Prolonged Induction of $p21^{Cip1/WAF1}$/CDK2/PCNA Complex by Epidermal Growth Factor Receptor Activation Mediates Ligand-induced A431 Cell Growth Inhibition

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Abstract. Proliferation of some cultured human tumor cell lines bearing high numbers of epidermal growth factor (EGF) receptors is paradoxically inhibited by EGF in nanomolar concentrations. In the present study, we have investigated the biochemical mechanism of growth inhibition in A431 human squamous carcinoma cells exposed to exogenous EGF. In parallel, we studied a selected subpopulation, A431-F, which is resistant to EGF-mediated growth inhibition. We observed a marked reduction in cyclin-dependent kinase-2 (CDK2) activity when A431 and A431-F cells were cultured with 20 nM EGF for 4 h. After further continuous exposure of A431 cells to EGF, the CDK2 activity remained at a low level and was accompanied by persistent G1 arrest. In contrast, the early reduced CDK2 activity and G1 accumulation in A431-F cells was only transient. We found that, at early time points (4–8 h), EGF induces $p21^{Cip1/WAF1}$ mRNA and protein expression in both EGF-sensitive A431 cells and EGF-resistant A431-F cells. But only in A431 cells, was $p21^{Cip1/WAF1}$ expression sustained at a significantly increased level for up to 5 d after addition of EGF. Induction of $p21^{Cip1/WAF1}$ by EGF could be inhibited by a specific EGF receptor tyrosine kinase inhibitor, tyrphostin AG1478, suggesting that $p21^{Cip1/WAF1}$ induction was a consequence of receptor tyrosine kinase activation by EGF. We also demonstrated that the increased $p21^{Cip1/WAF1}$ was associated with both CDK2 and proliferating cell nuclear antigen (PCNA). Taken together, our results demonstrate that $p21^{Cip1/WAF1}$ is an important mediator of EGF-induced G1 arrest and growth inhibition in A431 cells.

EPIDERMAL growth factor (EGF)[1] is a potent mitogen for most epithelial tissues. Binding of EGF to specific cell surface receptors stimulates formation of noncovalent homodimers (or oligomers) involving two (or more) EGF receptors, which activates receptor tyrosine kinase and results in transphosphorylation of tyrosine residues on the opposite member of the pair. The tyrosine autophosphorylated EGF receptors exhibit increased binding affinities for the *Src* homology 2 regions of substrate molecules. Substrate binding to receptors is followed by phosphorylation of the substrate molecules on tyrosine residues, which results in a cascade of biochemical steps involved in the mitogenic signal transduction pathways of cells (Carpenter, 1992; Ullrich and Schlessinger, 1990).

While EGF has a stimulatory effect on the proliferation of a wide variety of normal and malignant cells, the growth of some cultured tumor cell lines bearing high levels of EGF receptors was found, paradoxically, to be inhibited by exposure to exogenous EGF (Gill and Lazar, 1981; Barnes, 1982; Kawamoto et al., 1983; Filmus et al., 1985; Davidson et al., 1987; Ennis et al., 1989; Gross et al., 1991). The human epidermoid carcinoma cell line A431 expresses a high number of EGF receptors ($\sim 2 \times 10^6$/cell) and its growth in monolayer culture is inhibited by nanomolar concentrations of EGF that are mitogenic to other cell lines (Gill and Lazar, 1981; Barnes, 1982; Kawamoto et al., 1983; Fan et al., 1993*a*). EGF also inhibits anchorage independent growth of A431 cells in soft agar (Fernandez-Pol et al., 1986). Growth inhibition by EGF can be modified by blockade of EGF receptors with anti-receptor mAb. The concentration of mAb must be carefully titrated, since total receptor blockade by mAb also inhibits cell growth (Sato et al., 1983; Kawamoto et al., 1983, 1984; Ennis et al., 1989; Fan et al., 1993*a*). The biochemical processes by which EGF inhibits cell proliferation remain poorly understood. Low (picomolar) concentrations of EGF can stimulate A431 cell growth in culture (Kawamoto et al., 1984). Furthermore, treatment of athymic

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1. *Abbreviations used in this paper*: EGF, epidermal growth factor; CDK, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; Cip1, CDK-interacting protein 1; WAF1, wild-type p53-activated fragment 1.

mice with EGF enhances the growth of A431 cell xenografts (Ginsburg and Vonderhaar, 1985), suggesting that EGF-mediated inhibition of proliferation may be related to the stimulation of large numbers of receptors in cultured cells.

Recently, studies on the function of cellular proto-oncogenes indicate that the products of many of these genes play a critical role in cell cycle progression (Hunter, 1991; Weinberg, 1991; Hartwell and Kastan, 1994). Mammalian cell cycle traversal and cell division are controlled by the ordered activation of a group of related serine/threonine kinases, known as cyclin-dependent kinases (CDKs). The activities of these kinases are regulated by their levels of phosphorylation (Gu et al., 1993) and by their periodic association with positive regulatory subunits called cyclins (Sherr, 1993). Orderly progression through the cell cycle requires sequential formation of different activated cyclin–CDK complexes at appropriate phases of the mitotic cycle. In vertebrate cells, the predominant cyclin–CDK complexes required for traversal of the cell cycle are cyclin D–CDK4, cyclin E–CDK2, cyclin A–CDK2, and cyclin A/B–CDC2, acting at G1, G1/S, S, and G2/M, respectively (Sherr, 1993). Recently, a new group of negative regulators of the cell cycle, called CDK inhibitors or CDIs, has been discovered in mammalian cells. Among them are $p21^{Cip1/WAF1}$ (variously named CDK-interacting protein 1 [Cip1]\wild-type p53-activated fragment 1 [WAF1]\SDI1\CAP20) (El-Deiry et al., 1993; Gu et al., 1993; Gyuris et al., 1993; Harper et al., 1993; Xiong et al., 1993) and $p27^{KIP1}$ (Polyak et al., 1994*a*,*b*; Toyoshima and Hunter, 1994) that can bind to and inactivate CDK–cyclin complexes in mammalian cells (Sherr, 1993, 1994). $p21^{Cip1/WAF1}$ also binds to proliferating cell nuclear antigen (PCNA) and inhibits in vitro PCNA-dependent DNA replication (Flores-Rozas et al., 1994; Waga et al., 1994), which $p27^{KIP1}$ cannot do (Luo et al., 1995). It has been reported that the CDK and PCNA inhibitory activities of $p21^{Cip1/WAF1}$ are functionally independent. An amino–terminal domain of the $p21^{Cip1/WAF1}$ molecule inhibits cyclin/CDK kinases and a carboxy–terminal domain inhibits PCNA (Chen et al., 1995; Luo et al., 1995).

