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CELL-CYCLE-DEPENDENT MODULATION OF EGF-RECEPTOR-MEDIATED SIGNALING

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Summary. In A431 cells synchronized by treatment with thymidine, the level of EGF-stimulated tyrosine protein kinase activity in cells in S and G_2/M phases was reduced ~40% relative to that seen in cells in G_1. This decrease in receptor tyrosine protein kinase activity did not correlate with a decrease in cell surface EGF receptor expression,indicating that the reduced activity could not be attributed to receptor loss. EGF-stimulated PI 3-kinase activity was also reduced by ~60% during S phase as compared to G_1 phase. The change was not due to decreased PI 3-kinase expression since Western blot analyses indicated that cellular p85 levels remained constant throughout the cell cycle. These data suggest that the ability of EGF to stimulate biological responses varies during the cell cycle and implicate cell-cycle-dependent processes in the regulation of EGF-receptor-mediated signaling.

The epidermal growth factor receptor is a 170 kDa glycoprotein with an extracellular ligand binding domain, a single transmembrane domain, and an intracellular domain with intrinsic protein tyrosine kinase activity (1, 2). The binding of EGF to the external domain of the receptor induces receptor dimerization (3, 4) and stimulates its tyrosine protein kinase activity (5, 6). This promotes the activation of other signaling molecules such as phospholipase C (7, 8), phosphatidylinositol 3-kinase (9) and MAP kinase (10, 11). Ultimately, activation of the EGF receptor leads to mitosis.

Regulation of EGF receptor activity is crucial for the maintenance of controlled cell growth. Therefore a variety of mechanisms, including receptor down regulation and desensitization, have evolved to decrease EGF receptor function when cells are exposed to prolonged treatment with EGF. Because growth factors are thought to act principally by inducing quiescent cells to enter G_1 or to pass into S phase from G_1, we wondered whether the activity of growth factor receptors was also modulated in response to the cell cycle. We therefore examined EGF receptor-based signaling throughout the cell cycle. We report here that the ability of EGF to stimulate receptor tyrosine kinase activity and PI 3-kinase activity varies during the cell cycle, implicating cell cycle-dependent processes in the regulation of EGF-mediated signaling.

Materials and Methods

Materials. [γ^{32}P]ATP and [^{125}I] Protein A were obtained from Du Pont-New England Nuclear (Boston, MA). [^{125}I]-labeled EGF was prepared as described (12) using free ^{125}I purchased from

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Amersham. The Arg-Arg-Src peptide (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) was synthesized on an Applied Biosystems peptide synthesizer and purified by high performance liquid chromatography. Antibodies to the 85 kDa subunit of phosphatidylinositol 3-kinase were purchased from Upstate Biotechnology, Inc. Anti-phosphotyrosine IgG agarose (1G2 Agarose) was purchased from Oncogene Science.

Cell Culture and Synchronization Human A431 epidermoid carcinoma cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 3% fetal calf serum, 7% newborn bovine serum, and 2 mM glutamine. For synchronization, cells were transferred to medium containing 4 mM thymidine for 16 hours. The cells were released from the block by washing with serum-free DMEM and refeeding with normal medium containing 10% serum.

FACS Analysis. Cells were harvested by trypsinization and incubated with Kirshan's Reagent (0.05 mg/ml propridium iodide, 0.1% sodium citrate, 0.02 mg/ml ribonuclease A, 0.3% NP-40, pH 8.3) for 30 min at 4°. Analysis was performed on a Becton Dickinson flow cytometer and the data were analyzed using the CellFIT computer program.

Receptor Tyrosine Kinase Assay. Cells were harvested in 10 mM Tris, pH 7.5, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 100 μM sodium orthovanadate and homogenized using a Dounce homogenizer. The homogenate was centrifuged at 30,000 x g for 30 min at 4°. The membrane pellet was resuspended in 40 mM imidazole, pH 7.2, 250 mM NaCl, 10% glycerol. Membrane preparations were assayed for their ability to phosphorylate the synthetic peptide Arg-Arg-Src in the presence and absence of EGF as described by Pike (13).

