Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 4848–4850, August 1984
Cell Biology

Epidermal growth factor inhibits the synthesis of the nuclear protein cyclin in A431 human carcinoma cells

(polypeptide synthesis/cell proliferation/[^{35}S]methionine labeling/two-dimensional gel electrophoresis/A431 cell mutants)

RODRIGO BRAVO

European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, Federal Republic of Germany

Communicated by Diter von Wettstein, May 1, 1984

ABSTRACT **The effect of epidermal growth factor (EGF) on the synthesis of the nuclear protein cyclin and its relationship with cell proliferation has been studied in human carcinoma A431 cells. Quantitative two-dimensional gel electrophoretic analysis of [^{35}S]methionine-labeled polypeptides from EGF-treated and untreated A431 cells showed that the synthesis of cyclin (0.07% of total labeled protein) could be decreased to 20% (<0.01% of total labeled protein) by EGF at a concentration of 100 ng/ml. A good correlation between the effect of different concentrations of EGF on cyclin synthesis and A431 cell proliferation was found. A431-derived cells resistant to the growth-inhibitory effect of EGF showed no significant changes in cyclin synthesis after EGF treatment. Taken together, these results support the idea that cyclin may be an important protein that is involved in the control of cell proliferation.**

Epidermal growth factor (EGF) is a potent mitogen for a number of cell types in culture (1), though it inhibits proliferation of A431 human carcinoma cells (2–4). Several studies have been carried out to establish the possible role of EGF-stimulated tyrosine-specific protein kinase activity in the inhibition of proliferation in these cells (5–7). Even though growth inhibition is associated with high concentrations of activated EGF receptor/kinase, the mechanism(s) by which such activities affect cell proliferation remain unknown. It is possible that in A431 cells the effect of EGF resides in a biochemical step subsequent to the EGF-stimulated protein kinase activity.

Recently, there has been described a transformation-sensitive nuclear protein cyclin (M_r, 36,000), whose synthesis correlates directly with the proliferative state of the cells (8–10). This protein is present in very small amounts in normal nondividing cells or tissues but is synthesized by normal proliferating as well as transformed cells and tumors (11–16). The levels of cyclin fluctuate during the cell cycle, with a clear increase in the S phase (17).

Here I present evidence showing that a decrease in the synthesis of cyclin parallels the EGF-growth inhibitory effect on A431 cells but not on A431-derived clones resistant to the growth factor.

METHODS

Cells. Human epidermal carcinoma cells (A431) were kindly provided by G. Todaro. Cells were routinely grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics (penicillin, 100 units/ml; streptomycin, 50 μg/ml).

Labeling of Cells with [^{35}S]Methionine. Cells were grown in 0.25-ml flat-bottomed microtiter plates (Nunc) for at least 24 hr before labeling. They were labeled by replacing the normal medium with 0.1 ml of Dulbecco's modified Eagle's medium containing methionine at 1 mg/liter and supplemented with 10% dialyzed fetal calf serum in the presence of 100 μCi of [^{35}S]methionine (Amersham, SJ204; 1 Ci = 37 GBq) as described (18, 19).

Two-Dimensional Gel Electrophoresis. The procedures used are those previously described (18, 20) with some modifications (4). Briefly, the first-dimension separations (isoelectric focusing, IEF) were performed on 230 × 1.2 mm 4% (wt/vol) polyacrylamide gels containing 2% ampholytes (1.6% pH 5–7; 0.4% pH 3.5–10) at 1200 V for 20 hr. The second-dimension separations were performed in a 15% polyacrylamide gel (25 × 25 cm) at room temperature overnight. After the run, gels were processed for fluorography, dried, and exposed at −70°C (21). Approximately 10^6 trichloroacetic acid-precipitable cpm was routinely applied on a gel.

RESULTS

EGF-Induced Changes in the Rate of Synthesis of Cyclin in A431 Cells. For orientation and reference purposes, Fig. 1 shows the polypeptide pattern of asynchronous whole A431 cells labeled for 20 hr with a mixture of 16 ^{14}C-labeled amino acids. The area containing the polypeptides of interest has been enclosed in a box. Some major cytoarchitectural polypeptides are also indicated.

To determine the effect of EGF on cyclin synthesis, A431 cells were labeled with [^{35}S]methionine for 2-hr intervals every 2 hr after the addition of EGF to the culture medium at 100 ng/ml. Representative two-dimensional gels of these samples are presented in Fig. 2. A decrease in the synthesis of cyclin (0.07% of total labeled cellular protein) was detected 2–4 hr after addition of EGF and reached a minimum at 14–16 hr (0.01% of total cellular labeled protein). The synthesis of only a few other polypeptides was affected by EGF, and of these only IEF6 and -8 are shown on the various gels presented in Fig. 2. The synthesis of these polypeptides reached a maximum 8–10 hr after stimulation and remained constant as long as EGF was present in the culture medium. The synthesis of cyclin and polypeptides IEF6 and -8 returned to normal 30–36 hr after the growth factor had been removed from the medium (not shown). One-dimensional peptide mapping (22–24) of cyclin and polypeptides IEF6 and -8 has unequivocally demonstrated that the latter are not related to cyclin but are related to each other (not shown). IEF6 and -8 have not been observed in many cell lines so far studied.

