Signal transduction through epidermal growth factor receptor is altered in HeLa monolayer cells during mitosis

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Epidermal growth factor (EGF)-induced signalling was studied separately in the mitosis and G2-phases of HeLa monolayer cells presynchronized (1) by amethopterin inhibition and thymidine release or (2) by nocodazole. For comparison, cells were treated with the phorbol ester phorbol 12-myristate 13-acetate (PMA). In contrast with the observed responses effected by PMA, which seem to be independent of cell cycle and synchronization conditions, those induced by EGF are greatly influenced by both criteria. Synchronization with nocodazole abolished the EGF-induced stimulation of phosphoinositide hydrolysis in G2 as well as in mitotic cells although tyrosine phosphorylation of the EGF receptor and phospholipase C γ 1 could be shown to occur,

especially in G2 cells. Synchronization with amethopterin/thymidine showed that, in contrast with G2 cells, mitotic cells were not able to react to EGF with an increase in phosphoinositide hydrolysis although a certain degree of EGF receptor dimerization and autophosphorylation as well as tyrosine phosphorylation of phospholipase C γ 1 could still be shown to occur in mitosis. The results seem to indicate that the EGF pathway leading to a stimulation of phosphoinositide hydrolysis is attenuated at different levels and requires a cytoskeletal condition that is not present either after treatment (24 h) with nocodazole or during normal mitosis of a monolayer cell.

INTRODUCTION

On entry into mitosis, cells undergo a profound reorganization. A number of membrane functions have been shown to be altered or even arrested [1]. These include phagocytosis, pinocytosis and receptor recycling as shown for the transferrin receptor [2,3]. The transport of vesicles between organelles ceases [1,4]. Moreover, mitotic cells resist functional responses elicited by surface ligand–receptor interactions, as has been demonstrated for IgE receptor-mediated serotonin release and for isoprenaline-stimulated degranulation of salivary cells, indicating an altered transmembrane signalling [5,6]. Cell division seems to represent a period during which animal cells are largely withdrawn from exogenous regulatory processes and in cell culture become detached from the substrate in a reversible fashion.

We became interested in the influence on mitotic cells of extracellular ligands, epidermal growth factor (EGF) and the phorbol ester, phorbol 12-myristate 13-acetate (PMA), while studying the effects of both agents on the cell cycle, specifically on the transition of human cells from G2-phase to mitosis. Both agents have been found to inhibit cycling cells [7,8]. It has been shown in particular that EGF as well as PMA inhibits HeLa cells very rapidly in G2-phase and prevents the transition to mitosis [7,8]; neither agent, however, alters the actual passage of cells through mitosis. Therefore, cells in G2-phase seem to be sensitive to the action of EGF and PMA, whereas cells in mitosis behave as though they were refractory to these agents. This idea was supported by the observation that the fully active complex of mitosis-promoting factor in mitotic cells was insensitive to treatment with EGF or PMA [9–11]. The inhibition of the transition from G2 to mitosis, however, resulted from the fact that both agents prevent the activation of the kinase function of pre-MPF. In both cases the normal dephosphorylation of the catalytic subunit p34^{cdc2} at Tyr-15 of the otherwise correctly formed complex with cyclin B did not occur in G2-phase [9–11].

It was proposed earlier that EGF-induced inhibitory mediators derived from cellular phospholipids contribute to the mechanism of cell cycle inhibition in G2-phase [12,13], whereas cells in mitosis were either not producing them or were insensitive to them.

To understand the events at the G2-phase–mitosis transition in more detail we compared the signalling in mitotic cells with that of cells in G2-phase. HeLa monolayer cells were chosen for this study because this line permits the physical removal and separation of the less adherent mitotic fraction from firmly attached cells in other phases of the cell cycle. EGF is known to bind to its receptor, a 170 kDa transmembrane glycoprotein, causing receptor dimerization and activating the intracellular tyrosine kinase domain, which in turn promotes a plethora of reactions downstream (reviewed in [14–17]). The phorbol ester PMA acts primarily through the activation of certain protein kinase C isozymes. In HeLa cells in interphase, effective signalling by both agents is indicated by the appearance of phospholipase C and phospholipase D products [12,18]. We report here that PMA-induced activation of phospholipase(s) is not prevented by the mitotic state, whereas the EGF-induced activation of phospholipid hydrolysis does not operate properly in mitotic HeLa cells. Evidence for the attenuation of EGF-induced signal transduction in mitosis can be seen directly at the level of EGF receptor and phospholipase C γ 1, and indirectly for cytoskeletal components.

MATERIALS AND METHODS

Materials

Amethopterin and adenosine were obtained from Calbiochem (Bad Soden, Germany), EGF, PMA and nocodazole (stock solution in DMSO) were from Sigma (Deisenhofen, Germany), the radioactive precursors were obtained either from Amersham

Abbreviations used: A/T, A/T, amethopterin/thymidine; DTAF, dichlorotriazinyl amino fluorescein; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate.

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Buchler (Braunschweig, Germany) or from BioTrend Chemikalien (Cologne, Germany). The antibodies used were from the following sources: anti-(phospholipase C γ 1) polyclonal antibody from rabbit, and mouse monoclonal antibody against the extracellular domain of EGF receptor (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), mouse monoclonal antibody against the intracellular domain of EGF receptor (Biotrend, Cologne, Germany), mouse monoclonal anti-phosphotyrosine antibody 4G10 (UBI, Lake Placid, NY, U.S.A.), peroxidase or fluorochrome conjugated goat anti-rabbit or sheep anti-mouse IgG (Dianova, Hamburg, Germany). Normal goat serum was from BioTrend. The enhanced chemiluminescent detection system was from Amersham Buchler. Biotinylated EGF complexed to Texas Red streptavidin was from Molecular Probes (Leiden, The Netherlands).