Phosphatidylinositol 3-Kinase Assay. Cells were incubated in serum-free Dulbecco's Modified Eagle's Medium containing 1 mg/ml bovine serum albumin for 1 hr and were then treated with or without 50 nM EGF for 3 min. Cells were lysed in buffer containing 137 mM NaCl, 20 mM Tris, pH 8.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 400 μM sodium orthovanadate, 1 μg/ml leupeptin, and 1 μg/ml aprotinin and the lysates clarified by centrifugation at 13,000 x g for 10 min at 4°. Approximately 400 μg of lysate protein was immunoprecipitated using anti-phosphotyrosine antibody coupled to Sepharose. Phosphatidylinositol 3-kinase activity was measured essentially as described by Auger et al. (14).

Scatchard Analysis. A431 cells plated in 24 well dishes were incubated in 300 μl of binding media containing 25 pM [^{125}I]EGF and increasing concentrations of unlabeled EGF. Following incubation for 2 hr on ice, the binding media was aspirated and the cells washed extensively with Hanks Balanced Salt Solution. The cells were hydrolyzed in 1N NaOH, and the hydrolysate counted for ^{125}I in a LKB 1275 Minigamma gamma counter. The data were analyzed using the LIGAND computer program (15).

Results

A431 cells were synchronized by treatment with 4 mM thymidine for 16 hours and then refed with fresh media. At various times following refeeding, the position of the cells in the cell cycle was assessed by fluorescence activated cell sorting. As shown in Figure 1, immediately following treatment with thymidine, the majority of cells were in G_1 (Figure 1b). This is consistent with previous studies which demonstrated that treatment with thymidine causes cells to arrest in late G_1 phase (16). By 3 hours after release (Figure 1c), the cells had progressed into S phase. By 6 hours (Figure 1d), most of the cells were in G_2/M phase. By 12 hours following release, the cells had returned to G_1 (Figure 1f).

To determine whether EGF receptor function varies during the cell cycle, membranes were prepared from cells synchronized to different points in the cell cycle and assayed for their ability to phosphorylate the synthetic peptide, Arg-Arg-Src. As shown in Figure 2, basal receptor tyrosine kinase activity was not significantly different in cells at different points in the cell cycle. However, EGF-stimulated receptor tyrosine kinase activity was decreased by 40% 6 to 9 hours after release from the thymidine block. This corresponds to a point at which the cells are in the G_2/M phase of

