Transforming Growth Factor \( \beta \) Modulates Phosphorylation of the Epidermal Growth Factor Receptor and Proliferation of A431 Cells

Tzipora Goldkorn\(^2\) and John Mendelsohn
Memorial Sloan-Kettering Cancer Center and Cornell University Medical College, New York, New York 10021 [T. G., J. M.] and Yale University School of Medicine, New Haven, Connecticut 06510 [T. G.]

Abstract
Transforming growth factor \( \beta \) (TGF-\( \beta \)) increased the phosphorylation of the epidermal growth factor (EGF) receptor and inhibited the growth of A431 cells. Incubation with TGF-\( \beta \) induced maximal EGF receptor phosphorylation to levels 1.5-fold higher than controls. Phosphorylation increased more prominently (4-5-fold) on tyrosine residues as determined by phosphoamino acid analysis and antiphosphotyrosine antibody immunoblotting. The kinase activity of EGF receptor was also elevated 2.5-fold when cells were cultured in the presence of TGF-\( \beta \). The antiproliferative effect of TGF-\( \beta \) on A431 cells was accompanied by prolongation of G\(_0\)-G\(_1\) phase and by morphological changes. TGF-\( \beta \) augmented the growth inhibition of A431 cells which could be induced by EGF. In parallel, the specific EGF-induced increase in total phosphorylation of the EGF receptor was also augmented in the presence of TGF-\( \beta \). In cells cultured with TGF-\( \beta \), the phosphorylation of EGF receptor tyrosines induced by 20-min exposure to EGF was further increased 2-3-fold, suggesting additive effects upon receptor phosphorylation. EGF receptor activation by TGF-\( \beta \) is characterized by kinetics quite distinct from that induced by EGF and therefore appears to take place through an independent mechanism. The TGF-\( \beta \)-induced elevation in the phosphorylation of the EGF receptor may have a role in the augmented growth inhibition of A431 cells observed in the presence of EGF and TGF-\( \beta \).

Introduction
The most versatile growth factors with respect to functional diversity appear to be those of the TGF-\( \beta \) family. The TGF-\( \beta \) isomers are widely distributed throughout both embryonic and adult tissues, and receptors are to be found on most, if not all, cell types. Furthermore, TGF-\( \beta \) is capable of acting in a cell type-specific manner to either promote or inhibit the processes of proliferation, differentiation, and chemotaxis (1-4).

The 25,000-dalton type 1 TGF-\( \beta \) (TGF-\( \beta \), will here be referred to as TGF-\( \beta \)) is a homodimeric polypeptide which has been shown to exert a wide variety of effects, both stimulatory and inhibitory, on cell growth and differentiation (see Refs. 5-9 for reviews). TGF-\( \beta \) may act by binding to cell surface receptors that initiate the transduction of antimitogenic signals to the nucleus (10). At the cellular level, the action of TGF-\( \beta \) is characterized by changes in the expression of growth-regulatory genes (11, 12) and genes whose products mediate cell adhesion (13-15). As with other peptide hormones or growth factors that recognize specific cellular receptors, interaction with other molecules, subsequent to binding, may play an important role in the cellular response to TGF-\( \beta \). TGF-\( \beta \) also regulates the actions of many other growth factors and determines a positive or negative direction of these effects (3).

Recent reports have documented effects of TGF-\( \beta \) upon the RB protein. TGF-\( \beta \) and RB appear to function in a common growth-inhibitory pathway in which TGF-\( \beta \) acts to retain RB in the underphosphorylated, growth-suppressive state, so that cells (MvLu) are arrested in the late G\(_1\) phase (16). TGF-\( \beta \) was also demonstrated to inhibit skin keratinocyte proliferation when added during the G\(_1\) phase of the cell cycle, and it was suggested that RB mediates TGF-\( \beta \) regulation of \( c-myc \) gene expression and growth inhibition (17).

Recent findings suggest involvement of TGF-\( \beta \) in enhancing the expression of the EGF receptor gene (18), changing the number of EGF receptors expressed per cell, and changing binding affinities of EGF to its receptor (12, 19-21).

