Epidermal growth factor (EGF) stimulates proliferation of a wide variety of cultured cells. After binding to specific cell membrane receptors the hormone sets in motion a chain of events which lead eventually to cell replication (8, 12). A minimum interval of 12–15 h exists between EGF binding and the onset of DNA synthesis (2, 3, 10, 27, 31) which suggests that a major reorganization of the biochemical and structural machinery of the cell must occur. In fact, numerous biochemical and morphological changes have been described in response to EGF (for reviews see references 8 and 12), including increases in glycolysis (19), ion (48), amino acid (28), and hexose transport (4), membrane ruffling (7, 14), hormone and receptor uptake (9, 38), and protein tyrosine phosphorylation (11, 29). However, despite the accumulation of a great deal of information about what happens after EGF binding we are still far from understanding the complex program through which a cell prepares itself for a cycle of DNA replication and division in response to EGF or other mitogens.

It has been shown that colchicine induces DNA synthesis in quiescent cells (17, 52) and enhances the mitogenic effect of EGF and other growth factors (17, 22, 37, 42, 43, 52). Furthermore, taxol, a drug which prevents microtubule disassembly, has recently been shown to inhibit EGF and thrombin-stimulated DNA synthesis in mouse embryo cells (16). These studies suggest that microtubule disassembly or reorganization is involved in the regulation of cell replication.

We recently reported that EGF stimulates centrosomal separation in 3T3 and HeLa cells before DNA synthesis (50). The experiments described in the present report were aimed at clarifying the mechanism by which the cell brings about centrosomal separation in response to EGF.

MATERIALS AND METHODS

Cells

HeLa cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's modified Eagle's medium supplemented (DME) with 5% fetal and 5% newborn calf serum. Cells were synchronized at the G1/S interface by 48 h of serum deprivation (0.5% calf) in the presence of 2 mM hydroxyurea (1). Less than 2% of cells showed nuclear thymidine incorporation during a 2-h exposure (see below) whereas 50 to 55% showed incorporation in unsynchronized cells.
Autoradiography

To determine the nuclear labeling index (5), cells, grown on cover slips, were incubated with [3H]thymidine (1 μCi/ml) for 2 h. Cells were fixed in methanol at −20°C, air dried, and then treated with Kodak NTB-2 emulsion. After a 36-h incubation in the dark, cover slips were developed, fixed, and mounted on slides. Only nuclei with at least 20 dark grains were scored as labeled. For each condition, 200 cells were counted.

Immunofluorescence

Antibodies to MAPs were prepared by excising the appropriate stained band from SDS polyacrylamide gels of twice-cycled rat brain microtubule protein preparation, homogenizing it in complete Freund’s adjuvant (GIBCO) and injecting the suspension (30 μl of protein) into multiple subcutaneous sites on the back of a rabbit. After 1 mo the rabbit was boosted and then bled 10 d later. Serum was used at a dilution of 1:20 to 1:30 to stain centrosomes. At lower dilutions microtubules were stained as well and centrosomes were less easily identified. Centrosomal staining was abolished by preincubation of the diluted antiserum with MAPs, excised from a stained polyacrylamide gel (18) after electrophoresis (35) of two-cycle purified porcine brain microtubule protein. Preincubation of the antiserum with MAPs, tubulin or a gel slice containing no protein failed to block centrosomal staining by this antiserum.

To stain centrosomes, cover slips were removed from the medium, rinsed once in phosphate-buffered saline (PBS) at room temperature and then immersed in methanol at −20°C for 5 min. They were then removed, rinsed in PBS, and exposed to the first antibody for 30 min at 37°C in a humidified chamber. After rinsing three times in PBS, cover slips were overlayed with rhodamine conjugated goat anti-rabbit immunoglobulin 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 sealed with nail polish. Slides could be stored for up to 2 mo at 4°C without evident fading of fluorescence.

Cells were viewed with a Zeiss Photoscope III equipped with epifluorescence. If the distance between centrosomes was greater than their diameter, they were scored as separated. Mitotic cells (<5%) or those in which separation was equivocal (<3%) were not counted. At least 100 cells were scored per cover slip and for each condition at least two cover slips were scored on two separate occasions. Slides were coded and the observer was not aware of the treatment.
group. Values represent the mean ± the standard error of the mean (SEM) of replicate counts.