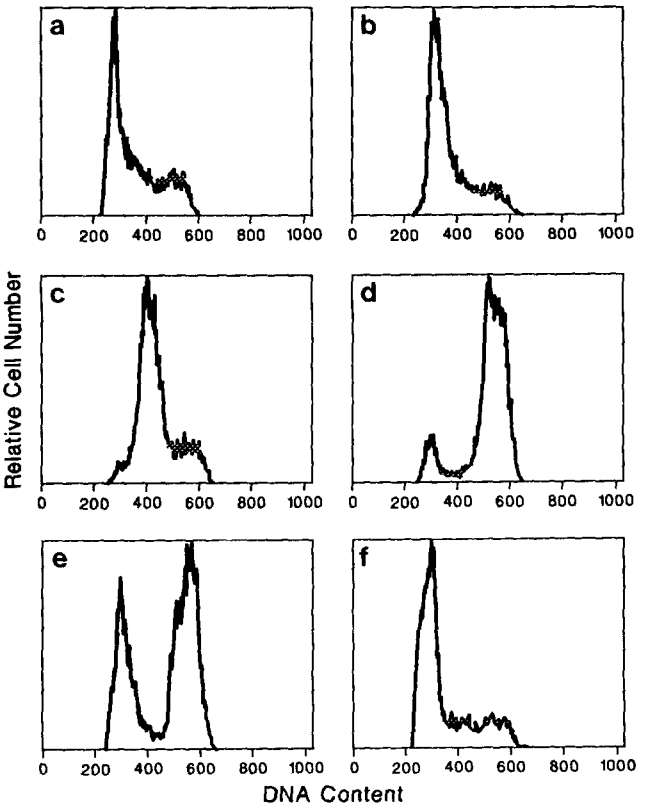

Figure 1. ***Fluorescence Activated Cell Sorting Analysis of Thymidine-Synchronized A431 cells.*** A431 cells were incubated in the presence or absence of 4 mM thymidine for 16 hours, refed with fresh medium, and allowed to continue through the cell cycle. At various times after refeeding, the cells were harvested and prepared for FACS analysis as described in Materials and Methods. Panel a (cycling cells) shows cells that were prepared identically except they were not synchronized by the addition of thymidine. Harvest times after refeeding are as follows: Panel b, 0 h; Panel c, 3 h; Panel d, 6 h; Panel e, 9 h; Panel f, 12 h.

the cell cycle. By 12 to 15 hours after removal of thymidine, when the cells have returned to G_1, EGF-stimulated peptide kinase activity had increased to levels seen immediately following thymidine treatment. These data suggest that the ability of EGF to stimulate receptor tyrosine kinase activity changes as cells progress through the cell cycle.

The observed differences in EGF receptor tyrosine kinase activity during the cell cycle were not due to changes in either total or cell surface expression of EGF receptors. Western blot analyses of lysates and membranes from synchronized cells revealed no difference in total receptor levels throughout the cell cycle (data not shown). To investigate whether cell surface expression of the EGF receptor varied during the cell cycle, Scatchard analyses of [^{125}I]-EGF binding to thymidine synchronized cells were performed. Receptor levels showed a downward trend as the cells progressed through the cell cycle, however, these changes in receptor number did not parallel the observed changes in EGF-stimulated receptor tyrosine kinase activity (Figure 3). For example, 6 hours after removal of thymidine, the ability of EGF to stimulate receptor tyrosine kinase activity was decreased by 40%, whereas EGF receptor number was reduced by only 10%. The affinity of

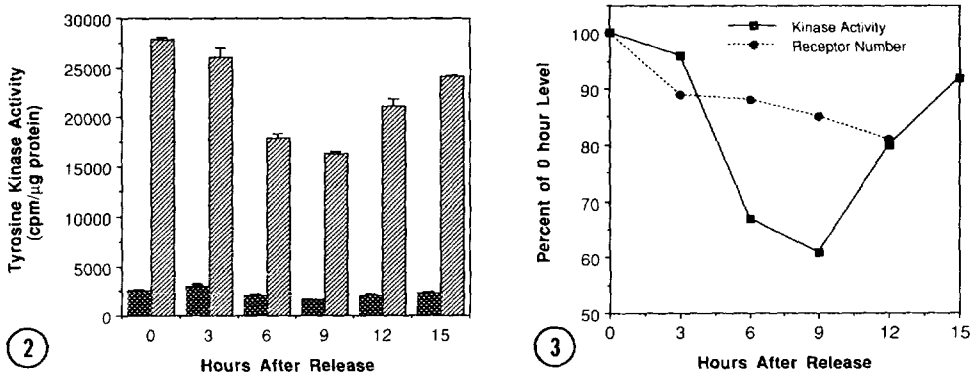

Figure 2. ***Receptor Tyrosine Kinase Activity of Thymidine-Synchronized Cells.*** A431 cells were treated with thymidine and released for the indicated lengths of time. The cells were harvested and membranes prepared as described in Materials and Methods. Samples were assayed for the ability to phosphorylate Arg-Arg-Src peptide in the absence (solid bars) or presence (hatched bars) of EGF. Data represent the mean +/- SD of triplicate determinations from a representative experiment.