In this report, we describe the effect of TGF-\( \beta \) treatment on several activities associated with the process of EGF receptor-induced signal transduction in the A431 epidermoid carcinoma cell line. We provide molecular insights into the mechanisms of action of TGF-\( \beta \). We present evidence to suggest that the antiproliferative effects of TGF-\( \beta \) in A431 cells may be related to modulation of intrinsic EGF receptor phosphorylation induced by incubation with TGF-\( \beta \).

Results
TGF-\( \beta \) Regulates A431 Cell Proliferation. Fig. 1 shows the effects of various concentrations of TGF-\( \beta \) on the growth rate of A431 cells during 3 days of culture. Incubation with TGF-\( \beta \) resulted in inhibition of the proliferative rate as compared with exponentially growing untreated cells. The extent of growth inhibition was con-
centration dependent and was maximized at 30 pm TGF-β. Thymidine incorporation into A431 cells (data not shown) was maximally inhibited on day 3 of TGF-β treatment. The growth inhibition of A431 cells by TGF-β was accompanied by morphological alterations (data not shown).

Flow cytometric cell cycle analysis performed on control and TGF-β-treated A431 cells demonstrated that TGF-β treatment increased the fraction of cells in G0–G1 phase and reduced the number of cells in S phase of the cell cycle (Table 1). After 24 h of treatment with TGF-β, there was little effect on the cell cycle distribution. However, after 48 h, 30 pm TGF-β substantially reduced the S phase of the cell cycle from 37.4% to 24.6% and increased the G1 phase from 48.8% to 66.4%.

In an epithelial tumor cell line with elevated EGF receptor, such as A431 cells, an optimal concentration of EGF is required to enhance proliferation (22). Higher concentrations of EGF can inhibit growth (23). As expected, and as shown in Fig. 2, addition of 5 nm EGF was antiproliferative for A431 cells. Moreover, when TGF-β and EGF were added together, the antiproliferative effects were additive, suggesting the possibility of cooperative interactions between these regulatory polypeptides in A431 cells.

Interestingly, the most prominent effect on the cell cycle distribution was also observed upon treatment with 30 pm TGF-β in the presence of EGF. After only 24 h of treatment in the presence of 30 pm TGF-β plus 10 nm EGF, the S phase contribution was reduced from 34.3% to 13.6% (Table 1). Treatment for only 24 h with either 10 nm EGF alone or with 30 pm TGF-β had almost no effect on the cell cycle distribution.

**TGF-β Regulates A431 Cell EGF Receptors.** The observation of added effects of TGF-β and EGF upon A431 cell proliferation led us to search for alterations in EGF receptor function following addition of TGF-β to the cultures. First, we examined the rate of EGF-induced turnover of the receptor, in the presence or absence of TGF-β. A431 cells were metabolically labeled with [35S] methionine, and then cultures were chased with unlabeled methionine in the presence of different concentrations of either TGF-β or EGF (Fig. 3). TGF-β stabilized the EGF receptor and increased its half-life, whereas EGF induced the expected accelerated degradation of its receptor. Interestingly, in the presence of both 30 pm TGF-β and 20 nm EGF, the half-life of the receptor was unchanged compared to control untreated A431 cells (Fig. 3).

A series of experiments was carried out to determine whether treatment of A431 cells with TGF-β resulted in changes in phosphorylation of the EGF receptor. First, the effect of TGF-β treatment on EGF receptor phosphorylation in intact cells was examined. A431 cells were cultured at different time points and were incubated with [32P] for the last 12 h prior to harvest to obtain equilibrium labeling of ATP. From 18–24 h on, there was an evident effect (24% increase in the cpm associated with EGF receptor), which at 48–72 h reached a maximum of 1.5-fold increase in total phosphorylation (Fig. 4; compare Lane A to Lane B). Activation of total phosphorylation of the EGF receptor by a 20-min exposure to EGF was 2-fold (Fig. 4; compare Lane A to Lane C), and by both TGF-β (72 h) and EGF (20 min) was about 3-fold (Fig. 4; compare Lane A to Lane D).