Cell culture and synchronization

HeLa cells were cultivated as monolayers as described elsewhere [7]. They contain approx. 1.4×10^5 EGF receptors per cell exhibiting high- and low-affinity binding sites [8]. The cells were synchronized in tissue culture quality plastic vessels (10^5 cells/cm 2) for 16 h with $1 \mu\text{M}$ amethopterin including $50 \mu\text{M}$ adenosine by the method of Mueller and Kajiwarra [19]. To release the blockage, thymidine ($10 \mu\text{g}$ per 10^6 cells) was added. When most of the synchronized cells were in late G2-phase, they were treated with EGF, PMA or, as control, with the respective solvents, PBS or acetone [0.2% (v/v) final concentration]. Alternatively, HeLa cells were synchronized in metaphase by a 24 h treatment with the antimicrotubule drug nocodazole ($2.5 \mu\text{M}$) [20]. After treatment with EGF or PMA the non-adherent mitotic cells were shaken off from the adherent remainder of the culture. The viability of mitotic cells was assayed by dye exclusion; it was more than 95% for both synchronization procedures. Synchrony was demonstrated by flow cytometric measurements of DNA distribution [21]. Non-adherent HeLa cells were obtained for control purposes by cultivation in uncoated plastic dishes. In a few instances A431 cells were used, which carry more than 10^6 EGF receptors per cell. These cells were cultivated as described earlier [8].

Analysis of phospholipid metabolites

For the analysis of inositol phosphates, cells were prelabelled for 24 h with *myo*-[2- ^3H]inositol (74 kBq/ml). Cells were further incubated for 2 h in the presence of 10 mM LiCl to prevent inositol phosphate breakdown. After treatment the dish was placed on ice and the non-adherent mitotic cells were shaken off and removed together with the medium. The adherent cells received 1 ml of ice-cold 10% (w/v) trichloroacetic acid. The non-adherent cells were centrifuged at 4 °C for 5 min in order to replace the medium with 1 ml of ice-cold 10% (w/v) trichloroacetic acid. The isolation of total inositol phosphates was performed as described earlier [19]. Radioactivity was measured in a liquid-scintillation counter.

For the measurement of phosphatidic acid, the medium together with the non-adherent cells was removed from the dish and 2 ml of ice-cold methanol was added. The medium with the non-adherent cells was pipetted into an ice-cold mixture of chloroform/methanol (1:1, v/v) containing 30 μl of 1 M HCl. The extraction of phospholipids was performed with chloroform/methanol (1:1, v/v) and 1 vol. of 1 mM EGTA in 1 M HCl [22]. Cellular lipids were separated on thin-layer plates (Silica gel G60) in a solvent system with the upper phase of iso-octane/water/ethyl acetate/acetic acid (20:100:130:30, by vol.). The quantification of phosphatidic acid was performed as described

[12,23]. The phospholipid phosphorus was determined by the method of Eibl and Lands [24]. For the determination of radioactively labelled phosphatidic acid in prelabelling experiments, cells were incubated with [$1\text{-}^{14}\text{C}$]arachidonic acid (7.4 kBq/ml) for the periods indicated. Lipids were extracted and separated as described above. The radioactivity on thin-layer plates was determined with a Linear Analyzer (Berthold, Wildbad, Germany).

Fluorescence microscopy

For fluorescence microscopy, cells were cultivated for 2 days. After removal of the medium the cells were gently rinsed with PBS and fixed for 15 min at room temperature with 0.5% freshly prepared formaldehyde/PBS, pH 7.4 [25–27]. Drying of cells between the steps was avoided. Fluorochromes were handled in the dark. The fixed cells were washed three times with PBS containing 1% (w/v) BSA.

For the detection of EGF binding the fixed cells were incubated for 1 h at 37 °C with a solution consisting of 0.2–1 $\mu\text{g}/\text{ml}$ EGF labelled with Texas Red in 20 mM Hepes-buffered PBS containing 1% (w/v) BSA, pH 7.4. For a control, cells were preincubated with 100 nM unlabelled EGF 15 min before and during the incubation with labelled EGF. Before embedding in Moviol solution, unbound EGF was removed with three washes of PBS.

EGF receptors exposed to the cell surface were analysed by staining of formaldehyde-fixed cells with monoclonal antibody against the surface epitope of EGF receptor. Cells marked with Texas Red-labelled EGF were treated for 20 min with 10% (v/v) normal goat serum in PBS/1% (w/v) BSA and incubated after three washes with PBS/1% BSA for 1 h with 1.25 $\mu\text{g}/\text{ml}$ mouse monoclonal anti-(EGF receptor) antibody in PBS/1% BSA at room temperature. After antibody removal and three washes with PBS/1% BSA the cells were incubated for 45 min with a 1:100 dilution of goat anti-mouse antiserum conjugated with the fluorescein-related fluorochrome dichlorotriazinyl amino-fluorescein (DTAF) in PBS/1% BSA. The cells were washed extensively and embedded. For control, incubations were performed without the first antibody.

The Moviol solution for embedding consisted of 0.13 g/ml Moviol 4-88 (Calbiochem) and 0.3 g/ml glucose in 0.13 M Tris/HCl, pH 8.5 [28], supplemented with 2.5% diazobicyclo[2.2.2]octane triethylenediamine (Sigma) [29,30]. Microscopy and photography of the cells were performed with an Axiophot Zeiss fluorescence microscope.