In the present study, we investigated the biochemical mechanism by which EGF inhibits cell cycle progression in A431 cells. To define the molecular events responsible for EGF-induced growth inhibition, we selected an A431 subpopulation, A431-F, that is resistant to EGF-induced growth inhibition. We provide evidence that $p21^{Cip1/WAF1}$ is induced by EGF and mediates EGF-induced cell cycle arrest and growth inhibition in A431 cells through its association with the cyclin/CDK2 complex and PCNA.

Materials and Methods

Reagents

EGF was obtained from Collaborative Research Inc. (Bedford, MA). Antibodies reactive with human CDK2, CDK4, cyclin E, cyclin A, cyclin D, and PCNA were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibody against $p21^{Cip1/WAF1}$ was from Pharmigen Biotechnology Inc. (San Diego, CA). Antibody against $p27^{KIP1}$ was generated by immunizing a rabbit with a mouse p27-GST fusion protein described previously (Polyak et al., 1994*a*). Anti-phosphotyrosine antibody PY69 was obtained from ICN Biomedicals Inc. (Irvine, CA). Tyrphostin AG1478 was kindly provided by Dr. Alexander Levitzki (Hebrew University of Jerusalem, Israel) and was dissolved in absolute DMSO (Levitzki and Gazit, 1995). All other chemicals were ordered from Sigma Chem. Co. (St. Louis, MO) unless specified elsewhere.

Cells and Cell Culture

A431 human vulvar squamous carcinoma cells have been described previously and are maintained in DMEM/Ham's F12 medium (1:1) with 0.5% fetal bovine serum (Fan et al., 1994). The A431-F cell subpopulation was selected by long-term exposure of A431 cells to a saturating concentration of monoclonal antibody (mAb) 225 (50 nM), which blocks receptor activation by ligand (Fan et al., 1993*a*). After three months of culture, 225 mAb was removed and cells were allowed to grow in the absence of 225 mAb. These selected cells have been shown to express about half the number of EGF receptors compared with parental A431 cells, evidenced by an ^{125}I–EGF receptor saturation binding assay, Western blot analysis using an antibody against the intracellular domain of the EGF receptor, as well as Northern analysis of EGF receptor mRNA expression. A431-F cells proliferate at a reduced rate and also are less sensitive to 225 mAb-mediated growth inhibition, compared with parental A431 cells (data not shown).

Cell Proliferation and Flow Cytometry

The cell proliferation assay was performed in six-well cell culture plates. A431 or A431-F cells were cultured with or without 20 nM EGF for 5 d with one change of growing medium and addition of EGF on day 3. After 5 d of culture, cells were harvested by trypsinization and counted with a Coulter counter.

For the flow cytometric analysis of DNA content, cells were cultured with or without 20 nM EGF for indicated time periods and then were harvested by trypsinization and fixed with 70% ethanol. Cells were stained for DNA by washing and suspending them in 2 ml PBS containing 50 μg propidium iodide/10^6 cells (Sigma) and 100 μg/ml RNase I (Sigma) and incubated for 6 h at room temperature. After filtering cells through 50–70-μm pore size nylon mesh, cell cycle distribution was analyzed by a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Histone H1 Kinase Assay

Cells were treated with EGF for indicated time periods and harvested by trypsinization. Cell lysates were prepared by sonication in HKM buffer (40 mM Hepes-KOH, pH 7.5, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride) or 0.5% NP-40 lysis buffer (described below) at 4°C. Freshly made and centrifuge-cleared cell lysates (200 μg) were subject to immunoprecipitation in 0.5% NP-40 lysis buffer for 1 h at 4°C in the presence of antibodies against CDK2, cyclin E, cyclin A or $p21^{Cip1/WAF1}$, followed by another 1-h incubation with immobilized protein A–agarose beads (Repligen Corp., Cambridge, MA). The beads were washed by centrifugation two times with NP-40 lysis buffer and underwent four additional washes with kinase assay buffer (20 mM Tris-HCL, pH 7.4, 7.5 mM $MgCl_2$, 1 mM dithiothreitol). Phosphorylation of histone H1 was measured by incubating the beads with 50 μl reaction mixture containing 30 μM lithium ATP (Boehringer Mannheim Corp., Indianapolis, IN), 10 μCi ^{32}P-γ-ATP (3,000Ci/mmol; New England Nuclear, Boston, MA), and 2 μg histone H1 (Boehringer Mannheim Corp., Indianapolis, IN) in kinase assay buffer for 30 min at 37°C. The reaction was stopped by boiling the sample in Laemmli SDS sample buffer for 5 min and samples were resolved by 12% SDS-PAGE. The gel was dried and subjected to autoradiography. Radiolabeled histone H1 bands were quantitated with a Phosphoimager and analyzed with ImageQuant software (Molecular Dynamics).

