

Mitosis-specific Negative Regulation of Epidermal Growth Factor Receptor, Triggered by a Decrease in Ligand Binding and Dimerization, Can Be Overcome by Overexpression of Receptor*

(Received for publication, January 8, 1997, and in revised form, April 29, 1997)

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The function of epidermal growth factor receptor (EGFR) was found to be negatively regulated in M phase in which it showed less phosphotyrosine content and reduced intrinsic kinase activity accompanied by retarded electrophoretic mobility owing to total hyperphosphorylation. Ligand-induced autophosphorylation and downstream signaling of EGFR were tightly suppressed in M phase due to a decrease in ligand binding affinity and the inability of epidermal growth factor (EGF) to induce receptor dimerization. There was no change in the number of surface-exposed EGF receptors between G₀/G₁ and M phases of the cell cycle. Hyperphosphorylation (due to serine and/or threonine phosphorylation) correlates with the unresponsiveness of cells to EGF-mediated stimulation of tyrosine phosphorylation in cells that express the normal or basal level of EGFR. This M phase-specific negative regulation was overcome by overexpression of EGFR, which was responsive to ligand throughout the cell cycle and revealed ligand-induced signaling in the M phase. These findings indicate that EGFR does not respond to ligand stimulation in M phase and suggest that a negative regulation of ligand-receptor interactions in M phase may control the normal function of receptor tyrosine kinase and that receptor overexpression will disrupt this cell cycle-dependent regulation of receptor tyrosine kinases.

breast (4), and ovary (5), strongly suggesting that the receptor tyrosine kinase plays a role in their progression. In addition, many cases of EGFR overexpression in cancer cells are accompanied by the expression of transforming growth factor α , a ligand for EGFR, suggesting the involvement of an autocrine activation mechanism in oncogenesis (6, 7). Second, the potent transforming ability of EGFR is confirmed by *in vitro* or *in vivo* experiments. For example, it was reported that both overexpression of EGFR (8, 9) and induction of a deletion mutant of EGFR (10, 11) conferred an EGF-dependent and an EGF-independent transformed phenotype on avian and mouse fibroblasts, respectively. Indeed, *v-erbB* oncogene encoding a truncated form of chicken EGFR led to erythroleukemia and fibrosarcomas in chicks (12–14). These reports have shown that enhancement of the mitogenic signal from receptor tyrosine kinase is sufficient to induce the progression of cancer. Although many mitogenic signal transduction cascades involving receptor tyrosine kinases have been defined (15, 16), the downstream mechanism of carcinogenesis still remains unclear.

On the other hand, a number of reports have suggested that malfunction of the cell cycle control mechanism is directly involved in carcinogenesis. For example, it is well documented that an essential function of the protein products of tumor suppressor genes, such as the retinoblastoma gene and the *p53* gene, whose abnormalities result in cancer progression, is to keep the cells in G₀ phase (17). Recently it has been shown that the cyclin-dependent kinase (cdk) inhibitors, p21 and p16 (which block the cell cycle progression at the check point of G₀ to G₁ or G₁ to S by inhibiting cdk activity), function as tumor suppressors (18–21). The above findings indicate that the normal cell cycle control mechanism is important for the prevention of cancer progression. Considering the fact that an essential function of receptor tyrosine kinases is to promote the progression of the cell cycle, carcinogenesis may be regarded as a result of disrupted cell cycle regulation. Because receptor tyrosine kinases are believed to promote the transition of cells from the resting state to cell division cycle, most cell cycle-related studies involving receptor tyrosine kinases have focused on the cell's entry into the cell cycle. Even though the structures and activities of numerous growth-related molecules, such as Abl (22), Src (23), p53 (24), and retinoblastoma gene product (25), have been shown to be regulated throughout the cell cycle, how receptor tyrosine kinases affect the remaining cell cycle stages or how they are regulated throughout the entire cell cycle has not been well studied.

In an attempt to understand the detailed mechanism of oncogenesis induced by enhanced tyrosine kinase activity, we investigated the relationship between the receptor tyrosine kinases and the entire cell cycle. Recently, we have reported

The epidermal growth factor receptor (EGFR)¹ (1) is a member of the *c-erbB* family of growth factor receptors possessing an intrinsic protein tyrosine kinase activity. There are several lines of evidence that implicate the enhanced activity of this growth factor receptor with the progression of cancer. First, numerous clinically related studies reported overexpression of EGFR in various human cancers such as bladder (2), brain (3),

* This work was supported by National Institutes of Health Grants CA58880 and CA60856, by U. S. Dept. of Defense Grant DAMD17-94-J-4315 (to M. C. H.), and by Cancer Center Core Grant CA16672. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a fellowship from the Sankyo Foundation of Life Science (Japan).

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¹ The abbreviations used are: EGFR, epidermal growth factor receptor; BS³, bis(sulfosuccinimidyl) suberate; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FCS, fetal calf serum.

that the structure and tyrosine kinase activity of another member of the EGFR family, ErbB-2, a protein product (185 kDa) of the *neu* or *c-erbB-2/Her 2* proto-oncogene (26–28), is regulated in a cell cycle-specific manner, and the disruption of this regulation is suggested to be involved in cellular transformation (29). The next question we asked was about the relationship between the cell cycle-dependent regulation of receptor tyrosine kinases and ligand-induced signaling. Since a direct ligand for ErbB-2 is still unknown, despite the reports of several candidates (30–33), we have investigated the structural and functional changes of another member of this receptor tyrosine kinase family, the EGFR tyrosine kinase, and its relation to ligand stimulation during the cell cycle.

In this report, we describe that in addition to the regulation of intrinsic kinase activity, ligand-induced signaling of EGFR is also tightly suppressed in the M phase at its basal level expression. However, although it showed structural modification in a cell cycle-specific manner, the overexpressed EGFR is still highly responsive to the ligand to induce ligand-mediated signaling in the M phase, suggesting that EGFR overexpression may overcome the M phase-specific negative regulation.

MATERIALS AND METHODS

Cell Culture—Cell lines, A431, rat-1, MDA-MB-468, Swiss 3T3 (SW3T3), and HBL-100 were obtained from American Type Culture Collection. Her-5 cells (derived from NR-6 cells by stable transfection with the human EGFR expression vector) were provided by Dr. H.-J. Kung (Case Western Reserve University). NR-6 is a Swiss 3T3 variant that lacks EGF receptors (28, 34). Cells were grown in Dulbecco's modified Eagle's medium mixed with F-12 (1:1; Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified 5% CO₂ atmosphere.

Chemicals and Antibodies—Human recombinant EGF was purchased from Upstate Biotechnology. Aphidicolin, Hoechst 33342, and Protein A-agarose were supplied by Boehringer Mannheim. Nocodazole and thymidine were bought from Sigma. IODO-GEN and BS³ were from Pierce. Radioactive Na¹²⁵I and [γ -³²P]ATP were obtained from Amersham Corp. Monoclonal anti-phosphotyrosine antibody PY20 (Transduction Lab.) and monoclonal anti-PLC- γ antibody (Upstate Biotechnology) were used for immunoblotting. Monoclonal anti-EGFR antibody RP-13 (Amersham Corp.) and rabbit anti-PLC- γ antisera (a gift from Dr. Y. Yarden, The Weizmann Institute of Science) were used for immunoprecipitation. Monoclonal antibody to EGFR (528) was obtained from Neo Markers, CA. Sheep polyclonal anti-EGFR antibody (Upstate Biotechnology) and polyclonal anti-Shc antibody (Transduction Lab.) were used for both immunoprecipitation and immunoblotting. A monoclonal antibody to *cdc2* (Santa Cruz, sc-54) and rabbit anti-mouse immunoglobulin G (Amersham Corp.) were also used.