Decrease in Synthesis of Cyclin Parallels a Decrease in Cell Proliferation in A431 Cells. Since inhibition of A431 cell proliferation depends on the concentration of EGF used (refs. 2, 3, and 7; see Fig. 3), it was important to determine whether the relative proportion of cyclin changed with the concentration of growth factor used. For this purpose, A431 cells were

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EGF, epidermal growth factor; IEF, isoelectric focusing.

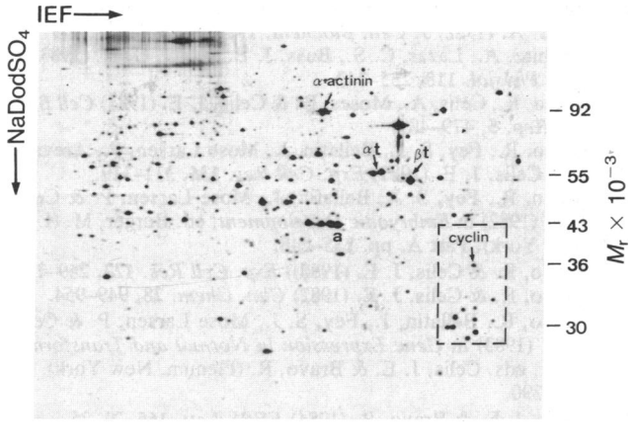

FIG. 1. Two-dimensional map (IEF) of asynchronous A431 cell polypeptides labeled for 16 hr with a mixture of 16 ^{14}C-labeled amino acids (Amersham, CBF 104) as previously described (12). Some of the major cytoarchitectural polypeptides are indicated for reference. a, Actin; αt, α-tubulin; βt, β-tubulin. The area within the broken lines is the fraction of the gels shown in Figs. 2 and 4.

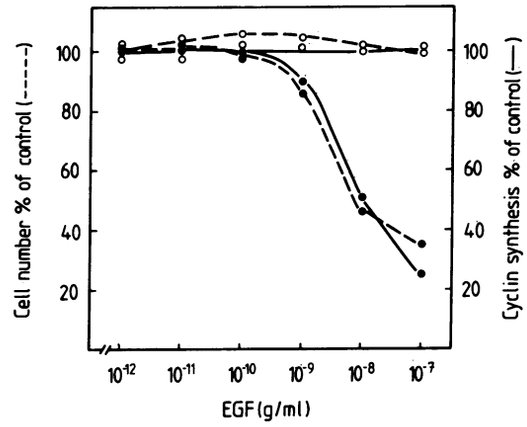

FIG. 3. Effect of different concentrations of EGF on cyclin synthesis and cell proliferation in A431 cells (●) and clone 16 (○). Data represent the number of cells present in dishes for each concentration of EGF compared to control dishes without EGF. The number of cells in control dishes was taken as 100%. Cells were counted at day 6. For the analysis of cyclin, cells were grown in the presence of EGF at the concentrations indicated for 5 days before labeling. Cells were labeled for 16 hr with [^{35}S]methionine in the presence of EGF.

grown for 5 days in the presence of different concentrations of EGF (1 pg/ml to 100 ng/ml) before labeling with [^{35}S]methionine (20-hr labeling). The quantitative analysis of these results is illustrated in Fig. 3. Clearly there is a good correlation between the levels of cyclin and A431 cell proliferation at the different doses of EGF used.

Cyclin Synthesis Is Not Affected in A431 Cells Resistant to the EGF Growth-Inhibitory Effect. To study further the correlation between cyclin synthesis and A431 cell proliferation, it was necessary to obtain clones resistant to the growth inhibitory effect of EGF. For this, parental A431 cells were treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and subsequently cultured in the presence of EGF as described (5). The effect of different concentrations of EGF (1 pg/ml to 100 ng/ml) on cell growth and cyclin synthesis of one of these clones, clone 16, is shown in Fig. 3. Clearly, the growth properties and cyclin synthesis of clone 16 are not affected by EGF. That the mutants responded to EGF is supported by the fact that polypeptides IEF6 and -8 showed changes in synthesis very similar to those observed in the parental A431 cells (see Fig. 4). Similar analysis of other independent clones resistant to the growth inhibitory effect of EGF gave very similar results (not shown). Moreover, these clones exhibited changes in cell morphology and actin distribution similar to those observed in the parental A431 cells treated with EGF (not shown).