Materials

EGF (Ultrapure) was purchased from Laref. Sodium azide, sodium fluoride, A23187, Cytochalasins B and D, and colchicine were from Sigma Chemical Co. (St. Louis, MO). Lumicolchicine was prepared according to the method of Wilson and Friedkin (55). Taxol was obtained from the National Institutes of Health Investigative Drug Branch (Bethesda, MD). Trifluoperazine was a gift from C. Kaiser (Smith, Kline & French, Suanyale, CA), chlorpromazine and chlorpromazine sulfoxide from A. Manian National Institutes of Mental Health (NIMH). W-5 and W-7 were from Caabco (Houston, Texas).

RESULTS

Time Course and Concentration Dependence of Centrosomal Separation

Using an antiserum directed against one of the high molecule weight microtubule associated proteins (MAPs), one or two closely spaced perinuclear centrosomes are easily visualized by indirect immunofluorescence in virtually all HeLa cells (Fig. 1a). Less than 10% of unstimulated cells show centrosomes separated by more than one diameter. Upon addition of EGF centrosomes split and migrate in opposite directions along the nuclear border (Fig. 1b). The time course and concentration dependence of centrosomal separation in response to EGF is shown in Fig. 2. Within 45 min of exposure to EGF, between 30 and 35% of unsynchronized (Fig. 2a) or synchronized (Fig. 2b) cells have separated centrosomes. This percentage does not significantly increase in unsynchronized cells even after 4 h of exposure (Fig. 2a) whereas almost 70% of synchronized cells showed separation at this time (Fig. 2b). Less than 10% of cells showed centrosomal separation 240 min after addition of 10 μl of DME lacking EGF.

The percentage of cells with separated centrosomes in synchronized cells is shown as a function of EGF concentration at 45 and 240 min in Fig. 2c. A concentration of EGF between 0.1 and 0.5 ng/mL induces a half-maximal response. This is similar to the concentration which induces half-maximal stimulation of DNA synthesis in fibroblasts (28) and other cells (8, 12).

Energy Requirement for Centrosomal Separation

Centrosomal separation is inhibited by sodium azide, dinitrophenol (DNP) and sodium fluoride (Table I), indicating that the process is energy dependent. Addition of glucose (1 mg/ml) to cells exposed to EGF in the presence of azide or DNP results in rapid separation to levels comparable to that seen in control cells but does not cause separation in cells exposed to sodium fluoride.

Involvement of Ca++ and Calmodulin

The calcium ionophore A23187 (45) stimulates centrosomal separation in HeLa cells to almost the same extent as a maximal concentration of EGF and the effect is blocked by trifluoperazine (Table II). The effect of EGF is completely blocked by trifluoperazine, chlorpromazine and W-7 (Table II), inhibitors of Ca++-Calmodulin action (25, 26, 36, 54), whereas chlorpromazine sulfoxide and W-5, which are relatively ineffective calmodulin inhibitors (25, 26, 36, 54), had no effect. The fact that the response to EGF plus A23187 is no greater than to either agent by itself is consistent with the suggestion that they work by a common mechanism. Dimethylsulfoxide (DMSO), the vehicle for A23187 (final concentration 0.1%), had no effect by itself or to enhance or inhibit the EGF effect. A23187 had no effect on the HeLa cell microtubule array as determined by indirect immunofluorescence using rabbit anti-tubulin antibody (not shown), indicating that centrosomal separation induced by the drug is not a consequence of microtubule disassembly (see below).
Role of Microfilaments

The observation that EGF-induced centrosomal separation is an energy requiring and calcium-calmodulin activatable process suggested that movement might be dependent upon microfilaments. Cytochalasins B and D, which destroy cellular microfilaments (20, 51), completely block centrosomal separation (Table III).

Role of Microtubules

Many cytoplasmic microtubules emanate from the centrosome and terminate at fixed points on or close to the cell membrane (21, 23, 24, 41, 44). Thus, centrosomes are a major microtubule organizing center (30). Looked at another way, microtubules might be expected to hold centrosomes in a fixed position within the cell, i.e., microtubules organize the centrosome. Colchicine and oncodazole, which depolymerize microtubules, cause centrosomal separation and augment the effect of EGF (Table IV). Lumicolchicine, which is structurally similar to colchicine but does not cause microtubule disassembly, has no effect either alone or in the presence of EGF. Taxol,
Which stabilizes microtubules (49) blocks centrosomal separation (Table IV).

Effects of Drugs that Inhibit EGF-induced Centrosomal Separation on Colchicine-induced Separation

Sodium fluoride, trifluoperazine (TFP), cytochalasin D, and taxol inhibited centrosomal separation in response to colchicine (Table V) suggesting that at least part of the mechanism by which centrosomes separate upon microtubule disassembly is similar to that of EGF. However, the inability to inhibit separation completely in this case using TFP or cytochalasin D suggests that a component of the process which occurs as a result of microtubule disassembly may not involve calmodulin or microfilaments.

