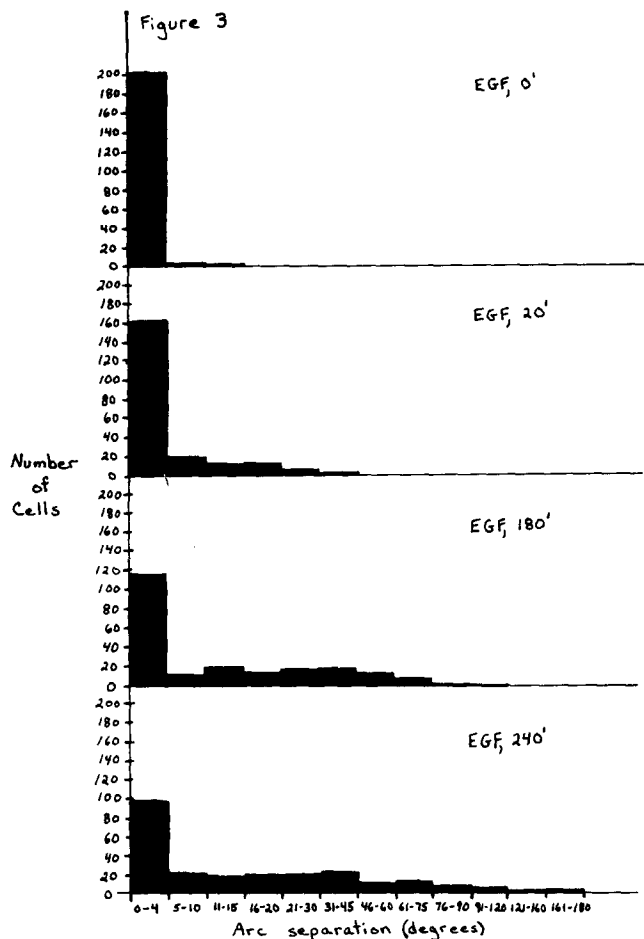


FIGURE 3 Frequency histogram of angular separation in synchronized HeLa cells at various times after EGF (100 ng/ml).

finding that EGF rapidly stimulates centrosomal splitting and separation is consistent with the suggestion that the microtubule reorganization occurs during the lag phase in response to EGF. The fact that centrosomal separation occurs before DNA replication in EGF-stimulated cells raises the possibility that such separation is a prerequisite for entrance of cells into S phase. Since microtubules radiate from the centrosome to the cell membrane, it seems likely that they act to maintain the centrosome in a relatively fixed position in the cell. If centrosomal separation is required for the subsequent DNA synthetic response to EGF, then drug-induced microtubule depolymerization, by releasing this constraint on centrosomal movement, would be expected to facilitate DNA synthesis and ultimately cell division in response to EGF.

In synchronized HeLa cells, centrosomes begin to separate in G_1 with centriolar replication and further separation during S and G_2 (34). In quiescent 3T3 cells stimulated to proliferate by serum, centrioles rapidly deciliate but centrosomes were noted to separate only at the onset of DNA synthesis some 15 h after serum addition (42). Thus, the interval between centrosomal separation and DNA synthesis may vary in different circumstances and the question of how centrosomal events and DNA synthesis are coordinated remains unanswered. It will therefore be important to determine the time of centrosomal separation in relation to DNA replication in a variety of circumstances to clarify the causal connection, if any, between these two events. It will also be of interest to understand how EGF brings about such a rapid and dramatic repositioning of centrosomes.

We thank Diane Bourke and Sandra Sousa for technical assistance.

This work was supported by an National Institutes of Health (NIH) grant (GM-22497) to P. Sherline. R. Mascardo is the recipient of a Juvenile Diabetes Foundation fellowship and P. Sherline of a Research Career Development Award from the NIH.

Received for publication 10 February 1982.

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