Cell lysis, immunoprecipitation and Western blotting

The non-adherent cells were removed with the medium and sedimented by centrifugation (5 min at 500 g and 4 °C). Adherent cells and sedimented cells were lysed by the addition of ice-cold buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% (w/v) Triton X-100, 1 mM EGTA, 1.5 mM MgCl_2 , 0.1 mM Na_3VO_4 , 50 mM NaF, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin and 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor [31]. The lysates were homogenized by sonication and centrifuged (10 min at 3000 g and 4 °C) to remove cell debris. Aliquots of the supernatant were removed for protein determination with the Bradford assay. For demonstration of EGF receptor dimerization, the so-called RIPA lysis buffer (1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS in PBS containing 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 0.1 mM Na_3VO_4 and 50 mM NaF) was used.

For immunoprecipitation of specific antigens, aliquots of cell lysates were diluted to 1.5 ml with a buffer consisting of 10 mM

Hepes, pH 7.5, 0.1 % Triton X-100, 0.1 mM Na_3VO_4 , 50 mM NaF and 10 $\mu\text{g}/\text{ml}$ aprotinin [31]. The samples were precleared for 2 h at 4 °C with 50 μl of Protein A-agarose (1:2 dilution; Boehringer Mannheim, Germany) and centrifuged at 4 °C in an Eppendorf centrifuge. Immunoprecipitation was by overnight incubation with 0.5 μg of antibody per mg of protein on a slow rotator. The mixture was incubated with 50 μl of a 1:2 diluted Protein A-agarose for 2 h at 4 °C and afterwards pelleted at 4 °C in an Eppendorf centrifuge and washed three times with 1 ml of immunoprecipitation buffer. The probe was brought to Laemmli buffer conditions and heated for 2 min at 95 °C.

For Western immunoblotting, usually 200 μg aliquots of cell lysate in Laemmli sample buffer were subjected to SDS/PAGE [8 % (w/v) gels or 3.75 % (w/v) gels] and transferred to Immobilon membranes (Millipore, Eschborn, Germany). After blocking with 2 % BSA in PBS/0.05 % Tween-20, membranes were probed with the desired antibody followed by exposure to peroxidase-conjugated secondary antibody and developed by the enhanced chemiluminescent detection system (ECL; Amersham Buchler). In some cases the blot was stripped with 0.2 M glycine, 0.1 % SDS and 1 % Tween-20, pH 2.2, to remove the antibodies. The specificity of the antibodies was tested by blocking the reaction with respective antigenic peptides.

Data presentation

Experiments were performed at least twice, in the case of biochemical measurements with triplicate samples. Representative results are demonstrated.

RESULTS

Cell synchrony and treatment conditions

For the comparison of the response of mitotic cells with that of cells in interphase, particularly those in G2-phase, two mechanistically different synchronization procedures were used to accumulate cells in the desired cell cycle phase. The actual treatment with EGF and PMA was conducted in synchronized cultures containing simultaneously cells in mitosis and G2-phase, i.e. *in situ*. Separation of cells was achieved *after* treatment by a mechanical shake-off. Thus we obtained predominantly mitotic cells (the non-adherent fraction) separated from the adherent population, which consisted mainly of G2 cells. A comparison of DNA histograms of these fractions is shown in Figure 1.

Treatment of cells with the antimicrotubule drug nocodazole was employed to accumulate cells in metaphase (Figure 1A). Microscopic analysis revealed that the proportion of metaphase cells in the non-adherent nocodazole fraction was approx. 85–90 %. The respective adherent fraction, which was nearly devoid of metaphase cells, consisted mainly of cells in G2-phase (Figure 1B). To inhibit cells at the G1–S border and in S-phase, cells were incubated for approx. 16 h with amethopterin, which causes a thymidine deficiency [19]. These cultures contained approx. 70 % cells at the G1–S border and about 30 % cells in S-phase. After the addition of thymidine, cultures continue through the cell cycle and reach a peak of mitotic activity by approx. 9–11 h. Because in this case the passage through mitosis is not stopped in metaphase this procedure also resulted in some non-adherent cells with the DNA content of divided cells (Figure 1C). In this case the non-adherent fraction contained approx. 60–65 % cells in metaphase, whereas the adherent fraction (Figure 1D) contained less than 10 % metaphase cells. These observations in combination with the DNA histograms indicate that the non-adherent fractions contained principally mitotic cells, whereas the adherent fractions consisted predominantly of cells in G2-

Figure 1 Cell cycle phase distribution of HeLa cell cultures

DNA histograms of nocodazole-synchronized cells (A, B) and of cells 10.5 h after release of amethopterin blockage (C, D) show the cell cycle phase distribution by flow cytometric measurements. The abscissa is the relative fluorescence intensity equivalent to DNA content; the ordinate is the relative cell number. (A, C) Cell cycle distribution of the non-adherent, loosely bound cell population; (B, D) distribution of the adherent cell population of the same cultures.

phase. For the sake of simplicity the terms adherent and non-adherent will be used further in combination with the synchronizing agent used. The non-adherent fraction contained in both cases more than 95 % viable cells as indicated by Trypan Blue exclusion.

The concentrations of EGF (10 nM) and phorbol ester PMA (100 nM) chosen for this study have been shown in dose-response relations to be maximally effective in HeLa cells with regard to phospholipid hydrolysis as well as cell cycle inhibition in G2-phase [12]. For EGF the responses are maximally expressed within 5 min, and for PMA within 20 min [12].