Cell Synchronization—Cells were chemically synchronized at each cell cycle stage. To arrest cells in the G₀/G₁ phase, cells (50–60% confluent) were serum-starved in DMEM/F12 with 0.5% FCS for 48 h (22). After serum starvation, cells were accumulated at pre-S phase by incubation in DMEM/F12 containing 10% FCS and aphidicolin (5 μ g/ml) for 24 h (35). The cells were then washed with medium and placed in DMEM/F12 containing 10% FCS and Hoechst 33342 (1 μ g/ml) for 24 h to be accumulated in G₂ phase (35). To synchronize cells in M phase, cells were incubated in DMEM/F12 with 10% FCS and nocodazole (0.4 μ g/ml) for 12–24 h. After nocodazole treatment, about 50–90% cells had a highly rounded mitotic morphology and were collected by mechanical shake-off (23). In the case of A431 cells, because of the difficulty of shaking off, 2 \times 10⁶ cells were incubated in DMEM/F12 with 10% FCS and nocodazole in 15-cm plates for 12–24 h, a condition in which more than 80% of the cells had a mitotic shape as determined by microscopy, and then were collected by scraping.

DNA Content Analysis—For DNA content analysis, trypsinized cells were fixed for 30 min at –20 °C in 70% ethanol, 30% phosphate-buffered saline (PBS) mixture. After staining with PBS containing 50 μ g/ml propidium iodide and 8 μ g/ml RNase A, cells were analyzed on an EPICS PROFILE flow cytometer (Coulter) as described (23). The results were analyzed with the MultiCycle computer program (Phoenix Flow System). For some experiments, rat-1 cells were blocked in G₁ phase with 4 mM thymidine in serum-free medium for 16 h, and the block was released by removing the drug (washing) and supplementing the medium with 10% FCS. After 9–12 h, changes in EGFR were

followed in cells (collected by a shake-off procedure) by DNA content analysis and/or electrophoresis.

Immunoblotting and Immunoprecipitation—After each treatment, cells were solubilized for 30 min on ice in lysis buffer (20 mM Na₂PO₄, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 100 mM NaF, and 2 mM Na₃VO₄), and the total protein concentration was determined using Bio-Rad protein assay kit. Electrophoretically separated proteins were transferred to a nitrocellulose membrane for 1 h at 3 mA/cm² using a semi-dry transblot system (Bio-Rad). After blocking with PBS containing 5% milk, membranes were incubated with primary antibodies for 1 h at room temperature in 3% bovine serum albumin/PBS followed by washing with 0.05% Tween 20 in PBS. After 30 min incubation with an appropriate secondary antibody conjugated with horseradish peroxidase and a subsequent washing with PBS-0.05% Tween 20, immunoblots were developed by the enhanced chemiluminescence reagent (Amersham Corp.) for 1 min and exposed to film (Hyper-film, Amersham Corp.).

For immunoprecipitations, 500 μ g to 1 mg of cell lysates were incubated with 1 μ g of antibody and 50 μ l of 50% protein-A agarose for 1 h. After several washes with 0.05% Tween 20/PBS, immunoprecipitates were separated by electrophoresis and analyzed.

In Vitro Dephosphorylation of EGFR—EGFR proteins were immunoprecipitated from 100 μ g of Her-5 cell lysates arrested in the G₀/G₁ or M phase as described above. After washing three times with PBS, the immune complexes on agarose beads were incubated in 100 μ l of 50 mM Tris-HCl, pH 9.2, and 5 mM MgCl₂ for 30 min at 30 °C in the absence or presence of 20 units of calf intestinal alkaline phosphatase (New England Biolabs). After washing, the proteins were eluted into gel sample loading buffer and immunoblotted with anti-EGFR polyclonal antibody after electrophoresis.

Radiolabeling—The IODO-GEN method (Pierce) was used to radioiodinate the proteins as follows. Human recombinant EGF (5 μ g) or rabbit anti-mouse immunoglobulin G (50 μ g) in PBS was mixed in an IODO-GEN-coated (~1 μ g/tube dissolved in chloroform and air-dried) tube with Na¹²⁵I (1 mCi). Following 10 min at 23 °C, tyrosine was added to a final concentration of 0.1 mg/ml, and the mixture was separated on a column of Excellulose GF-5 (Pierce). The specific activity varied between 2 and 5 \times 10⁵ cpm/ng.

Binding and Scatchard Analyses—Monolayers of cells in 24-well dishes were washed once with binding buffer (DMEM containing 0.1% bovine serum albumin and 20 mM HEPES). For ligand binding analysis, cells were incubated with 5 ng/ml ¹²⁵I-EGF in the same buffer, and unlabeled ligand, at different concentrations, was co-incubated with the radiolabeled ligand for 2 h at 4 °C. For Scatchard analysis, cells were incubated with different concentrations of ¹²⁵I-EGF for 2 h at 4 °C. The cells were then washed three times with ice-cold binding buffer and lysed in 0.5 ml of 0.1 N NaOH containing 0.1% SDS for 15 min at 37 °C, and the radioactivity was determined by using a γ -counter. Nonspecific binding was determined by the addition of 1 μ g/ml unlabeled EGF together with labeled EGF under the above incubation conditions. For some experiments, a mouse monoclonal antibody to EGFR or a control antibody was first used to bind the cells at 4 °C for 90 min, washed, and a radiolabeled second antibody against the mouse immunoglobulin G was used in binding.

Chemical Cross-linking Analysis—Cells synchronized in the G₀/G₁ or M phase were stimulated with or without EGF, collected in PBS by scraping, and then centrifuged. For some experiments cells were incubated with ¹²⁵I-EGF (10 ng/ml) at 4 °C for 2 h. After washing with PBS twice, cells were incubated with 1 mM bis(sulfosuccinimidyl) suberate (BS³) in PBS for 30 min at 4 °C with rocking. The cells were washed with buffer containing 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl and solubilized with PI/RIPA buffer (36).

In Vitro Immune Complex Kinase Assay—EGFR proteins from cell lysates were immunoprecipitated on protein-A agarose beads as described above. After washing four times, once with PBS, twice with 50 mM Tris-HCl, pH 7.5, 0.5 M LiCl, and once with kinase assay buffer (50 mM Tris-HCl, pH 7.5, 10 mM MnCl₂), immunoprecipitates were incubated in 50 μ l of kinase buffer for 20 min at room temperature with 10 μ Ci of [γ -³²P]ATP (specific activity >3000 Ci/mM, Amersham Corp.) and 10 μ g of enolase (37) as an exogenous substrate. Reactions were stopped by adding 10 μ l of 6 \times SDS-polyacrylamide gel sample loading buffer. After separation on a 7% SDS-polyacrylamide gel and drying, the phosphorylated proteins were visualized by autoradiography.

Phosphoamino Acid Analysis—After *in vitro* immune complex kinase assay, EGF receptor bands were excised and extracted from the gel followed by hydrolyzation in 6 N constant boiling HCl for 60 min at 110 °C as described previously (38). After drying, hydrolysates were