DISCUSSION

The studies presented in this report reveal important changes in the synthesis of cyclin after EGF treatment of human tumor A431 cells. EGF induced a decrease in the synthesis of cyclin at doses at which the growth factor inhibits cell proliferation. The magnitude of these changes is dose dependent, showing a good correlation with the parameters studied. For example, at a high dose of EGF (100 ng/ml)

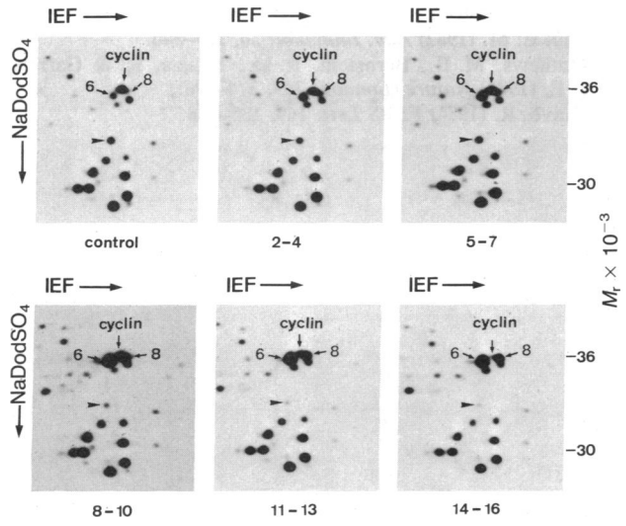

FIG. 2. Changes in cyclin synthesis induced by EGF. Cells treated with EGF (100 ng/ml) were labeled for 2 hr with [^{35}S]methionine at the times indicated (in hr) in each case. Only a small fraction of the gel is shown (see Fig. 1). The arrowhead indicates a tropomyosin-related polypeptide whose synthesis changes with cell transformation (12); 6 and 8, polypeptides IEF6 and IEF8 (see text).

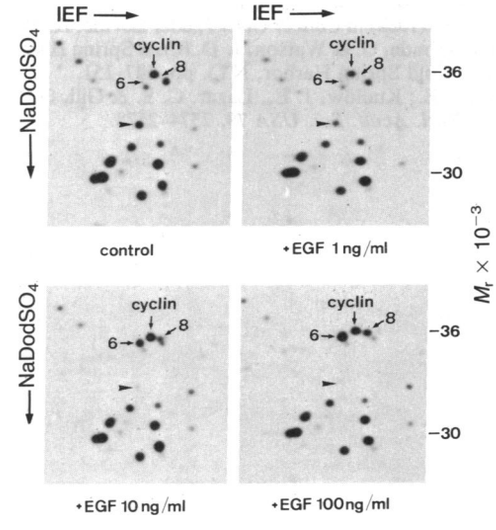

FIG. 4. EGF-induced changes in synthesis of polypeptides IEF6 and -8 in A431 clone 16. Cells were treated with different concentrations of EGF for 5 days before labeling. Cells were labeled for 16 hr with [^{35}S]methionine in the presence of EGF. The arrowhead indicates a tropomyosin-related polypeptide whose synthesis also changes in the parental A431 cells.

A431 cell proliferation is inhibited by 70% and the proportion of cyclin decreases by 80%.

The isolation of A431 cell variants resistant to the growth inhibitory effect of EGF proved to be of great value. These cells are able to respond in many respects to EGF as the parental cells do. They present the same changes in morphology, actin distribution, and synthesis of some polypeptides as those observed in the nonresistant A431 cells. However, no changes in the synthesis of cyclin was found at any of the doses of EGF used during these studies, even after several days of treatment. These results strongly support evidence obtained with the parental A431 cells that the growth inhibition correlates with a decrease in cyclin synthesis.

There is good evidence that the major alteration associated with the escape of variant cells from the growth-inhibitory effect of EGF is a decrease in the number of EGF receptors per cell and therefore of the EGF-stimulated tyrosine-specific protein kinase (7). In the continuous presence of EGF, the augmented level of phosphotyrosine is maintained in A431 cells for at least 6 hr. Thereafter, it starts to decline (25). As the effect of EGF on cyclin synthesis persists as long as the growth factor is present, it would seem that the activation of the tyrosine-specific protein kinase is not directly involved in controlling the synthesis of cyclin. Furthermore, none of the proteins previously identified as targets of the EGF-induced tyrosine protein kinase (25) corresponds to cyclin.

The results I have presented agree with previous observations which demonstrated that the proportion of the nuclear protein cyclin (8) correlates directly with the proliferative state of the cells (9–16). Recently, it has been shown that cyclin and the proliferating cell nuclear antigen (PCNA) (26–28) are the same protein (29). Also, a coordinated increase in cyclin and DNA synthesis after serum stimulation of quiescent 3T3 cells has been observed (30).