Phospholipid hydrolysis induced by EGF

One of the typical metabolic responses of cells to treatment with EGF results from the activation of phospholipase $\text{C}\gamma 1$ [32–35] and subsequent hydrolysis of phosphoinositides. The reaction is characterized by the products inositol phosphate and diacylglycerol [36]. For the analysis of inositol phosphates, cells were prelabelled during the synchronization procedure with radioactive myoinositol and treated with EGF for 5 min before the mechanical shake-off procedure, i.e. the adherent fraction and the non-adherent fraction were treated simultaneously in the same dish *before* their separation and analysis. For comparison, cultures have been included in the analyses that were blocked for 16 h with amethopterin but not released from blockage. The results of such an experiment are shown in Table 1. The adherent fraction obtained after amethopterin/thymidine (A/T) synchronization exhibited a more than 50 % increase in inositol phosphates in response to EGF, similar to the cells blocked at the G1–S border and in S-phase. In contrast, the non-adherent fraction obtained after A/T synchronization did not respond to EGF.

The second product of phosphatidylinositol hydrolysis, diacylglycerol, becomes rapidly phosphorylated in HeLa cells to yield phosphatidic acid [37–39], which was also analysed. To measure it, HeLa cells were prelabelled during the A/T synchronization

Table 1 Stimulation of inositol phosphate production by EGF treatment

After prelabelling of HeLa cells with myo -[3H]inositol and synchronization either with A/T or nocodazole (N), cells were treated for 5 min with 10 nM EGF. After separation of adherent and non-adherent cells the reaction was stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid. The inositol phosphates were isolated by anion-exchange chromatography, and the production of [3H]inositol phosphate was measured by scintillation counting. The results, given as percentages of control, represent the means $\pm$ S.D. for two independent experiments with three parallel treated cell cultures. The deviations of the controls treated with PBS and taken as 100% did not exceed $\pm$ 14%. For a further control, cultures synchronized in the S-phase with amethopterin have been analysed.

Cell fraction	Production of inositol phosphates (% of control)		
	A/T-synchronized	N-synchronized	Amethopterin
Adherent (G2)	165 $\pm$ 7	97 $\pm$ 18	—
Adherent (S-phase)	—	—	151 $\pm$ 22
Non-adherent (mitosis)	95 $\pm$ 9	98 $\pm$ 9	—

Table 2 Stimulation by EGF or PMA of phosphatidic acid production

[^{14}C]Arachidonic acid-prelabelled cell cultures were synchronized with A/T or nocodazole (N) and treated with EGF (10 nM, 5 min) or with PMA (100 nM, 20 min) and separated into adherent and non-adherent fractions. Lipids were extracted and separated for phosphatidic acid on thin-layer plates. Phosphatidic acid production was measured by a linear analyser. The results, given as percentages of control, represent the means $\pm$ S.D. for two independent experiments with three parallel treated cell cultures. The deviations of the controls treated with PBS (for EGF) or 0.2% (v/v) acetone (for PMA) and taken as 100% did not exceed $\pm$ 10%.

Cell fraction	Production of phosphatidic acid (% of control)			
	A/T-synchronized		N-synchronized	
	EGF	PMA	EGF	PMA
Adherent (G2)	147 $\pm$ 20	196 $\pm$ 12	113 $\pm$ 18	200 $\pm$ 7
Non-adherent (mitosis)	105 $\pm$ 10	173 $\pm$ 10	109 $\pm$ 7	183 $\pm$ 9

procedure with radioactive arachidonic acid and then, on treatment with EGF, the adherent and non-adherent cells were analysed for the appearance of radioactive phosphatidic acid. The results (Table 2) mirror those obtained with inositol phosphates (Table 1). The adherent fraction reacted with an increase in labelled phosphatidic acid of approx. 50% within 5 min. In contrast the non-adherent fraction did not show an increase in labelled phosphatidic acid after treatment with EGF. A comparable set of results was obtained by determination of the absolute amounts of phosphatidic acid (results not shown). Taken together, these results demonstrate that the non-adherent mitotic fraction, in contrast with the firmly attached G2 fraction, is unable to respond to EGF with an increase in phospholipid hydrolysis.

Overnight synchronization of HeLa cultures with nocodazole caused the adherent population not to respond to EGF with an increase in inositol phosphates or phosphatidic acid (Tables 1 and 2). This is in strong contrast with the reaction of adherent cells obtained by A/T synchronization. It was therefore not possible to attribute the lack of response to EGF in the non-adherent fraction of nocodazole-treated cells (Table 1) to the mitotic status itself. Treatment of cultures for only 2, 4 or 6 h with nocodazole was not sufficient to erase the EGF-induced increase in phospholipid hydrolysis (results not shown) although under these circumstances the interphase microtubular system should have disappeared [40].

During mitosis HeLa cells become round and lose contact with the substrate. To evaluate what kind of effect the simple detachment of cells might have, non-adherent asynchronous HeLa cells were treated with EGF. For this purpose cells were cultivated with their regular medium in non-coated plastic dishes (to prevent adherence) for a period of 7 h; for the last 6 h this was in the presence of radioactive arachidonic acid for prelabelling before they were treated without or with EGF for 5 min. These cells did not respond to EGF with an increase of labelled phosphatidic acid (results not shown), thus indicating that the physical condition of the cell in relation to its micro-environment represents an additional factor that contributes to the responsiveness of cell-surface receptors.

Phospholipid hydrolysis activated by PMA

To test the possibility that the mitotic state would itself influence phospholipase activation, experiments with PMA were performed. Treatment of asynchronous HeLa cells with PMA is known to lead to the formation of phosphatidic acid via two different pathways. One involves activation of phospholipase D, the other that of a phosphatidylcholine-specific phospholipase C and subsequent phosphorylation of diacylglycerol [41,42].