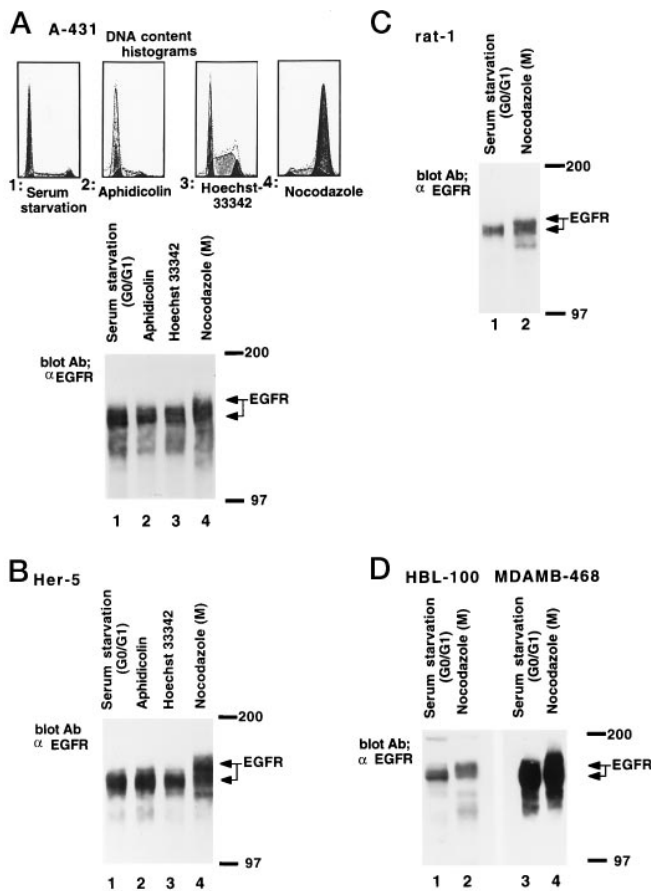


FIG. 1. DNA content analysis and changes in electrophoretic mobility of EGFR during cell cycle. *A*: upper panel, DNA content analysis of A431 cells. Cells were treated with the indicated chemicals to synchronize them at each stage of the cell cycle and trypsinized, and the DNA content analysis was done as described under "Materials and Methods." Lower panel, changes in electrophoretic mobility of EGFR in A431 cells. The total cell lysates from A431 cells synchronized at different stages of the cell cycle were analyzed, after electrophoresis, by Western blotting with a polyclonal anti-EGFR antibody (Ab). *B*, Her-5 cells, synchronized at different stages of the cell cycle, were analyzed for EGFR as described under lower panel in *A*. *C*, lysates from rat-1 cells arrested in G₀/G₁ or M phases were analyzed for EGFR as described above. *D*, HBL-100 and MDA-MB-468 cells were arrested in G₀/G₁ or M phases, and their lysates were analyzed for EGFR as described above. The results were confirmed in at least two independent experiments.

separated on thin layer chromatography plates with unlabeled phosphoamino acid standards by two-dimensional electrophoresis and then were exposed to x-ray film. The positions of phosphoamino acids were visualized by ninhydrin staining.

RESULTS

Cell Cycle-dependent Alteration of Structure and Function of EGFR—Since we have reported the altered electrophoretic mobility of ErbB-2 in a cell cycle-specific manner by means of serine and/or threonine phosphorylation with alteration of its kinase activity (29), we first examined whether the EGFR undergoes similar changes during the cell cycle. A human epidermoid carcinoma cell line, A431, known for its overexpression of EGF receptors ($\sim 2 \times 10^6$ /cell), was first chemically arrested in different stages of the cell cycle for DNA content analysis. As shown in the upper panel of Fig. 1A, serum starvation synchronized the cells in G₀/G₁, aphidicolin treatment accumulated the cells in pre-S phase and Hoechst in G₂, and nocodazole arrested them in M phase of the cell cycle as expected. When the EGF receptor protein from the A431 cell lysate was visualized by immunoblotting with anti-EGFR antibody, the 170-kDa EGF receptor band showed a retardation of

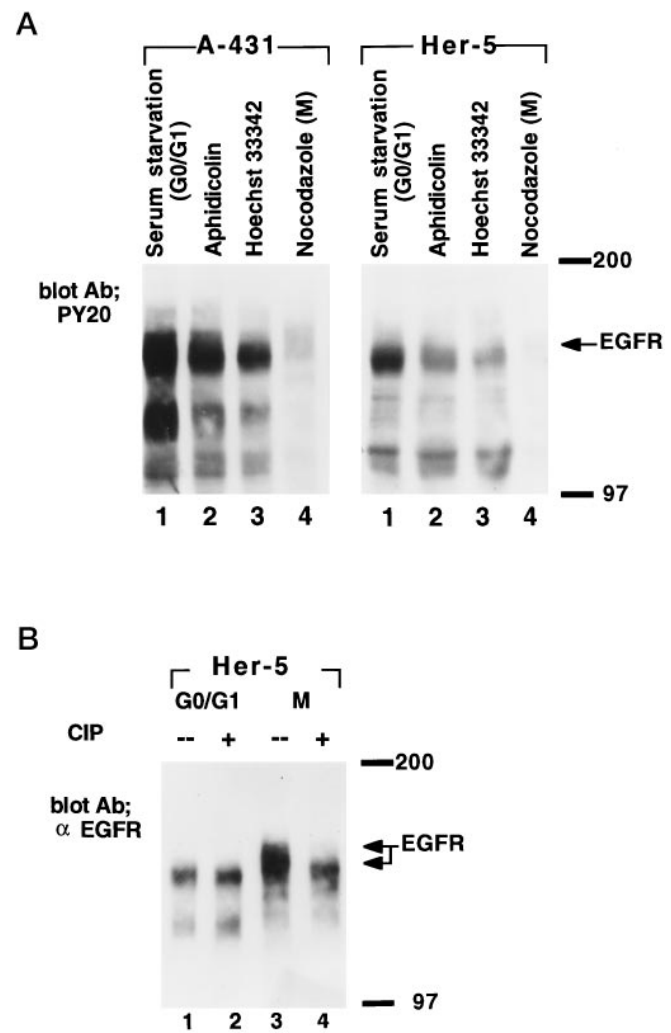


FIG. 2. Changes in tyrosine phosphorylation of EGFR and the effect of dephosphorylation on its electrophoretic mobility during cell cycle. *A*, the same cell lysates from A431 cells and Her-5 cells synchronized at different stages of the cell cycle used in the previous experiment (Fig. 1) were separated on a 6% SDS-polyacrylamide gel and then subjected to immunoblot analysis with a monoclonal anti-phosphotyrosine antibody (Ab). *B*, the EGFR was immunoprecipitated from Her-5 cells arrested in G₀/G₁ or M phases, after treatment with (+, lanes 2 and 4) or without (-, lanes 1 and 3) calf intestinal phosphatase (20 units CIP, New England Biolabs) as described under "Materials and Methods." Immune complexes were separated on a 6% SDS-polyacrylamide gel and analyzed by Western blot analysis using a polyclonal anti-EGFR antibody. Two independent experiments were done to ascertain these results.

its electrophoretic mobility notably in M phase (Fig. 1A) suggestive of cell cycle-dependent structural alterations in the EGF receptor. Similar results were observed in Her-5, a mouse fibroblast cell line that was made to express the human EGF receptor by integrating a human EGFR expressing vector into NR-6, a subline of SW3T3 (Fig. 1B), and in a breast cancer cell line, MDA-MB-468, that overexpresses the EGFR (Fig. 1D). To see whether the electrophoretic mobility changes are related to the level of EGFR expression, rat-1 cells, rat fibroblasts expressing a low level of EGFR (2.8×10^4 receptors/cell), were synchronized at G₀/G₁ and M phase. The electrophoretic mobility was retarded again upon nocodazole treatment (Fig. 1C), and similar results were obtained in HBL-100 (Fig. 1D), a mammary epithelial cell line expressing a basal level of EGFR, indicating that the electrophoretic mobility retardation of the EGF receptor in M phase is independent of its expression level. It is interesting to note that the retardation in electrophoretic