Figure 3. ***Comparison of EGF Receptor Protein Tyrosine Kinase Activity and EGF Receptor Number in Thymidine-Synchronized Cells.*** Receptor tyrosine kinase assays were performed in the presence of EGF. Results are presented as the mean of triplicate determinations expressed as percent of the activity at 0 hours. Receptor tyrosine kinase activity at 0 hours was 27,786 cpm per µg protein. EGF receptor number was obtained from Scatchard analyses performed on thymidine-synchronized cells, as described in Materials and Methods. Receptor number at 0 hours was 4.1 pmol receptor per mg protein. EGF receptor affinity following thymidine treatment and release was also determined from Scatchard analysis, and values are as follows: 0 h, 3.6 nM; 3 h, 3.2 nM; 6 h, 3.3 nM; 9 h, 2.8 nM; 12 h, 2.6 nM.

the receptor for EGF was similar at all times after release from the thymidine block (see legend to Figure 3). These results suggest that the differences observed in the ability of EGF to stimulate receptor tyrosine kinase activity at different points in the cell cycle are not due to changes in cell surface expression of the EGF receptor.

To further examine the cell cycle-dependence of EGF receptor function, we assessed the ability of EGF to stimulate phosphatidylinositol 3-kinase activity at different points in the cell cycle. As shown in Figure 4A, negligible amounts of phosphatidylinositol 3-kinase activity were immunoprecipitated from unstimulated cells and this basal phosphatidylinositol 3-kinase activity did not change significantly as the cells progressed through the cell cycle. By contrast, EGF-stimulated phosphatidylinositol 3-kinase activity changed markedly during the cell cycle. Immediately after release of cells from the thymidine block, EGF stimulated phosphatidylinositol 3-kinase activity greater than 80-fold. Three hours after release from the thymidine block when the cells were in S phase, EGF stimulation of PI 3-kinase activity was reduced by 60%. As the cells moved through G_2/M and back into G_1 12 hours after release, EGF-stimulated phosphatidylinositol 3-kinase activity returned to the higher levels seen immediately after release from the thymidine block. These data suggest that the ability of EGF to stimulate phosphatidylinositol 3-kinase activity is dependent upon the position of the cells within the cell cycle. Western blot analysis of extracts from thymidine synchronized cells indicated that the level of p85 did not change significantly throughout the cell cycle (Figure 4B) indicating that the differences were not due to changes in the level of phosphatidylinositol 3-kinase expression.

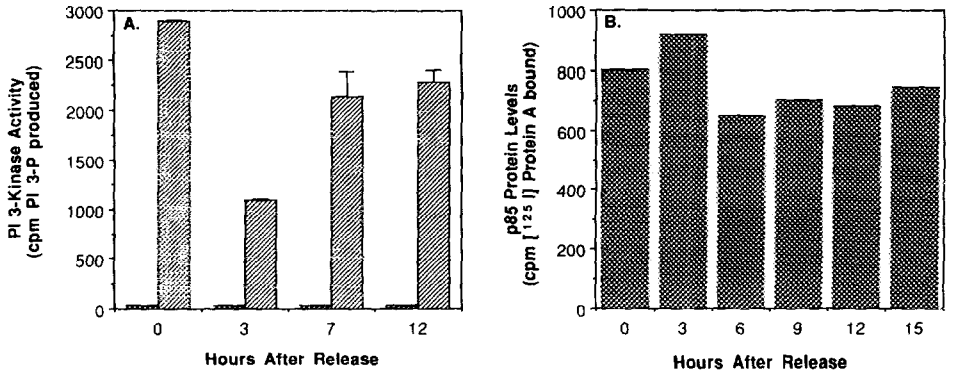

Figure 4. ***Phosphatidylinositol 3-Kinase Activity in Thymidine-Synchronized Cells.*** A. A431 cells were treated with thymidine and released for the indicated lengths of time. Cells were treated with (hatched bars) or without (solid bars) 50 nM EGF for 3 minutes, lysed, and assayed for PI 3-kinase activity as described in Materials and Methods. Results are expressed as mean +/- SD of duplicate determinations. B. Western Blot analysis was performed on extracts prepared from thymidine-synchronized cells using an antibody to the 85 kD subunit of PI 3-kinase. Antibody binding was quantitated with [^{125}I] Protein A. The bands corresponding to p85 were excised and counted for ^{125}I. Data from a representative experiment repeated two times are shown.