Immunological Analysis (Western Immunoblot and Immunoprecipitation)

Cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 25 μg/ml aprotitin) and sonicated at 4°C. The supernatants were cleared by centrifugation. Protein concentrations of the lysates were measured by the method of Bradford (Bradford, 1976). Equal amounts of protein were subjected to immunoprecipitation in the presence of various antibodies by the method previously described (Fan et al., 1993*b*, 1994). For Western immunoblot, equal amounts of protein were boiled in Laemmli SDS sample buffer, resolved by SDS-PAGE,

transferred to the nitrocellulose, and then probed with different antibodies, using ^{125}I–protein A as label (Fan et al., 1993*a,b*, 1994).

Northern Blot Analysis

Total RNA was prepared from cells by ultracentrifugation of cell homogenates in guanidine thiocyanate over cushions of cesium chloride. 10 μg of total RNA was loaded into each well, electrophoretically separated on 1% agarose/0.7% formaldehyde gel, and then Northern transferred to nitrocellulose by capillary blot. After baking at 80°C in a vacuum oven for 2 h, the blots were prehybridized in 7 mM Tris-HCl buffer, pH 7.4, containing 5% dextran sulfate, 48.5% formamide, 4× SSC, 40 μg/ml sonicated Salmon sperm DNA and 0.8× Deharts for 2 h at room temperature, followed by overnight hybridization at 42°C with an EcoR I-cut 1.0-kb human $p21^{Cip1/WAF1}$ cDNA construct (provided by Dr. Bert Vogelstein at Johns Hopkins University, Baltimore, MD) or with an EcoR I-cut 1.4-kb housekeeping gene GAPDH cDNA. Probes were labeled by random primer DNA synthesis according to the manufacturer's instructions (Pharmacia Biotech Inc., Uppsala, Sweden). The blots were washed three times with 0.1% SDS, 2× SSC at room temperature, followed by two to three washes with 0.1% SDS, 0.1% SSC at 50°C before being subjected to autoradiography.

Results

Effects of EGF on Cell Proliferation and Cell Cycle Arrest in A431 and A431-F Cells

We have shown previously that proliferation of A431 cells was inhibited by addition of nanomolar concentrations of EGF (Fan et al., 1993*a*; Kawamoto et al., 1983; Sato et al., 1983). To investigate the mechanisms by which EGF induces this growth inhibition, we derived an A431 cell subpopulation that was resistant to EGF-induced growth inhibition (see Materials and Methods). The A431 and A431-F cells were used for comparative studies of the effect of EGF upon sensitive and resistant cells with the same genetic backgrounds. Stimulation with exogenous EGF could activate the A431-F cell EGF receptor tyrosine kinase, assayed by receptor tyrosine autophosphorylation, suggesting the presence of functional EGF receptor kinase in these cells (data not shown). However, unlike the parental A431 cells, A431-F cells were found to be resistant to EGF-induced growth inhibition during 5 d of cell culture (Fig. 1 *A*). FACS analysis indicated that shortly after treatment with 20 nM EGF (9 h), both A431 and A431-F cells arrested in G1 phase. However, during prolonged EGF treatment (36 h), A431-F cells were released from G1 block while the parental A431 cells remained in G1 block, resulting in reduced entry into G2/M phase (Fig. 1 *B*).

Inhibition of CDK2 Activity Accompanies EGF-induced Cell Cycle Arrest

The transition from G1 into S phase in eukaryotic cells is governed by the activation of CDK2. We compared the expression and activities of CDK2 and its cyclin partners, cyclins E and A, in cultured A431 and A431-F cells treated with EGF. Fig. 2 *A* demonstrates that the activity of CDK2, assayed by in vitro histone H1 phosphorylation, was markedly inhibited without changing the amount of CDK2 protein (Fig. 3 *A*) after both type of cells were treated with EGF for 4–8 h. The suppression of CDK2 activity was sustained in A431 cells. In contrast, in A431-F cells, we observed only a transient inhibition of CDK2 activity (Figs. 2 *A* and 6 *B*). These results were consistent with FACS analysis data (Fig. 1 *B*) showing that EGF induces persistent G1 arrest in A431 cells, but only temporarily blocks G1 phase in A431-F cells.