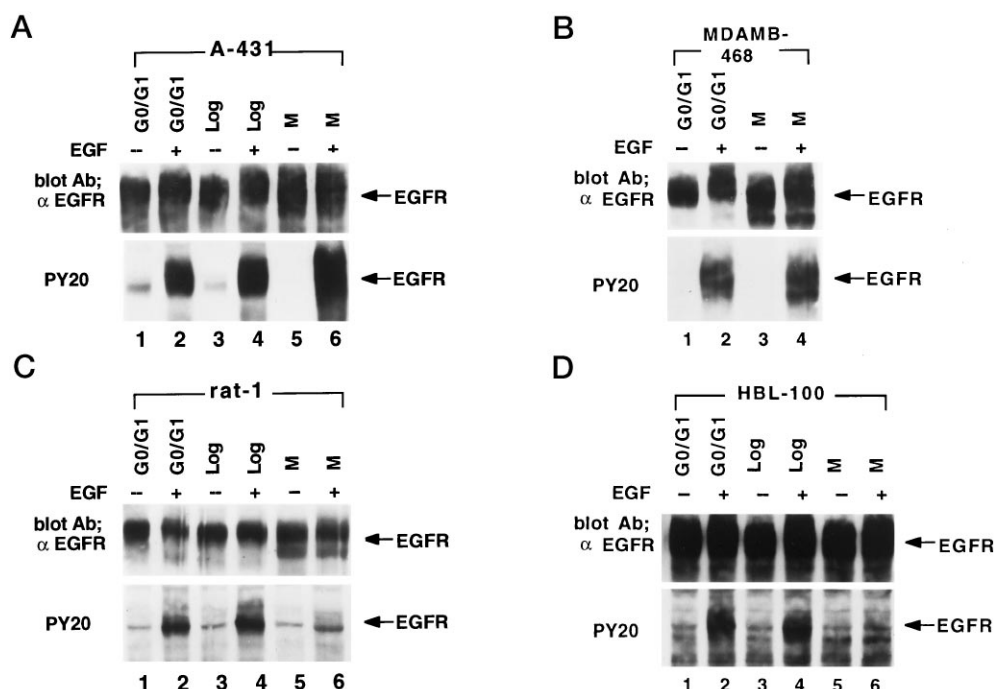


FIG. 3. EGF-induced autophosphorylation of EGFR in G₀/G₁ or M phase. A, A431 cells synchronized at G₀/G₁, logarithmic growth population, and M phase were treated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) EGF (100 ng/ml at 37 °C for 5 min) and were solubilized in lysis buffer. After separation on a 6% SDS-polyacrylamide gel, samples were blotted with either polyclonal anti-EGFR antibody (Ab) (upper panel) or monoclonal anti-phosphotyrosine antibody PY20 (lower panel). MDA-MB-468 cells (B), rat-1 cells (C), and HBL-100 cells (D) were analyzed as in A. Similar results were obtained when these experiments were repeated three times.

mobility of EGF receptor was observed in both the transformed cells (A431) and non-transformed cells (Her-5, rat-1).

To address the question of whether the tyrosine kinase activity of EGFR is also regulated in a cell cycle-specific manner, we next examined for an alteration of phosphotyrosine content in EGFR during the cell cycle which is thought to reflect changes in the gross functional activity of tyrosine kinase. When the protein extracts from A431 cells in different cell cycle stages were examined by immunoblotting with anti-phosphotyrosine antibody, EGFR showed different levels of phosphotyrosine content in a cell cycle-dependent manner: phosphotyrosine levels were highest in the G₀/G₁ phase, decreasing through the S and G₂ phases, and reaching their lowest level in the M phase (Fig. 2A). We also observed similar results with Her-5 cells that overexpress the human EGFR (Fig. 2A). Considering these results together with those of *in vitro* immune complex kinase assay which showed a reduced intrinsic kinase activity of EGFR in the M phase (Fig. 6A, lanes 1 and 3), we concluded that the tyrosine kinase activity of EGFR is regulated in a cell cycle-dependent manner and is least active in the M phase.

To investigate whether these mobility shifts are due to the hyperphosphorylation of the protein which is known to occur in other proteins (23, 25, 29, 39), we next examined the effect of phosphatase on the mobility shift of EGFR. The immunoprecipitated EGFR proteins from Her-5 cells arrested in G₀/G₁ or M phase were treated with calf intestinal alkaline phosphatase to remove all phosphates before analysis, and the mobility retardation in the M phase was no longer observed (Fig. 2B). These results confirm that this retardation in mobility is a result of overall hyperphosphorylation of EGFR.

All the above data indicate that EGFR undergoes a structural modification that is caused by altered phosphorylation of the receptor protein in a cell cycle-specific manner and that this modification is related to functional alteration, suggesting that the structure and function of EGFR are regulated in a cell

cycle-specific manner similar to ErbB-2. Our data also showed that this phenomenon is independent of the level of EGFR expression or transformation status of the cell line.

M Phase-specific Suppression of Ligand-induced Signal Transduction of EGFR at Basal Level Expression but Not at High Level Expression—We next asked whether this cell cycle-dependent regulation of EGFR had any effect on ligand-receptor interaction. To address this question we compared EGF-induced tyrosine autophosphorylation of EGFR in G₀/G₁ phase with that in the M phase. When the A431 cells were analyzed (Fig. 3A), EGFR showed significant EGF-induced tyrosine autophosphorylation in both phases (G₀/G₁ and M). It may be noted that in Fig. 3A, we intentionally used a short exposure time to show quantitative differences in the phosphorylation status between unstimulated and EGF-stimulated A431 cells although the unstimulated cells exhibited constitutive phosphorylation (Fig. 2A). We also checked the response of EGFR to transforming growth factor α in the A431 cells and observed similar results (data not shown). Similar results were obtained in a breast cancer cell line, MDA-MB-468, that overexpresses the EGFR (Fig. 3B). When the tyrosine phosphorylation level of EGF-stimulated EGFR on rat-1 cells synchronized in either G₀/G₁ or M phase was tested by immunoblotting (Fig. 3C), we observed significant EGF-induced tyrosine autophosphorylation on EGFR in both the G₀/G₁ phase and the asynchronous logarithmic growth population, but it was virtually undetected in the M phase. Similarly, EGF could not stimulate the tyrosine phosphorylation in M phase in HBL-100 (Fig. 3D), a mammary epithelial cell line expressing a basal level of EGFR. It is apparent from the above data that the ligand-induced autophosphorylation of EGFR is tightly suppressed in the M phase when it is expressed at basal level (rat-1 and HBL-100), but overexpressed EGFR (A431 and MDA-MB-468) can still respond to its ligands in the M phase. However, the different genetic backgrounds of the cell lines tested make it difficult to compare and interpret that way. To test our results in cells of

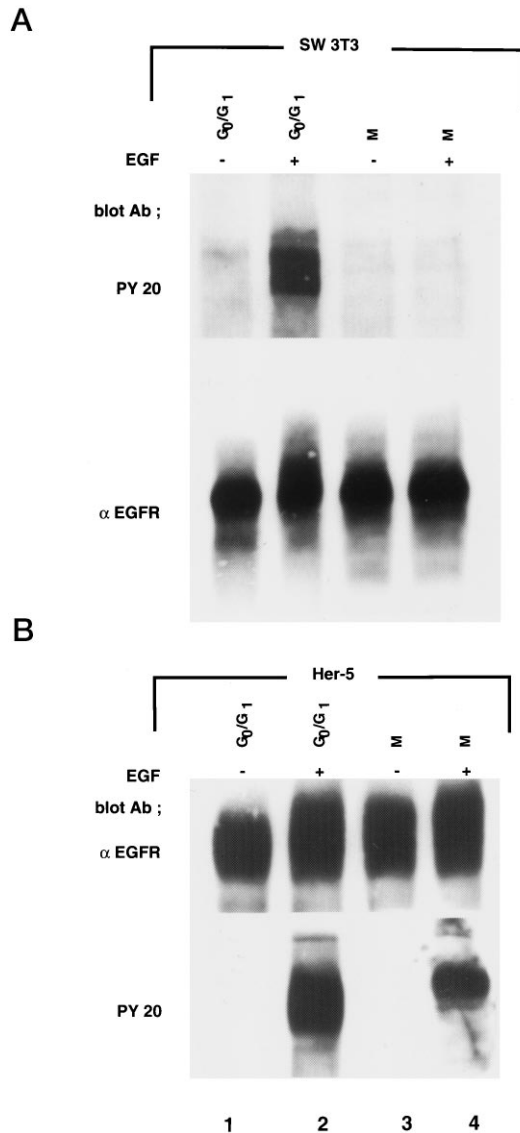


FIG. 4. Effect of overexpression of EGFR on EGF-induced autophosphorylation in G₀/G₁ or M phase. Parental SW3T3 cells (A) and the EGFR-transfected NR6 cells (Her-5) (B) were analyzed under the same experimental conditions described in Fig. 3. These results were reproduced in another independent experiment. Ab, antibody.