To assay the response of mitotic HeLa cells, cultures synchronized by the A/T procedure and prelabelled at the same time with radioactive arachidonic acid were treated with PMA (100 nM) for 20 min. Subsequently, the non-adherent cells were shaken off and analysed for labelled phosphatidic acid. The results (Table 2) show that the mitotic state of HeLa cells did not prevent the PMA-induced activation of phospholipid hydrolysis. The increase of labelled phosphatidic acid was approx. 1.7-fold. This responsiveness to treatment with PMA was also observed in cells synchronized with nocodazole. In the non-adherent as well as in the adherent fraction PMA caused an approx. 1.8-fold increase in labelled phosphatidic acid within 20 min (Table 2). Similar quantitative relationships were obtained by determination of the absolute amounts of phosphatidic acid (results not shown). These results indicated that in contrast with the action of EGF, neither the mitotic state itself nor the antimicrotubule drug nocodazole prevented the PMA-mediated stimulation of phospholipid hydrolysis. In other words, the failure of mitotic cells to respond to EGF seems to be related specifically to a disturbed EGF receptor pathway during cell division.

Surface exposure of EGF receptor

The difference between mitotic cells and interphase cells in their abilities to respond to EGF by a stimulation of phospholipid hydrolysis led to an examination of the surface exposure of the EGF receptor protein by fluorescence microscopy. These experiments were done *in situ*, i.e. under conditions in which the cells had been exposed to EGF for the assay of phospholipid metabolites. Cultures were fixed without permeabilization and tagged with antibodies against the extracellular domain of the EGF receptor protein as well as with EGF labelled with Texas Red. In these studies for control purposes we have included A431 cells, a human epidermal carcinoma cell line, which carries a larger number of EGF receptor molecules than HeLa cells [43,44]. Representative results are shown in Figure 2. It is evident that in both cell lines mitotic cells bind EGF, as indicated by a clearcut red margin (Figure 2). The intensity of staining was practically abolished by preincubation of cells with unlabelled EGF (100 nM) for 15 min at 37 °C before fixation. Binding of EGF to interphase cells is indicated by the broad although less intense reddish margin.

Figure 2 EGF binding and surface exposure of EGF receptors

Fixed HeLa and, as control, A431 cells were stained with Texas Red complexed EGF and with anti-(EGF receptor) antibody detected with DTAF-conjugated antiserum. Mitotic cells (arrowheads) and interphase cells were discriminated by cell morphology and staining with 4',6-diamidino-2-phenylindole (DAPI). Abbreviation: EGFR, EGF receptor.

In principle, comparable morphological results were obtained by the use of an antibody against the extracellular domain of the EGF receptor protein (Figure 2). It stains a clearcut margin in mitotic cells and a broader circumference in interphase cells in both lines. This analysis *in situ* does not permit quantification; the impression is, however, that the fluorescence of EGF as well as the staining of EGF receptor with antibody seems to be more condensed in mitotic than in interphase cells. Together these results demonstrate that in mitotic cells the extracellular domain of the EGF receptor protein is accessible at the cell surface for either EGF or a specific antibody.

EGF receptor protein

In order to characterize EGF receptor protein it was immunoprecipitated with specific antibody from equal protein amounts of lysates from HeLa cells obtained under various conditions, as shown in Figure 3. It became evident that regardless of the synchronization procedure used, the adherent as well as the non-adherent HeLa cells contained comparable amounts of the EGF receptor protein as detectable by immunoblotting with anti-

(EGF receptor) antibody (Figure 3A). The amount is smaller than in A431 cells, of which half of the lysate amount had been applied for comparison (Figure 3A).

Autophosphorylation of EGF receptor protein at tyrosine residues belongs to the early responses of cells treated with EGF [15,45,46]. To compare that of cells in G2-phase with that of mitotic cells, HeLa cells synchronized by the A/T procedure were treated for 5 min with EGF and subsequently separated into adherent and non-adherent fractions. Immunoprecipitates obtained from lysed cells with anti-(EGF receptor) antibody were separated on SDS/polyacrylamide gels, blotted and assayed with anti-phosphotyrosine antibodies. The EGF-treated adherent fraction exhibited a strong signal in the position of the EGF receptor, whereas the non-adherent fraction showed a rather weak signal (Figure 3B). Similar results were obtained by probing Western blots of respective cell lysates with antiphosphotyrosine antibodies (see below).

If the order of antibody application was reversed, immunoprecipitation with anti-phosphotyrosine antibody followed by Western blotting with anti-(EGF receptor) antibody, a comparable result was obtained. In this case, however, much

Figure 3 EGF receptor protein and phosphorylation state

(A) Amount of immunoprecipitated EGF receptor from 3 mg of protein lysates each in different cell cycle phases (lanes 1–4). As control, EGF receptor was precipitated from 1.5 mg of lysate of A431 cells (lane 5). (B) Western blotting of precipitated EGF receptor from 1.5 mg of lysate with anti-phosphotyrosine antibody; comparison of adherent cells with non-adherent cells from the same EGF-treated culture. (C) Immunoprecipitation with anti-phosphotyrosine antibody and immunoblotting with anti-(EGF receptor) antibody from 1.5 mg of lysate of adherent A/T-synchronized cells, of non-adherent and nocodazole-treated cells. As control, immunoprecipitation was also done from PBS-treated A/T-synchronized cells. Abbreviations: ad, adherent cells; n-ad, non-adherent cells; N, nocodazole; IP, immunoprecipitation; WB, Western blotting; EGFR, EGF receptor; anti-PY, antiphosphotyrosine; kD, kDa.

less EGF receptor protein was recovered from the non-adherent fraction by the anti-phosphotyrosine antibody than from the adherent fraction (Figure 3C); without EGF treatment the adherent fraction did not yield a detectable signal.