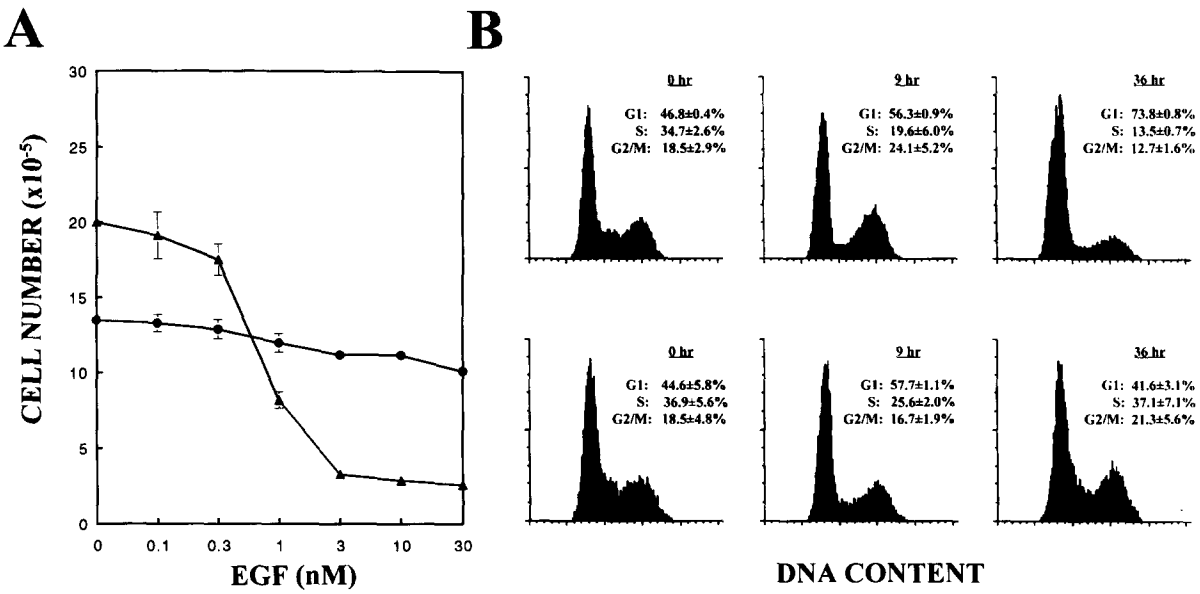

Figure 1. Effects of EGF on cell proliferation and cell cycle distribution in A431 and A431-F cells. (*A*) A431 (▲) and A431-F (●) cells were grown in six-well culture plates at 37°C in the presence of indicated concentrations of EGF. The medium and added EGF were replenished on day 3. Cell numbers were assayed with a Coulter counter after 5 d of culture and are expressed as the means of triplicates with SD (the SD is smaller than the symbol when not shown). The initial cell numbers for A431 and A431-F cells were 2.8 × 10^4/well and 3.4 × 10^4/well in the respective cultures. (*B*) Exponentially growing A431 (*top row*) and A431-F (*bottom row*) cells were treated with 20 nM EGF for 0, 9, or 36 h at 37°C, and their cell cycle distribution was analyzed by flow cytometric analysis of DNA content after staining with propidium iodide.

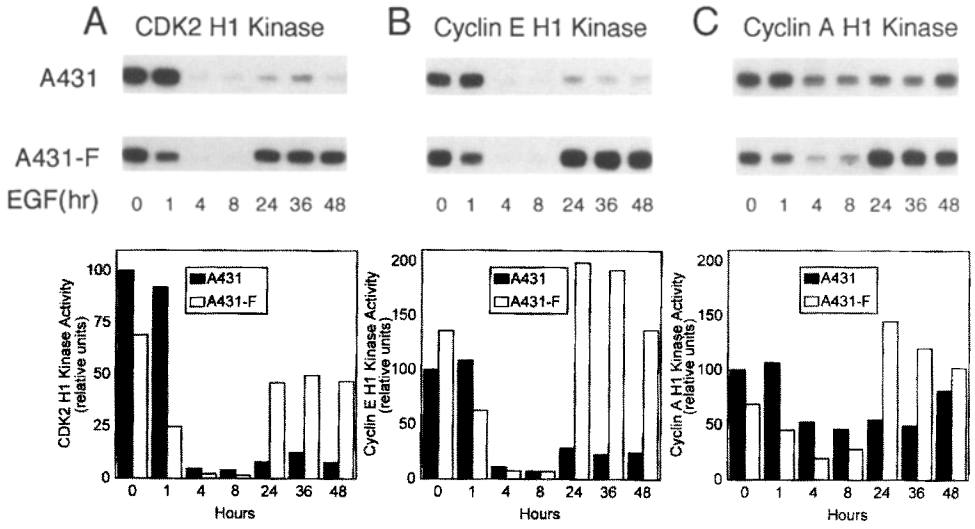

Figure 2. Effects of EGF on the histone H1 kinase activities of CDK2, and cyclin E- or cyclin A-associated kinase in A431 and A431-F cells. Cells were cultured in 20 nM EGF at 37°C for the indicated time periods, and then were harvested and sonicated. CDK2 or cyclin E- or cyclin A-associated histone H1 kinase activities were assayed by immunoprecipitation of cell extracts with antibodies against human CDK2, cyclin E or cyclin A, followed by incubating the immunoprecipitated beads with a reaction solution for the in vitro phosphorylation of histone H1 as described in Materials and Methods. Radiolabeled histone H1 bands were separated by 12% SDS–polyacrylamide gel electrophoresis, quantitated with a Phosphoimager and analyzed with ImageQuant software. The data are plotted in bar graphs.

It has been well-documented that CDK2 regulates the G1 to S phase transition through the formation of complexes with cyclin E and cyclin A. Therefore, we assayed the cyclin E- and cyclin A-associated histone H1 kinase activities after EGF treatment. The cyclin E-associated kinase assay on lysates of A431 and A431-F cells revealed an early marked inhibition after 4–8 h of EGF treatment, similar to that seen in the direct assays of CDK2 activity (Fig. 2 *B*). However, after further treatment of A431-F cells with EGF, the cyclin E-associated histone H1 activity rose to higher than the original levels (Fig. 2 *B*). This probably was due to the transient, partial A431-F cell synchronization in late G1 phase induced by EGF, resulting in accumulation of cyclin E (Fig. 3 *B*).

The cyclin A-associated histone H1 kinase activity also was inhibited by EGF in A431 cells, although less marked than CDK2 kinase activity or cyclin E-associated kinase activity (Fig. 2 *C*). In A431-F cells treated with EGF, inhibition of cyclin A-associated kinase activity was also transient. It should be noted that cyclin A can associate with both CDK2 and CDC2, and that both of these complexes can phosphorylate histone H1.

EGF treatment did not change the amount of either CDK2 or CDK4 protein (Fig. 3 *A*), but was accompanied by fluctuations in the amount of cyclins that did not correlate with the reduction in CDK2 activity. Cyclin E accumulated steadily in A431 cells, starting ~24 h after EGF treatment, but accumulation of cyclin E in A431-F cells was only temporary, declining after further treatment (Fig. 3 *B*). Since cyclin E protein accumulated during EGF treatment, other mechanisms must be invoked to explain the inhibition of cyclin E-associated CDK2 kinase activity in A431 cells and A431-F cells after short term EGF treatment, and the loss of this inhibition in A431-F cells after treatment for 24 h with EGF. In A431 cells, cyclin A levels showed no significant change until a late time point (48 h), when there was a reduction that could not explain the earlier fall in CDK2 activity. In A431-F cells, cyclin A content remained unchanged (Fig. 3 *B*).