the same genetic background, we used SW3T3 (Swiss 3T3) cells expressing low levels of EGFR and Her-5 cells (derived from NR-6 cells by stable transfection with the human EGFR expression vector since NR-6 is a Swiss 3T3 variant that lacks EGF receptors), and the comparative results are shown in Fig. 4. As we predicted, only the EGFR-transfected Her-5 cells showed EGF-induced autophosphorylation of EGFR in M phase although the extent was less than that in the G₀/G₁ phase (Fig. 4B), whereas the SW3T3 cells can be stimulated with EGF only in G₀/G₁ phase but not in M phase (Fig. 4A). This gives further credibility to our interpretation that EGF can induce tyrosine phosphorylation of EGFR in M phase in cells that overexpress it. Since our results in M phase were based upon those obtained from the nocodazole-treated cells, we used other approaches to correlate the cell cycle stage and the EGF-induced autophosphorylation of EGFR. First, the rat-1 cells were treated with nocodazole, and the metaphase cells were collected by a shake-off procedure, which is a standard procedure used to separate the cells in metaphase from G₂ phase cells (23). The results shown in Fig. 5 demonstrate that the EGF-induced tyrosine phosphorylation was minimal in the shake-off cells (*i.e.* M-

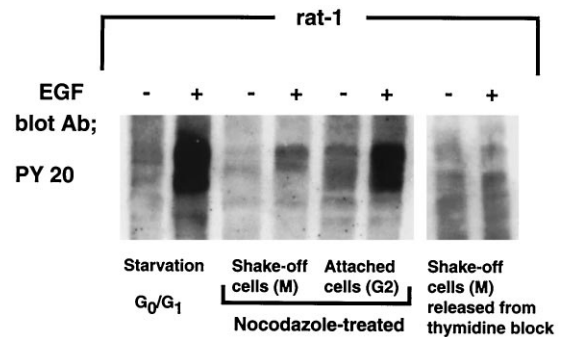


FIG. 5. EGF-mediated response in nocodazole-treated rat-1 cells or those released from a thymidine block. The cells (rat-1) that underwent serum starvation (G₀/G₁) or nocodazole treatment (attached cells or the shake-off cells) or the shake-off cells collected after a 10-h release from a thymidine block were treated with (+) or without (-) EGF under the same conditions described for Fig. 3. The experiment was repeated two times. Ab, antibody.

phase cells), but the attached cells (G₂ phase) did respond strongly to EGF (in spite of being treated with nocodazole), clearly suggesting that the effects we observed in the shake-off cells were not due to the nocodazole treatment *per se* but due to their cell cycle stage. A similar approach was used by us earlier for ErbB-2 (29). Second, we tested the EGF-mediated response by an alternative procedure to synchronize rat-1 cells in M phase after a 10-h release from a G₁ block using 4 mM thymidine (16 h). The results from the Western blot using an anti-phosphotyrosine antibody indicated an undetectable response to EGF in M phase (Fig. 5). These results are similar to the minimal response obtained from the nocodazole-treated shake-off cells (Fig. 5) and suggest that the EGF-induced tyrosine phosphorylation of EGFR is undetectable or minimal in M phase in cells that express basal level of EGFR as confirmed by the two different approaches used. Western blot of the same membrane using an anti-EGFR antibody detected no change in the EGFR levels in the presence or absence of EGF (data not shown).

EGF Can Enhance Intrinsic Kinase Activity as Well as Induction of Transphosphorylation of Substrates Both in the G₀/G₁ Phase and in the M Phase in A431 Cells—Next, we examined whether the autophosphorylation of EGFR induced by EGF in the M phase in A431 cells has any effect on the intrinsic kinase activity of EGFR. When the intrinsic kinase activity of EGFR from A431 cells stimulated with EGF in culture was examined by *in vitro* immune complex kinase assay, it showed significant increase of both autophosphorylation and transphosphorylation activity in the M phase as well as in the G₀/G₁ phase when compared with unstimulated controls (Fig. 6A, lanes 1–4). We also tested the cell lysates from Her-5 cells and observed similar findings as in A431 cells (data not shown). On the other hand, when rat-1 cells were examined by the same procedure, EGF-induced enhancement of intrinsic kinase activity of EGFR in the M phase was not significant compared with what we observed in the G₀/G₁ phase (Fig. 6A, lanes 5–8). To confirm that increased phosphorylation accurately reflects enhanced intrinsic tyrosine kinase activity and not serine/threonine kinase activity, we performed phosphoamino acid analysis of the *in vitro* phosphorylated EGFRs shown in Fig. 6B. This result indicates that EGF-induced autophosphorylation of EGFR in the M phase in A431 cells is accompanied by enhanced intrinsic tyrosine kinase activity.

To test whether the EGF-induced autophosphorylation in the M phase was able to be transferred to a downstream signal transduction cascade in A431 cells, EGF-induced transphosphorylation of substrates for EGFR was examined. When the

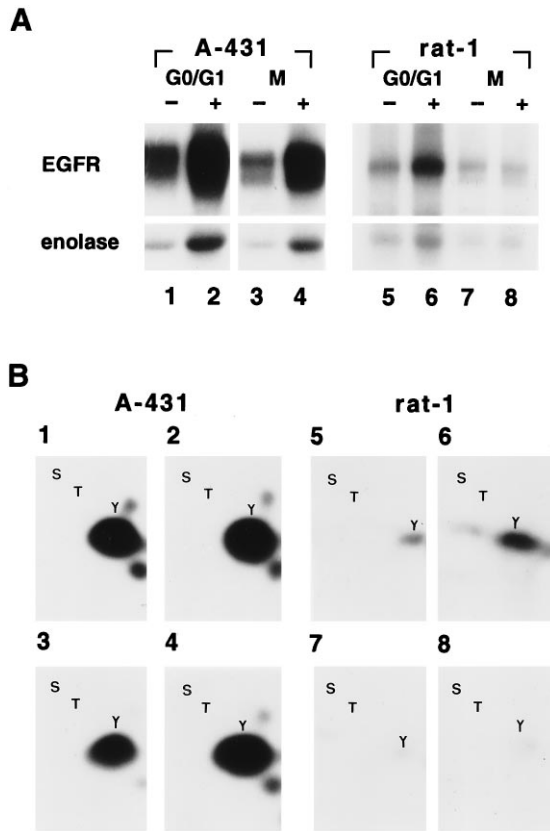


FIG. 6. Effect of EGF stimulation on the intrinsic kinase activity of EGFR in G₀/G₁ or M phase. *A*, after synchronization either in G₀/G₁ (lanes 1 and 2) or M phase (lanes 3 and 4), A431 cells were treated with (lanes 2 and 4) or without (lanes 1 and 3) 100 ng/ml human recombinant EGF (Upstate Biotechnology) for 5 min at 37 °C. After washing and solubilization, EGFR was immunoprecipitated. Using an *in vitro* immune complex kinase assay, EGFR autophosphorylation (upper panel) and transphosphorylation of enolase by EGFR (lower panel) were analyzed. The rat-1 cells were also analyzed by the same procedure described above (lanes 5 and 6, G₀/G₁ phase; lanes 7 and 8, M phase; lanes 5 and 7, without EGF stimulation; lanes 6 and 8, with EGF stimulation). *B*, the *in vitro* phosphorylated EGFR from each experiment in *A* was examined by phosphoamino acid analysis as described under "Materials and Methods" to confirm that the phosphorylation of EGFR of each experiment is mainly due to tyrosine phosphorylation. Each number corresponds to a single lane in *A*. Positions of phosphoserine and phosphothreonine standards are indicated by *S* and *T*, respectively, in panels 1 and 5. *Y* indicates ³²P-labeled phosphotyrosine. The experiments described under *A* and *B* were repeated at least twice.

phosphotyrosine content of immunoprecipitated PLC- γ , an *in vivo* substrate for EGFR, from A431 cells in the G₀/G₁ and M phases with or without EGF stimulation were tested by immunoblotting, it was shown that EGF was able to induce the tyrosine phosphorylation of PLC- γ and to allow its binding to EGFR in both phases (Fig. 7A). Next, the phosphotyrosine-containing proteins from rat-1 cells were immunoprecipitated with anti-phosphotyrosine antibody and then examined by immunoblotting with anti-PLC- γ antibody. We detected PLC- γ only in the G₀/G₁ phase after EGF stimulation (Fig. 7B), suggesting that the EGF-induced tyrosine phosphorylation occurred only in the G₀/G₁ but not in the M phase. We also tested another *in vivo* substrate for EGFR, Shc, which is thought to mediate EGF-induced mitotic signal by a pathway different from that of PLC- γ (Fig. 7C). In addition to the EGF-induced tyrosine phosphorylation, we also observed EGF-induced mobility shift of Shc both in the G₀/G₁ and M phases in A431 cells but only in the G₀/G₁ phase in rat-1 cells (Fig. 7C). When Her-5 cells were tested, results similar to those for A431 cells were observed (data not shown). These results suggest that EGF-