The consequences of EGF treatment of cells synchronized with nocodazole were analysed in Western blots of cell lysates. The adherent fraction showed an anti-phosphotyrosine-positive signal at the position of the EGF receptor (results not shown) that was only slightly weaker than that obtained with the adherent fraction of A/T-synchronized cells after treatment with EGF (Figure 3C). The non-adherent nocodazole cells, however, gave

Figure 4 Dimerization of EGF receptor protein

A/T-synchronized cells were treated with EGF (5 min, 10 nM) and further incubated with or without cross-linking agent (EDAC, 15 mM, 15 min). Immunoprecipitations of the EGF receptor protein from 2 mg of RIPA lysates of adherent and non-adherent cell populations were separated by SDS/PAGE [3.75% (w/v) gel] and transferred to the poly(vinylidene difluoride) membrane. The blots were probed with anti-(EGF receptor) antibody (A) or anti-phosphotyrosine antibody (B). Molecular masses (in kDa) are shown at the left. Abbreviations: IP, immunoprecipitation; WB, Western blotting; EGFR, EGF receptor; anti-PY, antiphosphotyrosine.

an even weaker signal than the non-adherent fraction of A/T-synchronized cells. With the antiphosphotyrosine antibody it was not possible to immunoprecipitate enough material from the non-adherent EGF-treated nocodazole cells for detection with anti-(EGF receptor) antibody in Western blots (Figure 3C).

EGF receptor dimerization

It has been shown that EGF induces dimerization of EGF receptor protein in intact cells [47–49] and it has been suggested that this process is required for transmembrane signalling. To test the possibility that dimerization was influenced by the mitotic state of cells, HeLa cells were synchronized by the A/T procedure and treated without or with EGF for 5 min. Subsequently, half of the culture plates were exposed to the cross-linking agent 1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide (EDAC) (15 mM) for 15 min at 37 °C by the method of Cochet et al. [48]. The non-adherent fraction was shaken off and separated from the adherent cells and identical amounts of lysed protein were immunoprecipitated with anti-(EGF receptor) antibody, separated by SDS/PAGE and transferred to poly(vinylidene difluoride) membrane. Detection of EGF receptor protein by the use of anti-(EGF receptor) antibody showed that EDAC elicited an EGF receptor-positive band above 300 kDa only in EGF-treated cells (Figure 4A). The adherent fraction gave a relatively

Figure 5 Phospholipase C γ 1

(A) Immunoprecipitation of phospholipase C γ 1 (PLC γ 1) from 2 mg of lysates of PBS-treated cells and immunoblotting with anti-PLC γ 1 antibody gave the same amount of PLC γ 1 in all cell cycle phases tested. From non-adherent cells an unknown protein of approx. 60–64 kDa co-precipitated with PLC γ 1 and was an antigen for the anti-PLC γ 1 antibody. (B) The tyrosine phosphorylation state of PLC γ 1 protein from 1 and 3 mg of lysate of EGF-treated cells was compared after immunoprecipitation by antiphosphotyrosine antibodies and immunoblotting with anti-PLC γ 1 antibodies. (C) PLC γ 1 protein was immunoprecipitated by anti-PLC γ 1 antibodies from 2 mg of cell lysate of nocodazole-synchronized (lanes 1–4) and A/T-synchronized cells; the tyrosine phosphorylation state was tested by anti-phosphotyrosine antibodies. The positions of molecular mass markers (in kDa) are shown at the left. Abbreviations: IP, immunoprecipitation; WB, Western blotting; ad, adherent cells; n-ad, non-adherent cells; N, nocodazole; anti-PY, antiphosphotyrosine.

strong signal; that obtained from the non-adherent fraction was comparatively weak. Signals above 300 kDa in a similar relation were also obtained by the use of anti-phosphotyrosine antibodies (Figure 4B). Owing to the low efficiency of the cross-linking reaction this technique has not previously been considered suitable for quantitative determination [48]. However, because in our experiments adherent and non-adherent cells were treated simultaneously in the same dish under identical conditions, the results do seem to reflect quantitative relations at least to some extent (the experiment was performed three times with essentially identical results). The results indicate that EGF-induced dimeri-

Figure 6 EGF-induced phosphorylations of proteins

After SDS/PAGE of 400 μ g of protein lysate and transfer to the membrane, the blot was tested with anti-phosphotyrosine antibody. Proteins of approx. 170 kDa [EGF receptor, tested by anti-(EGF receptor) antibodies] and 155 kDa [phospholipase C γ 1 (PLC γ 1), tested by blotting with anti-PLC γ 1 antibody] and unknown proteins of approx. 120 and 100 kDa were strongly phosphorylated in adherent cells treated for 5 min with EGF. Abbreviations: WB, Western blotting; ad, adherent cells; n-ad, non-adherent cells; N, nocodazole; EGFR, EGF receptor; anti-PY, antiphosphotyrosine; kD, kDa.

zation of EGF receptor protein does occur in the non-adherent fraction but to a smaller degree than in the adherent fraction and thus reflects the different degree of EGF receptor autophosphorylation. In nocodazole-synchronized cultures the EGF-induced dimerization of the receptors could be detected in the adherent fraction and also, but to a smaller extent, in non-adherent cells (results not shown). This indicates that the EGF-induced dimerization is not prevented by the mitotic state of the cells.