Effects of Agents that Inhibit Centrosomal Separation on DNA Synthesis

Each of the drugs that inhibited centrosomal separation in response to EGF also inhibited DNA synthesis in response to EGF in serum deprived cells (Table VI). As has been reported in other cell types (17, 22, 37, 42, 43, 52) colchicine enhanced nuclear thymidine incorporation by itself and augmented the EGF effect.

Discussion

The objective of the present study was to elucidate the mechanism by which EGF stimulates centrosomal separation. The experiments support the following conclusions: (a) The process occurs rapidly (within 45 min) and at low concentration of EGF comparable to that required to support growth of HeLa cells in a defined medium (30) and to stimulate DNA synthesis in most cells (8, 12); (b) The signal for centrosomal separation involves calcium-calmodulin and the process is energy requiring and is dependent upon microfilament integrity; (c) Microtubules are not involved in centrosomal movement and, in fact, restrain or inhibit it.

These observations are consistent with the hypothesis that, upon binding to its receptor, EGF causes an increase in the cytosolic calcium which activates calmodulin. The calcium-calmodulin complex activates myosin light chain kinase (13, 25, 33, 39) which stimulates microfilaments attached to the centrosome to contract with consequent centrosomal separation. This formulation accounts for inhibition by energy inhibitors since ATP would be required for both myosin phosphorylation and for an actomyosin-based contractile event; for stimulation by the ionophore A23187 which would raise intracellular calcium (45), for inhibition by TFP, chlorpromazine, and W-7, which would block calcium-calmodulin action (13, 25, 26, 33, 36, 39, 54) and for inhibition by cytochalasins which destroy cellular microfilaments (20, 51). The observation of numerous microfilaments in the pericentrosomal region (34, 46) is consistent with the hypothesis that both workers (56) have not observed microfilaments there. It is of interest that calmodulin has been found to be highly concentrated in the pericentriolar region of interphase 3T3 cells (I. Pastan, personal communication), a strategic location for mediating the effects of calcium on microfilaments or microtubules associated with the centrosome.

The fact that the effects of A23187 and EGF are not additive and that both are blocked by TFP supports the contention that they are working through a common mechanism and suggests that the calcium level achieved in the presence of EGF alone is sufficient for maximal activation of separation. Nevertheless, in the absence of any published data which directly documents a rise in the free intracellular calcium concentration in response to EGF, the conclusion that calcium mediates the action of EGF in this situation must be considered tentative.

The inhibition of centrosomal separation by taxol and its stimulation by colchicine and oncodazole suggests that microtubule disassembly, perhaps in the pericentriolar region, is necessary for centrosomal movement. However, if EGF induces such disassembly, it is either incomplete or it does not occur in all cells, since colchicine and oncodazole substantially augment the EGF effect (Table IV).

The mechanism we suggest for EGF-induced centrosomal separation relies in large part upon pharmacological evidence

Table V

Effect of Agents that Inhibit EGF-induced Centrosomal Separation on Colchicine-induced Separation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Separated centromeres*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12 ± 1.07</td>
</tr>
<tr>
<td>EGF (45 min)</td>
<td>28 ± 1.48</td>
</tr>
<tr>
<td>Colchicine (45 min)</td>
<td>40 ± 1.60</td>
</tr>
<tr>
<td>NaF (15 min) followed by Colchicine (45 min)</td>
<td>12 ± 1.48</td>
</tr>
<tr>
<td>Cytochalasin D (15 min) followed by Colchicine (45 min)</td>
<td>23 ± 1.69</td>
</tr>
<tr>
<td>TFP (15 min) followed by Colchicine (45 min)</td>
<td>18 ± 1.18</td>
</tr>
<tr>
<td>Taxol (15 min) followed by Colchicine (45 min)</td>
<td>12 ± 1.23</td>
</tr>
</tbody>
</table>

Cells (100,000/ml) were plated on cover slips in DME containing 5% fetal and 5% newborn calf serum 36 h before the experiment. Media was replaced with PBS containing the compounds indicated. Colchicine was added after 15 min. Concentrations: Colchicine, 10 μM; EGF, 100 ng/ml; NaF, 0.5 mM; Cytochalasin D, 20 μM; TFP, 10 μM; Taxol, 1 μM. Immunofluorescence and quantitation of separation were as described in Materials and Methods.

*Values determined ± SEM.
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