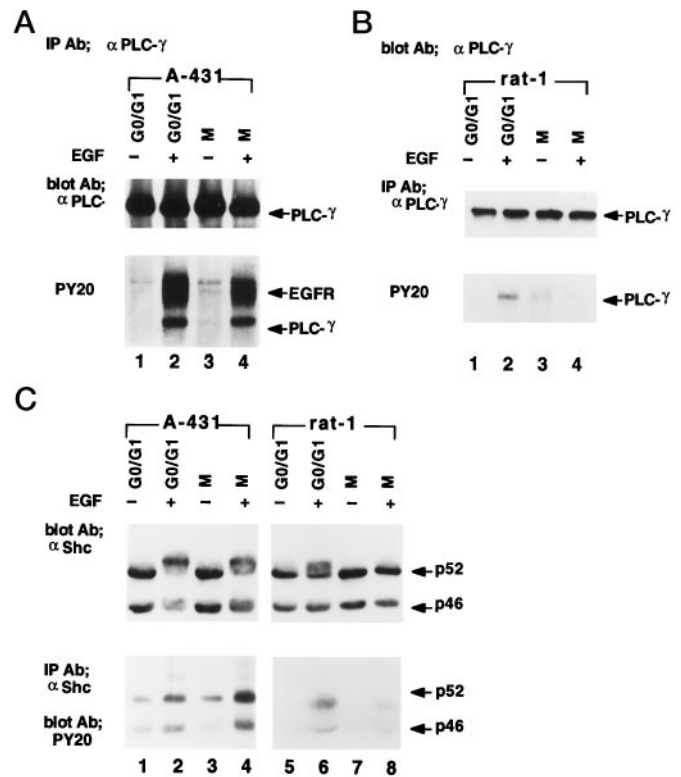


FIG. 7. EGF-mediated signaling in G₀/G₁ and M phase. *A*, A431 cells in G₀/G₁ or M phase treated with (lanes 2 and 4) or without (lanes 1 and 3) EGF were used for immunoprecipitation (IP) with anti-PLC- γ antiserum. After electrophoretic separation and transfer of the lysates, the membrane was blotted with either anti-PLC- γ monoclonal antibody (Ab) (upper panel) or anti-phosphotyrosine antibody PY20 (lower panel). *B*, proteins immunoprecipitated with either anti-PLC- γ serum (upper panel) or monoclonal anti-phosphotyrosine antibody PY20 (lower panel) from rat-1 cell lysates (1 mg of protein) prepared from cells arrested in G₀/G₁ or M phase and treated with (lanes 2 and 4) or without (lanes 1 and 3) EGF stimulation underwent electrophoresis and subsequent immunoblotting with anti-PLC- γ monoclonal antibody. *C*, 50 μ g of cell lysates used in *A* were separated on a 10% SDS-polyacrylamide gel and examined by immunoblot analysis with anti-Shc polyclonal antibody to see the mobility shift after EGF stimulation (upper panel). The alteration of phosphotyrosine content of Shc proteins was also examined by immunoprecipitation with anti-Shc polyclonal antibody, followed by immunoblotting with anti-phosphotyrosine antibody PY20 (lower panel) as described (*A*). Lanes 1–4, A431 cells; lanes 5–8, rat-1 cells; lanes 1, 3, 5, and 7, without EGF stimulation; and lanes 2, 4, 6, and 8, with EGF stimulation. These results were confirmed in two other independent experiments.

induced autophosphorylation of EGFR in the M phase in A431 cells is able to trigger a downstream signal transduction cascade but that EGF fails to induce a signal through EGFR in the M phase in rat-1 cells.

The Mechanism of Suppression of EGF-induced Autophosphorylation in the M Phase in rat-1 Cells—Our data suggest that there is an M phase-specific negative regulation (failure to enhance tyrosine phosphorylation and signaling in response to ligand stimulation) in cells that have normal or low level of EGFR expression, whereas the cells that overexpress EGFR overcome this regulation and respond to the ligand. Since the ligand-mediated response differed between the cells that do and do not overexpress EGF receptor, it was of interest to study the mechanism of the M phase-specific negative regulation in these cells. To begin with, we followed the ligand-dependent changes in dimerization during cell cycle, and for this purpose the A431 cells were synchronized in the G₀/G₁ or M phase, treated with or without EGF, chemically cross-linked, and then examined by immunoblotting with anti-EGFR antibody. Dimers were detected in G₀/G₁ phase and M phase with EGF

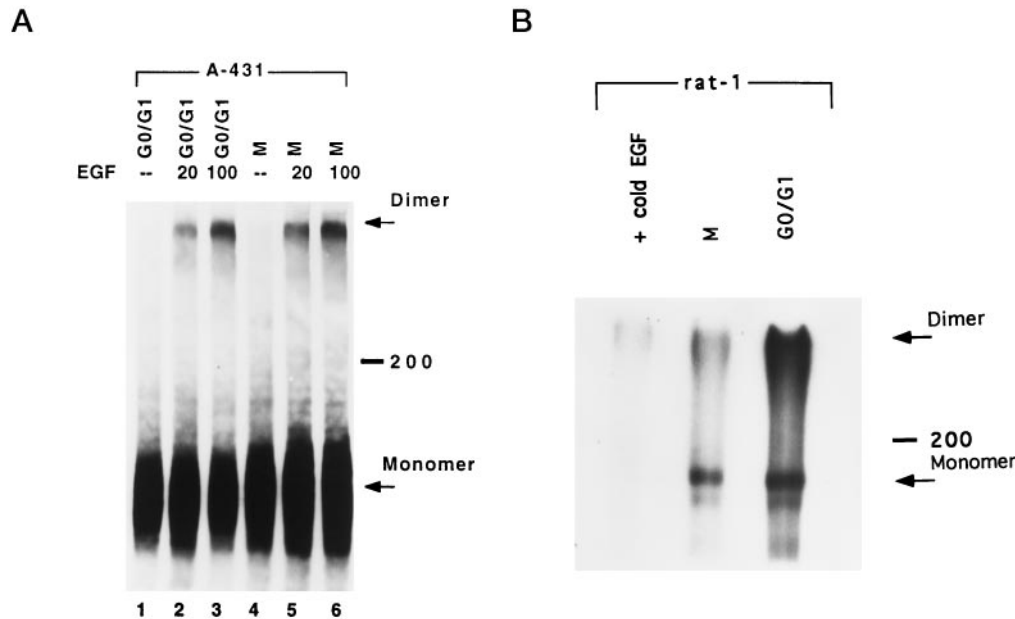


FIG. 8. **Lack of EGF-induced dimerization in M phase in rat-1 cells but not in A431 cells.** A, cross-linking of EGF in G₀/G₁ or M phase in A431 cells. Cells synchronized in G₀/G₁ or M phase were treated with or without EGF. After cross-linking of cell surface proteins with BS³, cells were lysed and separated on a 4.5% SDS-polyacrylamide gel, followed by blotting with polyclonal anti-EGFR antibody. Lanes 1 and 4, without EGF stimulation; lanes 2 and 5, stimulated with 20 ng/ml EGF; and lanes 3 and 6, stimulated with 100 ng/ml EGF. B, cross-linking of ¹²⁵I-EGF in G₀/G₁ or M phase in rat-1 cells. Cells synchronized in G₀/G₁ (1×10^6 cells/well) or M phase (2×10^6 cells/well) were treated with 10 ng/ml ¹²⁵I-EGF in PBS in 6-well tissue culture dishes, incubated at 4 °C for 2 h. Unlabeled EGF (1 μg/ml) was added to one of the wells together with the labeled EGF (+cold EGF). Incubation was continued for 30 min with BS³ (1 mM in PBS), and the cells were lysed and separated on a 5% SDS-polyacrylamide gel, and after drying the gel the protein bands were visualized by autoradiography. These experiments were repeated at least two times with similar results.

stimulation (Fig. 8A), and dimerization increased with EGF stimulation in a dose-dependent manner, and the increase was comparable in both G₀/G₁ phase and M phase (Fig. 8A, lanes 1–6). We used a similar approach in rat-1 cells to cross-link them in the presence or absence of EGF, and the immunoblotting with an anti-EGFR antibody detected the dimers in the presence of EGF in G₀/G₁, but the dimers were not detectable in M phase in the presence or absence of EGF (data not shown). In addition, we used a more sensitive assay to detect the differences in dimerization in G₀/G₁ phase and M phase. Accordingly, ¹²⁵I-EGF was cross-linked to rat-1 cells synchronized in G₀/G₁ or M phase, and the results shown in Fig. 8B indicated a lack of dimerization in M phase, although the dimers could be seen clearly in G₀/G₁ and the bands disappeared in the presence of excess unlabeled EGF indicating the specificity of these results.