Cyclin D3, which is expressed in A431 cells, showed a modest increase after 4 h treatment with EGF and subsequently returned to the original levels in both A431 and A431-F cells (Fig. 3 *B*). At no time did it decrease.

Taken together, these observations suggest that cell cycle arrest and prolonged inhibition of CDK2 activity were not explained by changes in the protein levels of either cyclins or cyclin-dependent kinases in A431 cells. Therefore, we explored whether CDK2 inhibitor(s) were involved in EGF-induced cell cycle arrest in these cells.

Induction of the p21$^{Cip1/WAF1}$ Cell Cycle Inhibitor by EGF Treatment in A431 and A431-F Cells

We examined whether EGF treatment can regulate ex-

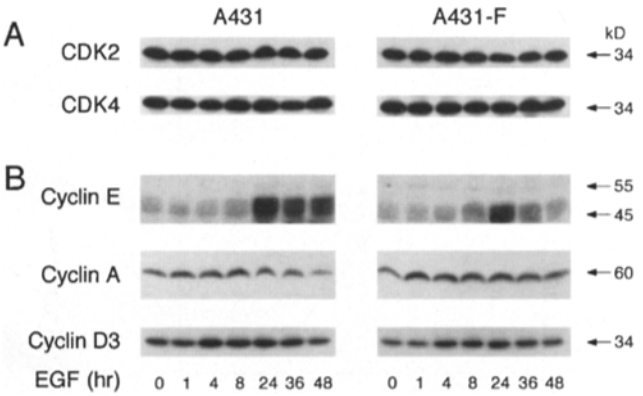

Figure 3. Expression of CDKs and cyclins in A431 and A431-F cells after EGF treatment. Cells were cultured in 20 nM EGF at 37°C for the indicated time periods. Cells were lysed as described in Materials and Methods and boiled in Laemmli SDS sample buffer. Equal amounts of protein were loaded onto (*A*) 15% or (*B*) 12% SDS–polyacrylamide gels and resolved by electrophoresis. Western immunoblot analyses were performed with antibodies directed against human CDK2, CDK4, cyclin E, cyclin A, or cyclin D3, using ^{125}I–protein A as label.

pression of $p21^{Cip1/WAF1}$ or $p27^{KIP1}$. Fig. 4 *A* shows that the basal expression of $p21^{Cip1/WAF1}$ mRNA was very low in both A431 and A431-F cells and could be strongly induced after 1–2 h of EGF treatment. However the expression of $p21^{Cip1/WAF1}$ mRNA was reduced in A431-F cells compared with A431 cells after >24 h of treatment. The amount of $p21^{Cip1/WAF1}$ protein increased after 2-h EGF treatment, in parallel with the expression of $p21^{Cip1/WAF1}$ mRNA. This was coincident with the observed early G1 cell cycle arrest and inhibition of CDK2 activity in both A431 and A431-F cells (Fig. 2). $p21^{Cip1/WAF1}$ protein reached a peak in A431 cells after 4–8 h EGF treatment and decreased modestly afterwards, but remained at elevated levels over a 5-d period of treatment with EGF (Fig. 4 *B*). In A431-F cells, the rise in $p21^{Cip1/WAF1}$ protein levels after 4–8 h of treatment with EGF was followed by a sharp decline at time points beyond 8 h (Fig. 4).

We also explored whether $p27^{KIP1}$ could be involved in the sustained inhibition of CDK2 activity in parental A431 cells exposed to EGF treatment. We observed the presence of $p27^{KIP1}$ in A431 and A431-F cells, but no significant increases in the content of $p27^{KIP1}$ protein or $p27^{KIP1}$/CDKs complexes were found during the period of EGF treatment, even after 5 d (data not shown).

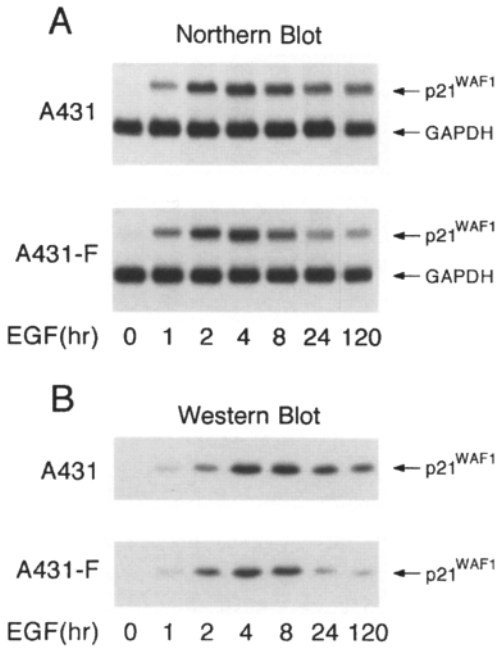

Figure 4. Induction of $p21^{Cip1/WAF1}$ mRNA and protein expression by EGF in A431 and A431-F cells. Cells were treated with 20 nM EGF at 37°C for the indicated time periods. Lysates were prepared for RNA assays and protein assays as described in Materials and Methods. (*A*) 10 μg total RNA in each lane was separated by 1% agarose/0.7% formaldehyde gel electrophoresis. The expression of $p21^{Cip1/WAF1}$ mRNA was assayed by Northern blot hybridization with human $p21^{Cip1/WAF1}$ cDNA fragment, as well as a cDNA fragment of human house keeping gene GAPDH to show the quantitation of loaded RNA. (*B*) The expression of $p21^{Cip1/WAF1}$ protein was assayed by resolving lysates containing equal amounts of protein by electrophoresis, followed by transferring samples to nitrocellulose and immunoblotting blots with a rabbit anti-$p21^{Cip1/WAF1}$ antibody, using ^{125}I–protein A as label.