Phospholipase C γ 1

Phospholipase C γ 1 is one of the substrates of EGF receptor kinase. Phosphorylation of phospholipase C γ 1 at tyrosine is involved in the activation of this enzyme [32–35]. The analysis of the cell lysates by SDS/PAGE and immunoblotting with anti-(phospholipase C γ 1) antibody showed that irrespective of the synchronization procedure the adherent fraction as well as the non-adherent fraction of cells contained comparable amounts of phospholipase C γ 1 protein. This result was confirmed by immunoprecipitation with anti-(phospholipase C γ 1) antibody and Western blot analysis with the same antibody (Figure 5A). Immunoblotting with anti-phosphotyrosine antibodies revealed that EGF treatment caused a phosphotyrosine-positive signal at the position of phospholipase C γ 1 (approx. 150 kDa) in the adherent fraction of cells synchronized by the A/T procedure (Figure 5B), but surprisingly also in that obtained with nocodazole (Figure 5C). In addition to the EGF receptor signal by anti-phosphotyrosine antibody, in A/T-synchronized cells proteins at approx. 120 and 100 kDa were tyrosine-phosphorylated in response to EGF (Figure 6) that were not detected in adherent cells synchronized with nocodazole.

The results presented in this study indicate that (1) EGF causes tyrosine phosphorylation of phospholipase C γ 1 (and possibly other proteins) predominantly in adherent cells correlated with the phosphorylation of EGF receptor protein; and (2) EGF-induced tyrosine phosphorylation of phospholipase C γ 1 in adherent cells is accompanied by an EGF-stimulated phospholipid hydrolysis only in A/T synchronization but not in nocodazole-treated cells. In the non-adherent fraction the weakness of EGF-induced tyrosine phosphorylation of phospholipase

Table 3 Summary of the responses of synchronized HeLa cells to EGF and PMA

Because the protein amounts of EGF receptor and of phospholipase C γ 1 were not altered by treatment with EGF these parameters have been omitted from the table. An entry of 0 indicates no stimulation. Abbreviation: n.d., not done.

Synchronization		EGF binding	EGF receptor		Phospholipase C γ 1 Tyr phosphorylation	Stimulation of phospholipid hydrolysis by:	
Method	Cycle phase		Dimerization	Tyr phosphorylation		EGF	PMA
A/T	G2 (adherent)	Yes	++ ++	++ ++	++ ++	++ ++*	++ ++
	Mitosis (non-adherent)	Yes	++	++	++	0*	++ ++
Nocodazole (24 h)	G2 (adherent)	n.d.	++	++ +	++ +	0**	++ ++
	Mitosis (non-adherent)	n.d.	+	(+)	(+)	0**	++ ++

* Non-adherent cells (interphase) did not respond to EGF with stimulation of phospholipid hydrolysis.

** Short-term treatment with nocodazole of cells for 6 h or less did not interrupt EGF-induced phospholipid hydrolysis.

C γ 1 is correlated with the lack of phospholipid hydrolysis under the same conditions. Together the results show that tyrosine phosphorylation of phospholipase C γ 1 is a necessary but not sufficient condition for the activation of phosphoinositide hydrolysis. A schematic summary of the main observations in this study is given in Table 3.

DISCUSSION

Treatment of HeLa cells with EGF or PMA causes a delay in G2-phase but not in mitosis, i.e. at the G2–M transition cells become resistant to the cell cycle-delaying efficacy of both compounds. Comparable observations have been made with A431 cells [12,13]. One possibility is that the proposed inhibitory mediators mobilized from membrane phospholipids by activation of phospholipases in response to EGF and to PMA [12,13] contribute to the G2 delay but are no longer generated as the cell enters mitosis. Alternatively, at the G2–M transition the cell could become refractory to the action of these mediators. The results presented in this study with cells in G2-phase and in mitosis indicate that both possibilities are realized. With EGF the EGF receptor transmembrane signalling does not function properly in dividing cells and thus phospholipid hydrolysis does not take place. Treatment of mitotic cells with PMA, in contrast, caused phospholipid hydrolysis but the process of cell division seemed to be insensitive to it and to its products. The disruption of the EGF signal transduction to phospholipase C γ 1 in mitosis could only be demonstrated if the cells were synchronized without an antimicrotubule drug. Synchronization of HeLa cells with nocodazole was not applicable because the drug blocked EGF signal transduction even in cells in interphase. As pointed out below, the analysis of effects of EGF and PMA in cells synchronized with nocodazole did yield data that help to explain the role of cytoskeletal elements for an efficient activation of phospholipase C γ 1 by EGF.

The PMA-induced phospholipid hydrolysis is not greatly influenced either by nocodazole or by the cell cycle staging. Nocodazole-induced changes of the microtubular system and subsequent alterations of the cytoskeleton seen after several hours of treatment with nocodazole [40] as well as natural rearrangements of the microtubular and cytoskeletal systems during mitosis do not interfere with the response to PMA. The activation of phospholipases by PMA seems to be independent of changes in cytoskeletal structures.

The response to EGF of cells synchronized with nocodazole points to a specific involvement of cytoskeletal elements in the

activation of phospholipase C γ 1. A pretreatment of cells with nocodazole for a few hours did not inhibit EGF-induced phospholipid hydrolysis. These observations indicate that a breakdown of the interphase microtubular system usually seen on short-term treatment with nocodazole [40] is not sufficient to disturb EGF signalling. Long-term treatment with nocodazole, however, as required for synchronization, abolished the EGF-induced phospholipid hydrolysis completely in G2-phase as well as in mitosis.