Since the decrease in dimerization is likely to be related to changes in ligand binding, it was necessary to ascertain whether EGF binding was affected under these conditions. As expected, when ¹²⁵I-EGF was displaced by different concentrations of unlabeled EGF in A431 cells (Fig. 9A), we did not find differences in binding in either G₀/G₁ or M phase. Consistent with the lack of dimerization in M phase in rat-1 cells treated with EGF, it was found that there is a decrease in ligand binding in M phase compared with the cells in G₀/G₁ phase (Fig. 9C). The difference in binding between G₀/G₁ and M phases observed in rat-1 cells but not in A431 cells could potentially arise from differences on their cell surfaces, in number, and/or existence of EGF receptors. To assess the existence and level of surface-exposed EGF receptors, we employed a binding assay of a monoclonal anti-receptor antibody to A431 and rat-1 cells. The results shown in Fig. 9, B and E, indicated no change in the number of antibody binding sites, which reflects the number of EGF receptors, between G₀/G₁ and M phases in both A431 and rat-1 cells. Similar results were obtained by this assay in SW3T3 and Her-5 cells (data not

shown) suggesting that the number of receptors do not change between G₀/G₁ and M phases, whether the cells express low or high number of EGF receptors and whether they are transformed or not. Furthermore, we performed Scatchard analysis of ¹²⁵I-EGF binding to rat-1 cells, and the results indicate a 4-fold decrease in binding affinity in M phase, and as expected, the number of receptors remained unaltered between G₀/G₁ and M phases (Fig. 9D). These results suggest that inhibition of dimerization and ligand binding affinity is involved in the M phase-specific suppression mechanism of EGF-induced autophosphorylation and transphosphorylation in cells expressing a basal level of EGFR, and this suppression is overcome by overexpression of EGFR.

DISCUSSION

Our study demonstrated that EGF receptor undergoes structural and functional alterations in different phases of the cell cycle. The salient features of our results suggest that EGF-induced autophosphorylation and downstream signaling of the EGFR are tightly suppressed in M phase due to a decrease in ligand binding affinity and the inability of EGF to induce receptor dimerization in cell lines expressing a low or normal level of EGFR. However, overexpression of EGFR apparently reverses this negative regulation.

Structural and Functional Changes in EGFR during Cell Cycle Are Influenced by Serine and/or Threonine Phosphorylation—The structural modifications of EGFR could be inferred by the observed retardation of its electrophoretic mobility with cell cycle progression (Fig. 1). Differential phosphorylation is known to shift the electrophoretic mobility of the non-receptor tyrosine kinases, Src and Abl, showing retarded migration in M phase (22, 23). The structural changes in ErbB-2 tyrosine kinase in M phase reported earlier by us are similar to the present results (29). The retardation of electrophoretic mobility in EGFR in M phase is due to changes in the phosphorylation status of the receptor as the retardation was no longer observed

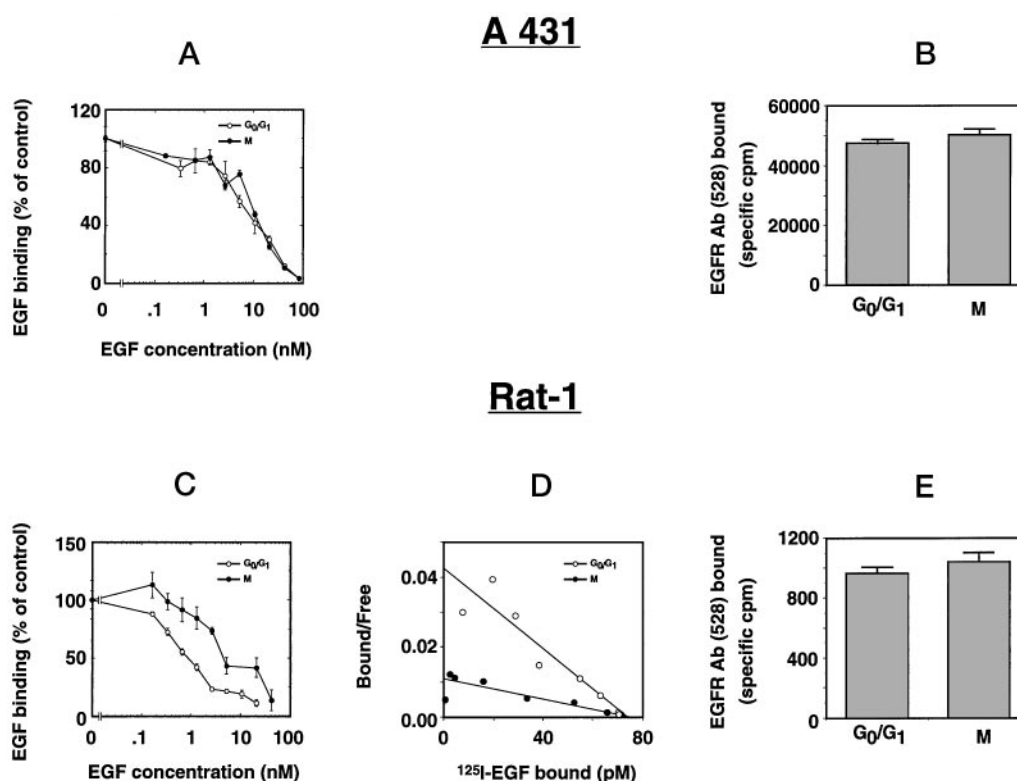


FIG. 9. Binding of EGF or an EGFR antibody to cells in G₀/G₁ or M phase. **A**, binding of EGF to A431 cells synchronized in G₀/G₁ or M phase. The cells synchronized in G₀/G₁ or M phase were incubated at 4 °C for 2 h with 5 ng/ml ¹²⁵I-EGF in binding buffer in the presence of increasing concentrations of unlabeled EGF. After washing, the cells were solubilized in 0.1% NaOH/SDS, and the radioactivity was counted in a γ -counter, and the nonspecific binding was subtracted from the total binding. **B**, surface level of EGF receptors on A431 cells synchronized in G₀/G₁ or M phase. Equal number of cells (1×10^4 cells/well in 24-well dishes) synchronized in G₀/G₁ or M phase were incubated at 4 °C for 90 min in binding buffer containing 5 μ g/ml mouse monoclonal antibody (Ab) to EGFR-(528). After washing, the amount of bound antibody reflecting the level of surface-exposed EGF receptors was determined by incubating the cells at 4 °C for 90 min with ¹²⁵I-radiolabeled rabbit antibody to mouse immunoglobulin G and determination of the bound radioactivity. For control, the cells were incubated with an antibody to cdc2, and the background binding was subtracted. **C**, binding of EGF to rat-1 cells synchronized in G₀/G₁ or M phase. The experimental conditions were the same as described in **A**. **D**, Scatchard analysis of ¹²⁵I-EGF binding to rat-1 cells. Cells synchronized in G₀/G₁ or M phase were incubated for 2 h at 4 °C with different concentrations of ¹²⁵I-EGF as described under "Materials and Methods." Means of triplicate determinations are shown, and the results were analyzed by the Scatchard method after subtracting the nonspecific binding determined in the presence of 1 μ g/ml unlabeled EGF. **E**, surface level of EGF receptors on rat-1 cells synchronized in G₀/G₁ or M phase. Equal number of cells synchronized in G₀/G₁ or M phase were incubated with a mouse monoclonal antibody to EGFR-(528) under the same experimental conditions described for **B** and analyzed. Means of triplicate determinations are shown, and the bars represent standard errors. The experiments were repeated two times.