Taken together, the data suggest that cyclin is involved in the effect of EGF on A431 cell growth inhibition. This protein could be an important component that regulates cell proliferation and its activity may be associated with events related to DNA replication (14, 30).

1. Gospodarowicz, D., Greenburg, G., Bialecki, H. & Zetter, B. R. (1978) *In Vitro* **14,** 85–118.
2. Gill, G. N. & Lazar, C. S. (1981) *Nature (London)* **293,** 305–307.
3. Barnes, D. W. (1982) *J. Cell Biol.* **93,** 1–4.
4. Bravo, R. (1984) in *Cancer Cells 1*, eds. Levine, A., Topp, W., van de Woude, G. & Watson, J. D. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 147–151.
5. Buss, J. E., Kudlow, J. E., Lazar, C. S. & Gill, G. N. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2574–2578.
6. Gill, G. N., Burns, D. E., Lazar, C. S., Lipshitz, A. & Cooper, J. A. (1982) *J. Cell. Biochem.* **19,** 249–257.
7. Lipshitz, A., Lazar, C. S., Buss, J. E. & Gill, G. N. (1983) *J. Cell. Physiol.* **115,** 235–242.
8. Bravo, R., Celis, A., Moses, D. & Celis, J. E. (1981) *Cell Biol. Int. Rep.* **5,** 479–489.
9. Bravo, R., Fey, S. J., Bellatin, J., Mose Larsen, P., Arevalo, J. & Celis, J. E. (1981) *Exp. Cell Res.* **136,** 311–319.
10. Bravo, R., Fey, S. J., Bellatin, J., Mose Larsen, P. & Celis, J. E. (1982) in *Embryonic Development*, ed. Burger, M. (Liss, New York), Part A, pp. 235–248.
11. Bravo, R. & Celis, J. E. (1980)) *Exp. Cell Res.* **127,** 249–260.
12. Bravo, R. & Celis, J. E. (1982) *Clin. Chem.* **28,** 949–954.
13. Bravo, R., Bellatin, J., Fey, S. J., Mose Larsen, P. & Celis, J. E. (1983) in *Gene Expression in Normal and Transformed Cells*, eds. Celis, J. E. & Bravo, R. (Plenum, New York), pp. 263–290.
14. Celis, J. E. & Bravo, R. (1984) *FEBS Lett.* **165,** 21–25.
15. Celis, J. E., Bravo, R., Mose Larsen, P., Fey, S. J., Bellatin, J. & Celis, A. (1984) in *Two-Dimensional Gel Electrophoresis of Proteins: Methods and Applications*, eds. Celis, J. E. & Bravo, R. (Academic, New York), pp. 307–362.
16. Garrels, J. I. & Franza, R. (1984) in *Cancer Cells 1*, eds. Levine, A., Topp, W., van de Woude, G. & Watson, J. D. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 137–146.
17. Bravo, R. & Celis, J. E. (1980) *J. Cell Biol.* **84,** 795–802.
18. Bravo, R., Small, J. V., Fey, S. J., Mose Larsen, P. & Celis, J. E. (1982) *J. Mol. Biol.* **154,** 121–143.
19. Bravo, R. (1984) in *Two-Dimensional Gel Electrophoresis of Proteins: Methods and Applications*, eds. Celis, J. E. & Bravo, R. (Academic, New York), pp. 3–36.
20. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021.
21. Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56,** 335–341.
22. Cleveland, D., Fischer, S., Kirschner, M. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106.
23. Fey, S. J., Bravo, R., Mose Larsen, P., Bellatin, J. & Celis, J. E. (1981) *Cell Biol. Int. Rep.* **5,** 491–500.
24. Fey, S. J., Bravo, R., Mose Larsen, P. & Celis, J. E. (1984) in *Two-Dimensional Gel Electrophoresis of Proteins: Methods and Applications*, eds. Celis, J. E. & Bravo, R. (Academic, New York), pp. 169–189.
25. Hunter, T. & Cooper, J. A. (1981) *Cell* **24,** 741–752.
26. Miyachi, K., Fritzler, M. J. & Tan, E. M. (1978) *J. Immunol.* **121,** 2228–2235.
27. Takasaki, Y., Deng, J. S. & Tan, E. M. (1981) *J. Exp. Med.* **154,** 1899–1909.
28. Tan, E. M. (1982) *Adv. Immunol.* **33,** 167–240.
29. Mathews, M. B., Bernstein, R. M., Franza, R. & Garrels, J. E. (1984) *Nature (London)* **309,** 374–376.
30. Bravo, R. (1984) *FEBS Lett.* **169,** 185–188.