The analysis of G2 cells obtained with nocodazole revealed that despite the lack of EGF-induced phospholipid hydrolysis a substantial tyrosine phosphorylation of the EGF receptor in response to EGF as well as of phospholipase C γ 1 had occurred. Tyrosine phosphorylation of the EGF receptor on binding of EGF is indicative of the activation of its kinase function, and phospholipase C γ 1 has been shown to be a substrate of the EGF receptor. Phospholipase C γ 1 can be phosphorylated at three different tyrosine residues, one of which seems to be important for the activation of this enzyme [35]. Therefore a treatment for several hours with nocodazole seems to interrupt events downstream of tyrosine-phosphorylated phospholipase C γ 1 that seem, however, to be required for the enzyme to hydrolyse phospholipids within the cell. It has been proposed that on phosphorylation by receptor protein tyrosine kinases, phospholipase C γ 1 is targeted via its SH3 domain to cytoskeletal components, possibly dynamin [50], a microtubule-associated protein with stimutable GTPase activity, and that such interaction might allow the enzyme to translocate to and interact with phospholipid substrates [51–53]. It is likely that this step does not occur because of nocodazole-induced changes in the cytoskeleton of cells in interphase. It is possible that profound changes in the cytoskeleton such as occur naturally in mitotic cells without drug treatment contribute to silencing phospholipase C γ 1 during cell division.

The experiments performed with HeLa cells presynchronized with nocodazole, although valuable for the interpretation for the involvement of cytoskeletal elements, did not furnish an answer to the initial question of EGF receptor signalling in mitotic cells. It should be noted that in a study on IgE receptor-mediated events in mitotic cells, nocodazole synchronization was also omitted because it inhibited serotonin release from interphase RBL cells [5]. Therefore those authors also used another synchronization method and completed it also by the shake-off procedure for mitotic cells. The shake-off procedure with HeLa cultures presynchronized with amethopterin/thymidine yields a less homogeneous population of non-adherent cells than from nocodazole-

presynchronized cells. In addition to metaphases, cells from later mitotic stages seem to detach, as indicated by cells with the DNA content of G1 cells. It was not possible to determine the extent to which this heterogeneity among mitotic cells might influence the results on the status of the EGF receptor and phospholipase C γ 1. It is evident, however, that despite the observed heterogeneity no EGF-inducible phospholipid hydrolysis was measurable with these cells, i.e. the EGF-induced response was interrupted in this predominantly mitotic population. The dimerization of EGF receptor observed in nocodazole-arrested HeLa cells confirms that dimerization can occur in metaphase.

In cells synchronized by inhibition of DNA replication and subsequent release it is shown that, in contrast with G2 cells, EGF-treated mitotic cells failed to yield phosphoinositide hydrolysis products although the EGF receptor seems to be exposed at the surface and capable of binding EGF. The failure is accompanied by a decrease in (1) EGF receptor dimerization, (2) tyrosine phosphorylation of EGF receptor, and (3) phospholipase C γ 1 phosphorylation at tyrosine (see Table 3).

In the mitotic cell an interference with the EGF receptor signalling cascade can occur simultaneously at different points, leading to a stepwise attenuation of the cascade, resulting in a fall in phospholipase C γ 1-catalysed phospholipid hydrolysis. The analysis *in situ* of EGF binding as required for this study, i.e. the incubation of cultures containing at the same time attached cells in G2-phase and less adherent cells in mitosis, did not permit a separate quantification but showed clearly that mitotic cells bound labelled EGF in an easily detectable and specific manner. It cannot be excluded that the binding capacity is altered as the cell undergoes division. If a normal binding of EGF is assumed, the first decrease detected is that seen with EGF receptor dimerization. The reason is not clear. An altered cell surface geometry, changes in biophysical membrane properties during mitosis or mitosis-specific modification of EGF receptor itself might play a role. Dimerization is important for the intermolecular autophosphorylation of EGF receptor and the subsequent gain in kinase activity. If it is decreased, as seen in mitotic cells, it might lead to a decreased EGF receptor tyrosine phosphorylation. An additional decrease in tyrosine autophosphorylation of EGF receptor might result from an attenuation of the kinase function of the EGF receptor during mitosis. It has been proposed that phosphorylation of EGF receptor at Ser-1002 by the catalytic subunit p34^{cdc2} of mitosis-promoting factor is responsible for the desensitization of the EGF receptor [54,55]. A cell cycle-dependent decrease in the tyrosine kinase activity of the EGF receptor has been described in G2/mitotic cells but there was no discrimination between G2-phase and mitotic cells in this work [56]. The various measures that the mitotic cell takes to decrease the kinase activity of the EGF receptor lead to a decrease in the phosphorylation of phospholipase C γ 1 at tyrosine residues on treatment with EGF and in turn to a decreased capacity to be activated. A final step to silence phospholipase C γ 1 results from the completely altered state of the cytoskeleton during mitosis, which might prevent the enzyme from gaining access to its substrate.

The observation that non-adherent HeLa cells in metaphase cannot be stimulated with EGF to hydrolyse phospholipids, either through phospholipase C γ 1 as shown here or through phospholipase A $_2$ as reported by Berlin and Preston [57], reflects the lack of response of mitotic HeLa cells in both cases. In other words, the failure to react to treatment with EGF is correlated with a non-adherent state, no matter how it is achieved, whether through internal means and measures of a cell required to distribute its components equally to the daughter cells or by external culture conditions chosen to prevent cells from at-

tachment. In both cases cytoskeletal conditions required for the activation of phospholipase C γ 1 did not seem to be present and might thus present a common deficiency. If this were the case the non-adhesion of a mitotic cell would be responsible for its lack of response. In this view the problem of the difference between the reactions of the interphase cell and the mitotic cell would be diminished, at least partly, to the degree of substrate adherence. It cannot be excluded, however, that parts of the EGF receptor signalling pathway that do not require a specific condition of the cytoskeleton are less affected by the mitotic state, comparable with the responsiveness to PMA, which is unaffected by mitosis. It should be added that the spatial compartmentation, which is partly determined by cytoskeletal integrity, has also been considered important [58,59] in another part of EGF receptor signalling, the generation of phosphatidylinositol 4,5-bisphosphate, thus reinforcing the conclusions drawn from our observations.

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