after an *in vitro* dephosphorylation of the receptor using a phosphatase (Fig. 2B). It is most probably due to serine and/or threonine phosphorylation of the receptor since our results do not support the involvement of tyrosine phosphorylation in this phenomenon as there was a declining trend in tyrosine phosphorylation of EGFR with cell cycle progression in cells that overexpress EGFR (Fig. 2A). Recently it has been reported that EGFR undergoes ligand-independent serine/threonine phosphorylation upon entry into the cell cycle (40). Both EGFR and ErbB-2 are reported to be phosphorylated on serine and threonine residues *in vivo* in a serum-dependent manner with accompanying reduction in tyrosine kinase activity without elevation of phosphotyrosine content (37, 41). Protein kinase-C is known to phosphorylate serine and threonine residues on both the EGFR (42, 43) and ErbB-2 (44). It has also been reported that the desensitization of EGFR that occurs rapidly after its binding to EGF can be accounted for, in part, by Ser-1046/7 phosphorylation on the receptor, a substrate for the calmodulin-dependent protein kinase II *in vitro*. (45). Cdc2, a serine/threonine kinase which is a key regulator of several growth-related proteins and is most active in the M phase (46, 47), can phosphorylate serine residues on EGFR and lead to reduction of its tyrosine kinase activity (48, 49). Our data in this report clearly show that EGFR also has a cell cycle-dependent regulatory mechanism, suggesting that such a mech-

anism is common among the receptor tyrosine kinases and is not unique to a particular tyrosine kinase. The suggestion that the function of the receptor tyrosine kinases that promote the cell division cycle are under negative regulation through serine/threonine hyperphosphorylation indicates the existence of a feedback regulation between tyrosine and serine/threonine phosphorylation during cell cycle.

EGF-induced Tyrosine Phosphorylation and Signaling Are Suppressed in M Phase in Cells Expressing a Basal Level of EGF Receptors but Not in Cells That Overexpress Them—A novel part of this study is related to the receptor responsiveness to the ligand being regulated in a cell cycle-specific manner under different levels of EGFR expression. Hyperphosphorylation (due to serine and/or threonine phosphorylation) correlates with the unresponsiveness of cells to EGF-mediated stimulation of tyrosine phosphorylation in cells that express the normal or basal level of EGFR. Our data clearly indicate that the M phase hyperphosphorylated EGFR (in rat-1 and HBL-100 cells) is associated with a decrease in affinity (Fig. 9), dimerization (Fig. 8), tyrosine phosphorylation (Fig. 3), and signaling (Fig. 7). Taken together, the results strongly suggest that hyperphosphorylation is the primary cause of inhibition in the cells that express low or basal level of EGFR. Consistent with our results, many reports indicated the involvement of serine or threonine phosphorylation in the negative regulation

of EGFR resulting in a decrease in ligand-mediated binding affinity and tyrosine phosphorylation. For instance, protein kinase C-mediated serine/threonine phosphorylation of the EGFR resulted in the reduction of both tyrosine kinase activity and ligand binding affinity (30, 50, 51). EGF stimulation is known to induce not only tyrosine phosphorylation but also serine/threonine phosphorylation on EGFR under conditions of desensitization (52).

However, when the cells overexpress EGFR, the receptor somehow escapes this negative regulation in M phase, since ligand binding, dimerization, and the ability to stimulate tyrosine phosphorylation were not very different between G₀/G₁ and M phases in A431 cells (Figs. 3, 8, and 9). Several possibilities exist that may allow the overexpressed receptor to escape from the M phase-specific negative regulation. Perhaps a minor fraction of the receptors that may not be detected by Western blotting (Fig. 3) escapes hyperphosphorylation and thus can respond to ligand stimulation in M phase. Alternatively, all the receptors may be hyperphosphorylated, and the hyperphosphorylated EGFR is still associated with a residual ability to respond to ligand. The residual ability of each receptor to respond to ligand (if put together) can be very high since both dimerization and tyrosine kinase activation of EGFR follow the second order kinetics with respect to EGFR concentration (53). For instance, we would expect 10⁴-fold increase in activity when EGFR concentration is increased 100-fold (A431 cells have ~2 × 10⁶ receptors/cells, whereas rat-1 cells have ~2.8 × 10⁴ receptors/cell). Of course, the real situation could be more complicated than the two possibilities outlined above. The proposed mechanisms provide a plausible interpretation for the escape of EGFR from the negative regulation in M phase under conditions of overexpression. Consistent with our results Newberry and Pike (54) observed no change in the ligand binding affinity of EGF receptors with cell cycle progression in A431 cells. However, contradictory to our results, they reported a decrease in tyrosine kinase activity with increase in time after releasing A431 cells from a thymidine block, and it is not known whether this discrepancy is due to their use of a synthetic substrate to assess the tyrosine kinase activity. Similar to our results showing no changes in the number of surface-exposed EGF receptors on A431 and rat-1 cells (Fig. 9, B and E) between G₀/G₁ and M phases, the number of receptors were the same at all stages of the cell cycle in LIM 1215, a human colon cell line (55). Our results showing that EGF-mediated downstream signaling is enhanced in A431 cells in M phase (Fig. 7) are consistent with an upward trend of phosphatidylinositol 3-kinase activity in later stages of the cell cycle in A431 cells (54). Since both A431 and MDA-MB-468 cells are derived from human cancer tissues, we cannot eliminate the possibility that these cell lines have a specific mechanism that can inhibit phosphorylation of any specific serine and/or threonine residues, which may be important for the negative regulation in the M phase, or that they have another mechanism that can eliminate the negative regulation derived from serine/threonine phosphorylation, independent of EGFR overexpression. However, our finding that Her-5 cells, which are not cancer cells and whose EGFR expression level is intermediate between that of rat-1 cells and that of A431 cells, partially escape from this suppression suggests that overexpression itself may be sufficient to overcome this suppression. Since it has been shown that even in the absence of ligand the dimerization and enhancement of tyrosine kinase activity of ErbB-2 will occur and that such occurrence simply depends on the level of receptor expression (56), we speculate that when the EGF receptors are phosphorylated on serine/threonine residues which may suppress the dimerization, the elevation of the receptor density may

overcome its suppressive effects in the presence of the ligand.

Mitogenic signals are required in the initial stages of the cell cycle (G₁) for the induction of growth and proliferation, and cells at this point would be expected to be very responsive to stimulation by growth factors. Mitogenic signals are not required once the cells have crossed G₁/S transition and are committed to undergo mitosis and complete the cell cycle. At this stage, growth factors are not expected to induce response to that extent seen at the initial stage. Therefore, the observation that cells with normal or low levels of EGFR do not respond to the growth factor in M phase may be a reflection of the changes in cell's need for growth signals at different stages of the cell cycle. However, overexpression of EGFR appears to deregulate this fine balance. Our results also provide a new way to understand how EGFR overexpression may contribute to development of human cancer, *i.e.* EGFR-overexpressing cancer cells can be constitutively activated by ligand stimulation regardless of their cell cycle stage. However, the normal cells would respond to ligand stimulation in a tight, cell cycle-dependent, regulation. Further studies on the detailed mechanism of this regulation may help us to understand better the combined role of receptor tyrosine kinases and their ligands in different cell cycle stages and also the role of receptor overexpression in cellular transformation and/or growth regulation.

Acknowledgments—We are grateful to Dr. H.-J. Kung for the gift of the Her-5 cell line and to Dr. Y. Yarden for the anti-PLC- γ antiserum.

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