Inhibition of EGF-induced $p21^{Cip1/WAF1}$ by Tyrphostin, an EGF Receptor Tyrosine Kinase Inhibitor

To confirm the causal role of EGF receptor activation in the induction of $p21^{Cip1/WAF1}$, we employed an EGF receptor tyrosine kinase inhibitor, tyrphostin AG1478, to examine its effect on levels of EGF-induced $p21^{Cip1/WAF1}$ protein. Fig. 5 *A* shows that preincubation of cells with tyrphostin AG1478 caused a dose-dependent inhibition of tyrosine phosphorylation on A431 cell proteins induced during a 4-h incubation with EGF. In Fig. 5 *B*, parallel cell lysates were examined for $p21^{Cip1/WAF1}$ protein expression, and it was observed that induction of $p21^{Cip1/WAF1}$ also was inhibited by AG1478. The prevention of the EGF-mediated increase in $p21^{Cip1/WAF1}$ expression by a specific inhibitor of EGF receptor tyrosine kinase strongly suggests the observed induction of $p21^{Cip1/WAF1}$ was a consequence of EGF receptor activation by ligand.

Association of $p21^{Cip1/WAF1}$ with Cyclin/CDK2 Complex

To further explore whether the observed inhibition of CDK2 activity was attributable to the physical association of $p21^{Cip1/WAF1}$ with CDK2-cyclins, we immunoprecipitated the $p21^{Cip1/WAF1}$ from lysates of cells either treated with EGF or left untreated for periods of 5 h and 5 d, and then immunoblotted the electrophoretically separated

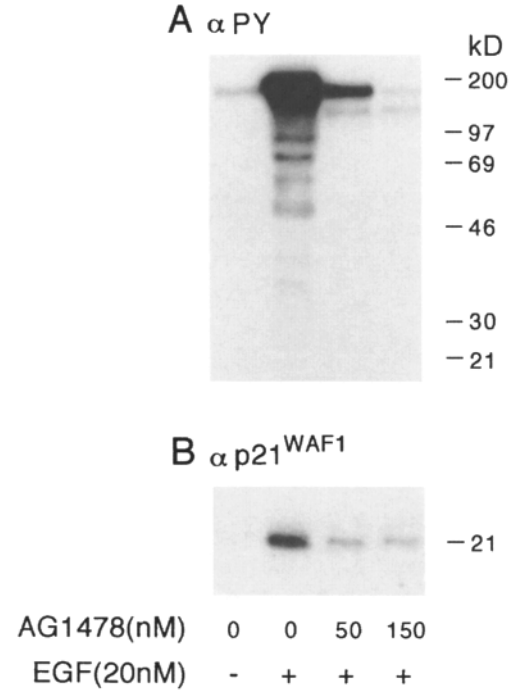

Figure 5. Effect of tyrphostin on EGF-induced of $p21^{Cip1/WAF1}$ protein expression. A431 cells were pretreated with 0, 50, or 150 nM tyrphostin AG1478 in an equal amount of DMSO (final concentration 0.5%) for 2 h at 37°C, followed by further 4 h incubation with or without 20 nM EGF as indicated. Cell lysates were prepared and boiled in Laemmli SDS buffer. Equal amounts of protein were loaded onto (*A*) 10% or (*B*) 12% SDS–polyacrylamide gels and resolved by electrophoresis. Western immunoblot analyses were performed with antibodies directed against (*A*) phosphotyrosine (*PY*), or (*B*) $p21^{Cip1/WAF1}$.

$p21^{Cip1/WAF1}$-immunoprecipitates with anti-CDK2 antibody. Fig. 6 reveals that CDK2 was coimmunoprecipitated by anti-$p21^{Cip1/WAF1}$ antibody, indicating their in vivo association. Increased amounts of $p21^{Cip1/WAF1}$/CDK2 complex were found after A431 and A431-F cells were treated for 5 h with EGF (Fig. 6 *A*). During further incubation of both cell types, the association of CDK2 with $p21^{Cip1/WAF1}$ (Fig. 6 *A*) parallelled expression of $p21^{Cip1/WAF1}$ mRNA and protein (Fig. 4), with a far greater reduction in the A431-F cells that displayed a return of CDK2 kinase activity to high levels (Figs. 2 *A* and 6 *B*).

In A431-F cells, in which EGF did not inhibit proliferation and cells traversed the cell cycle after a transient block in G1 phase, $p21^{Cip1/WAF1}$/CDK2 complexes decreased (Fig. 6 *A*) and the activity of cyclin/CDK2 was largely restored (Figs. 2 *A* and 6 *B*). However, the small amount of CDK2 that remained associated with $p21^{Cip1/WAF1}$ (Fig. 6 *A*) was not active (Fig. 6 *B*). In A431 cells, in which activation of EGF receptors by ligand persistently inhibited cell cycle traversal, the complexing of CDK2 with $p21^{Cip1/WAF1}$ was maintained at high levels (Fig. 6 *A*) with inactivation of the bound CDK2 (Fig. 6 *B*). This resulted in a sustained reduction of CDK2 kinase activity (Figs. 2 and 6 *B*), which maintained G1 phase arrest of the cell cycle (Fig. 1) in the EGF treated A431 cells.

At the early time points after EGF treatment, the $p21^{Cip1/WAF1}$/CDK4 complex was also increased in both A431 and A431-F cells, but the amounts of this complex decreased in both cell types after further treatment with EGF (data not shown), suggesting that the $p21^{Cip1/WAF1}$/CDK2 complex is more important than the $p21^{Cip1/WAF1}$/CDK4 complex in EGF-mediated growth inhibition of A431 cells.