It was important to address the question of whether the increase in phosphorylation of the EGF receptor in A431 cells might merely be a reflection of changes in the levels of receptor expression. This was assessed by comparing steady-state levels of EGF receptor content and EGF receptor phosphorylation after exposure to TGF-β. Cells were labeled in parallel cultures with [32P], or with [35S] cysteine, in the presence or absence of different concentrations of TGF-β, for 72 h. To assess the response to EGF, cells were treated for the last 20 min of culture with 20 nm EGF. Lysates were prepared and immunoprecipitated with 528 anti-EGF receptor mAb. After SDS gel separation, the [32P]-labeled EGF receptor bands and the [35S]-labeled receptor bands were excised and counted. Fig. 5A demonstrates a TGF-β-induced rise in specific phosphorylation of EGF receptors, which was further augmented by addition of EGF during the last 20 min of culture. It is apparent that EGF stimulation of A431 cells precultured with varying concentrations of TGF-β resulted in an approximately 3-fold further enhancement of the specific phosphorylation of receptors. This indicates additive effects of the two ligands.

In parallel, the cell lysates of cultures labeled with [32P] were precipitated with TCA to examine the levels of total protein phosphorylation. Growth in the presence of var-

---

**Table 1 Cytofluorometric analysis**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time of treatment (h)</th>
<th>Cell cycle distribution (%)</th>
<th>G1</th>
<th>S</th>
<th>G2-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td></td>
<td>49.2</td>
<td>34.3</td>
<td>16.5</td>
</tr>
<tr>
<td>30 pm TGF-β</td>
<td>24</td>
<td></td>
<td>52.5</td>
<td>30.8</td>
<td>16.8</td>
</tr>
<tr>
<td>10 nm EGF</td>
<td>24</td>
<td></td>
<td>56.0</td>
<td>27.9</td>
<td>16.1</td>
</tr>
<tr>
<td>30 pm TGF-β + 10 nm EGF</td>
<td>24</td>
<td></td>
<td>64.1</td>
<td>13.6</td>
<td>22.3</td>
</tr>
</tbody>
</table>

* Representative of three experiments.
ing concentrations of TGF-β did not affect the total protein phosphorylation at different time points (Fig. 5B). Therefore, TGF-β effects upon phosphorylation of EGF receptors are not merely general cellular effects but are specific for the receptors.

The next experiments were designed to measure changes in the phosphorylation of specific amino acids in the EGF receptor in response to TGF-β treatment. As shown in Fig. 6 and Table 2, the phosphorylation of all three amino acids, tyrosine, threonine, and serine, was increased after 72 h of culture with TGF-β. However, there was a relative increase in phosphorylation of tyrosine residues at the expense of serine and threonine. Increases in phosphorylation of all three amino acids were also observed after the short 20-min treatment with 20 nM EGF, with a prominent relative increase in P-Tyr. When the cells were exposed to both TGF-β and EGF, the relative content of P-Ser, P-Thr, and P-Tyr was similar to that observed in cultures exposed to EGF alone, but the absolute amount of each phosphoamino acid was further increased by nearly 2-fold. TGF-β increased the phosphorylation of tyrosine 4-fold, EGF caused a 9-fold increase, and the two factors together resulted in a nearly 16-fold increase in P-Tyr.