Discussion

The data presented here demonstrate that the ability of EGF to stimulate receptor tyrosine kinase activity varies as the cells move through the cell cycle. Cells in late S and G_2/M phase showed a clear decrease in EGF-stimulated receptor tyrosine kinase activity when compared to cells in G_1. Analyses of cell surface EGF receptor number throughout the cell cycle indicated that the changes in receptor tyrosine kinase activity cannot be attributed solely to changes in receptor number. Thus, these data suggest that there are cell cycle-dependent alterations in the EGF receptor itself.

The ability of EGF to stimulate phosphatidylinositol 3-kinase activity was also reduced in a cell cycle-dependent manner. The stimulation of phosphatidylinositol 3-kinase activity by EGF in S phase was 60% less than the stimulation observed during G_1. Interestingly, the decrease in EGF-stimulated phosphatidylinositol 3-kinase activity occurred slightly earlier in the cell cycle than the maximal decrease in receptor tyrosine kinase activity. There are several possible explanations for this observation. First, cell cycle-dependent changes in phosphatidylinositol 3-kinase itself, other than p85 expression, might affect its ability to be stimulated via the EGF receptor. Alternatively, changes in the EGF receptor may occur that are not related to its tyrosine kinase activity but may alter its ability to interact with and activate the phosphatidylinositol 3-kinase.

The observation that cells apparently modulate their responsiveness to EGF as they transit the cell cycle is not surprising. For cells in G_1, mitogenic signals are required for the induction of growth and proliferation. Thus, cells at this point in the cell cycle would be expected to be very responsive to stimulation by growth factors. Once cells have entered S phase, they are committed to undergo mitosis and complete the cell cycle. At this point, mitogenic signals are not required, and therefore growth factors might be expected to induce only a minimal response. Similarly, cells

in mitosis are occupied with cell division, and as a result might be less responsive to growth factor stimulation. Thus, the alteration of responsiveness to EGF at specific points in the cell cycle may directly reflect the cell's need for growth signals at that particular point in the cycle.

Cell cycle-dependent changes in the activities of a number of signaling molecules have been reported. For example, the activity of MAP kinase has been shown to increase in G_1 and M phases(17). In addition, Burg *et al* (18) demonstrated that the protein tyrosine kinase PTK72 could be activated in cells blocked in G_1/S phase but not in cells blocked in G_2/M. The protein tyrosine phosphatase PTP1B was recently shown to be phosphorylated on serine residues during G_2/M and this correlated with a 30% decrease in its tyrosine phosphatase activity (19). The activities of other hormone receptors also appear to be modulated during the cell cycle. Calcitonin was found to stimulate cAMP production during G_2 but not during S phase and the calcitonin receptor was found to couple to a cholera toxin-sensitive G protein during G_2 but to a pertussis toxin- sensitive G protein during S phase (20). Thus, in addition to the EGF receptor and the phosphatidylinositol 3-kinase, a variety of proteins involved in the transmission of growth and regulatory signals show some type of cell cycle-dependent regulation of function. This suggests that linkage of the activity of signaling molecules to the cell cycle is a widespread form of regulation and raises the question of how this linkage is achieved.

We have shown previously that $p34^{cdc2}$ is capable of phosphorylating the EGF receptor *in vitro* and decreasing its tyrosine protein kinase activity (21). The observation that EGF receptor tyrosine kinase activity is decreased in cells in G_2/M, a point at which $p34^{cdc2}$ kinase activity would be high, is consistent with the possibility that EGF receptor function *in vivo* is modulated by this or a related kinase.

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