Association of $p21^{Cip1/WAF1}$ with Proliferating Cell Nuclear Antigen (PCNA)

Since it has been reported that $p21^{Cip1/WAF1}$ also binds to PCNA and inhibits in vitro PCNA-dependent DNA replication (Waga et al., 1994; Flores-Rozas et al., 1994; Chen et al., 1995), we investigated whether the increased $p21^{Cip1/WAF1}$ in EGF-treated A431 cells was also associated with PCNA. No significant changes of PCNA levels were found in both A431 and A431-F cells incubated with EGF for 5 d (Fig. 7 *A*). However, the increased $p21^{Cip1/WAF1}$ levels in EGF-treated A431 cells (Fig. 7 *B*) was accompanied by increased association with PCNA (Fig. 7 *C*). In A431-F cells, the rise and subsequent fall in $p21^{Cip1/WAF1}$ levels (Fig. 7 *B*) was accompanied by a rise and fall in $p21^{Cip1/WAF1}$ associated PCNA (Fig. 7 *C*).

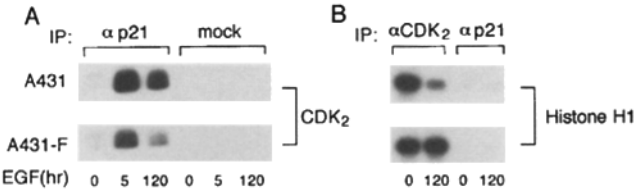

Figure 6. Association of CDK2 with $p21^{Cip1/WAF1}$ and the associated histone H1 kinase activity. Cells were treated with 20 nM EGF at 37°C for indicated time periods. (*A*) Equal amounts of A431 and A431-F cell lysates were subjected to the immunoprecipitation with rabbit anti-$p21^{Cip1/WAF1}$, or with rabbit anti–mouse IgG antibody (for nonspecific immunoprecipitation). The immunoprecipitates were boiled in Laemmli SDS buffer and loaded onto 15% SDS–polyacrylamide gels. After electrophoresis, the samples were transferred to nitrocellulose, followed by Western immunoblot analysis with anti-CDK2 antibody. (*B*) CDK2 histone H1 kinase activity and $p21^{Cip1/WAF1}$-associated histone H1 kinase activity were assayed by immunoprecipitation of equal amounts of A431 and A431-F cell lysates with antibodies against CDK2 or $p21^{Cip1/WAF1}$, followed by incubating the immunoprecipitated beads with a reaction solution for the in vitro phosphorylation of histone H1 as described in Materials and Methods. Radiolabeled histone H1 bands were separated by 12% SDS–polyacrylamide gel electrophoresis.

Discussion

The human A431 squamous carcinoma cell line is unusual in that physiologic concentrations of EGF inhibit proliferation in culture (Gill and Lazar, 1981; Barnes, 1982; Kawamoto et al., 1983). In the presence of 5–20 nM exogenous EGF, the proliferation of cultured A431 cells is decreased by 80% compared to untreated cells. We have shown previously that EGF activates two regulatory sites in A431 cell cycle traversal. The most prominent block was in G1 phase, preventing entry into S phase, and the other block was in G2 phase, inhibiting entry into mitosis (MacLeod et al., 1986). In mammalian cells, CDKs associated with cyclins D, E, and A have been implicated in the control of G1 to S phase transition. In the present study, we observed that EGF-induced cell cycle arrest was accompanied by marked inhibition of CDK2 activity, suggesting that CDK2 is the downstream target of the biological effect of EGF in arresting cell cycle traversal. Analysis of cyclin E- and cyclin A-associated kinase activities further demonstrated that CDK2 inhibition can account for the observed G1 block in A431 cells. A431-F cells, which exhibit resistance to EGF-induced inhibition of growth, showed a transient and rapidly reversible inhibition of CDK2 activity.

Cyclin levels have been shown to be rate-limiting factors for G1 progression in mammalian cells and many studies show that synthesis of cyclins, especial D type cyclins, may be the target of physiological signals that control cell pro-

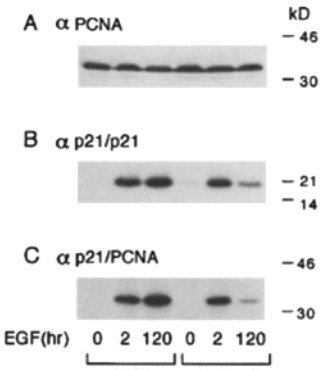

Figure 7. Effect of EGF on the expression of PCNA protein and its association with $p21^{Cip1/WAF1}$. A431 and A431-F cells were treated with 20 nM EGF at 37°C for the indicated time periods. (*A*) The expression of PCNA was measured by loading cell lysates containing equal amounts of protein onto 12% SDS–polyacrylamide gels, followed by electrophoresis and Western immunoblot analysis with a monoclonal antibody directed against human PCNA. In *B* and *C*, cell lysates were first subjected to the immunoprecipitation with rabbit anti-$p21^{Cip1/WAF1}$ antibody. The immunoprecipitates were boiled in Laemmli SDS buffer, split into two equal parts, loaded onto (*B*) 13.5% or (*C*) 12% SDS–polyacrylamide gels, and resolved by electrophoresis. Western blot analyses were then performed with antibodies directed against human (*B*) $p21^{Cip1/WAF1}$ or (*C*) PCNA.

liferation (Hunter and Pines, 1994; Jiang et al., 1993; Ohtsubo and Roberts, 1993; Quelle et al., 1993; Won et al., 1992). Therefore, we explored whether the observed inhibition of CDK2 could be attributed to reduced cyclin expression. Interestingly, we found that the cyclin D3, which is the predominant D cyclin expressed in A431 cells, was slightly increased after 4-h incubation with exogenous EGF and returned to baseline levels after 48 h of culture with EGF. Similar changes in cyclin D3 levels were found in EGF-resistant A431-F cells. Cyclin E levels were persistently increased for up to 48 h in A431 cells cultured with EGF. In contrast, A431-F cells treated with EGF showed a temporary increase of cyclin E followed by a decline towards baseline levels, as the cells were released from a transient G1 arrest. Since cyclin E is synthesized starting in late G1 phase, the differences between A431 and A431-F cells in their responses to EGF could be explained by persistent G1 arrest in A431 cells and only temporary G1 arrest in A431-F cells. The expression of cyclin A was reduced in A431 cells after 48 h incubation with EGF but no significant change of cyclin A was found in A431-F cells. Cyclin A is synthesized primarily during S phase of the cell cycle and the reduced percent of S phase cells in A431 cells arrested in G1 by EGF treatment may explain the reduced levels of cyclin A observed in these cultures. The levels of the three cyclins never reached values below those observed prior to treatment of cultures with EGF. Taken together, these observations suggest that changes in the expression of cyclins after EGF treatment are the consequence, rather than the cause, of the reduced CDK2 activity induced by EGF.

Recent research from a number of laboratories suggests that $p21^{Cip1/WAF1}$ is a universal inhibitor of cyclin-dependent kinase. The catalytic activity of each member of the cyclin-dependent kinase family can be inhibited by $p21^{Cip1/WAF1}$, although their relative affinities vary with each enzyme (Xiong et al., 1993; Gu et al., 1993; Harper et al., 1993). The regulation of $p21^{Cip1/WAF1}$ under different physiological situations may be through p53-dependent or p53-independent pathways. For example, when cell DNA is damaged by γ-radiation or chemotherapeutic agents, $p21^{Cip1/WAF1}$ may be induced through the p53-dependent pathway (El-Deiry et al., 1993; Xiong et al., 1993; Johnson et al., 1994). Cells with damaged DNA that have a normal p53 gene will undergo G1 arrest before the initiation of DNA synthesis; if cells can pass the G1 checkpoint because of an abnormal p53 gene, they may become apoptotic (El-Deiry et al., 1994). When resting cell cultures are stimulated by mitogens, such as growth factors or serum, or when cells are undergoing differentiation, a p53-independent transient induction of increased $p21^{Cip1/WAF1}$ may occur, and this temporary elevation has been postulated to serve as an internal control mechanism, preventing the cells from entering S phase prematurely (Harper et al., 1993; Michieli et al., 1994; MacLeod et al., 1995; Parker et al., 1995). In the present study, we found that, as an early response to EGF treatment for 4–8 h, $p21^{Cip1/WAF1}$ is markedly induced in both EGF-sensitive A431 and EGF-resistant A431-F cells. This corresponds to the timing of G1 arrest on the flow cytometry analysis of both cell types (Fig. 1). The changes in $p21^{Cip1/WAF1}$ expression appear to be independent of p53, since this gene is mutated in A431 cells (Kowk et al., 1994). Our data fit the model that $p21^{Cip1/WAF1}$ levels present a threshold that cyclin–CDK complexes must overcome before the cell can enter into S phase (Xiong et al., 1993; Zhang et al., 1994). At early time points after exposure of both A431 and A431-F cells to EGF, $p21^{Cip1/WAF1}$ is induced and causes growth arrest by functioning to inhibit the activity of the cyclin/CDK2 complex (Figs. 2 and 6) and as noted above, this could serve as an internal control mechanism (cell cycle brake) to prevent the cells from entering S phase prematurely. In the EGF-sensitive A431 cells, $p21^{Cip1/WAF1}$ remains persistently upregulated at high levels and associates with and inhibits cyclin/CDK2 for up to 5 d of incubation (Fig. 6), while in the A431-F cells the reduction of $p21^{Cip1/WAF1}$/CDK2 complex permits cell cycle traversal and cell proliferation.

We also have demonstrated that EGF-induced $p21^{Cip1/WAF1}$ is associated with PCNA, a protein essential for DNA replication. This provides a second mechanism, by which elevated $p21^{Cip1/WAF1}$ may act to inhibit cell cycle traversal in EGF-treated A431 cells.

Preliminary data from our laboratory and others have demonstrated induction of $p21^{Cip1/WAF1}$ by EGF in DiFi colon adenocarcinoma cells (unpublished observation), MDA468 human breast adenocarcinoma cells (Jiang, A., T. J. Thomas, C. A. Faaland, and T. Thomas. 1995. *Proc. Am. Assoc. Cancer Res.* 36:580.) and ME180 human cervical squamous carcinoma cells (Brown, C., M. Rubin, T. DeBlasio, H. Masui, and J. Mendelsohn. 1995. *Proc. Am. Assoc. Cancer Res.* 36:34). Each of these cell lines is inhibited by nanomolar concentrations of exogenous EGF. We conclude that $p21^{Cip1/WAF1}$ is involved in the general mechanism of EGF-induced growth inhibition, although other unknown regulatory molecules could also play important roles.

Several hypotheses have been proposed to explain the possible mechanisms by which EGF inhibits the growth of malignant cell lines expressing high numbers of receptors. These include EGF effects on the organization of cytoskeletal elements that regulate mitotic events (Barnes, 1982); the energy expenditure necessary for phosphorylation, internalization, degradation and synthesis of a large numbers of receptors (Kaplan et al., 1990); and prolonged phosphorylation of lipocortin-1 (Karasik et al., 1990). Our demonstration that sustained elevation of $p21^{Cip1/WAF1}$ is important in mediation of EGF-induced A431 cell inhibition provides a focus for further mechanistic studies of this "paradoxical" response, as well as further studies of the control of cell cycle progression by growth factors. We are currently pursuing the causal relationship between EGF receptor activation and selective $p21^{Cip1/WAF1}$ induction in studies of downstream substrates of the EGF receptor tyrosine kinase, and in studies of EGF-mediated activation of the promoter for the $p21^{Cip1/WAF1}$ gene and changes in synthesis and catabolism of $p21^{Cip1/WAF1}$.

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