In order to confirm the capacity of TGF-β to increase the levels of tyrosine phosphorylation, Western immunoblot analysis was used to determine the relative amount of P-Tyr per receptor. A431 cell cultures were exposed to TGF-β and/or EGF under the previously described experimental conditions, and equal amounts of protein from the whole cell lysates were subjected to SDS-PAGE analysis. Fig. 7 (upper panel) demonstrates an autoradiogram of a typical experiment. Paired Western blots were immunodetected with either antiphosphotyrosine antibody (Fig. 7A) or RKII anti-EGF receptor polyclonal antibody (Fig. 7B). The antiphosphotyrosine immunoblots of the lysates from TGF-β-treated cells showed only changes in the intensity of the 170-kilodalton band related to EGF receptor. A 6-fold increase in tyrosine phosphorylation of EGF receptor was induced by the TGF-β stimulation. The count ratios of PY69/RKII, cpm obtained with antiphosphotyrosine blotting (PY69) and with anti-EGF receptor (RKII) blotting, rose from 220 to 1275 (Table 3; compare A to B). This stimulation of tyrosine phosphorylation was further increased 4–5-fold when EGF was added to the cells for 20 min after incubation with TGF-β (Table 3; compare B to D). EGF alone, during a 20-min treatment, stimulated its receptor tyrosine phosphorylation 9-fold (Table 3; compare A to C). These data support the findings in Fig. 6 and Table 2 and clearly demonstrate additive effects of TGF-β and EGF on EGF receptor tyrosine phosphorylation.

Subsequent studies assessed the kinetics of the effect of TGF-β on EGF receptor tyrosine phosphorylation. The lower panel of Fig. 7 and Table 4 show that 1.7-fold stimulation was achieved after 30-min incubation with a maximally effective TGF-β concentration (100 pM), and a maximal effect reaching 8.3-fold over control levels occurred at 3 h. In contrast, EGF receptor tyrosine phosphorylation reached peak levels after only 20-min incubation, and a maximally effective EGF concentration (20 nM) stimulated the phosphorylation of EGF receptor tyrosines to 11.7-fold over control levels at the 20-min time point. These studies demonstrate that the kinetics of TGF-β-stimulated tyrosine phosphorylation of EGF receptor is different from that stimulated by EGF.

Although these experiments clearly demonstrate changes in EGF receptor tyrosine phosphorylation in A431 cells cultured with TGF-β, it was important to measure directly the effect of TGF-β treatment on the autophosphorylation activity of the tyrosine kinase of the EGF receptor. Therefore, cultures of A431 cells were incubated with TGF-β, and the EGF receptors were isolated from cell lysates by immunoprecipitation. The level of tyrosine kinase activity was assessed by assaying the capacity of immunoprecipitated EGF receptors to incorporate 32P from labeled γ-ATP into receptor tyrosine.
residues. After only 30 min of A431 cell culture with TGF-β, the kinase activity rose by 25%, and after 3 h of culture in the presence of TGF-β, the kinase activity was elevated to 2.2-fold higher than its basal level observed in the absence of TGF-β (from 66,000 cpm to 155,000 cpm) (Fig. 8). After 72 h of culture with TGF-β, kinase activity was 3-fold greater than the basal level (data not shown).

Discussion

TGF-β was originally described as a growth factor which interacted with TGF-α to produce phenotypic transformation of NRK fibroblasts (7). It can stimulate proliferation of some fibroblasts, and it has been found to inhibit proliferation of epithelial and lymphoid cells (3). In cells normally stimulated by EGF to proliferate, a role of TGF-β as a negative growth regulator was hypothesized. In these cells, EGF-induced mitogenesis could be blocked by TGF-β with subsequent accumulation of cells in the G0–G1 phase of the cell cycle. The result was failure to synthesize DNA and block of EGF-induced c-myc expression (20, 21, 24). It was also suggested that growth inhibition by TGF-β is modulated by changes in EGF receptor expression and affinity (20, 25).

In fibroblastic cells, TGF-β appears to stimulate growth by an indirect mechanism (11). Treatment of monolayer cultures of AKR-2B cells with TGF-β caused a rapid induction of c-sis mRNA, followed by the appearance of platelet-derived growth factor. Thus, TGF-β may stimulate growth indirectly via the mitogenic polypeptide platelet-derived growth factor. By contrast, TGF-β is a potent growth inhibitor in all normal epithelial cells studied. The growth of many neoplastic epithelial cells is also inhibited by TGF-β. Inasmuch as the antiproliferative effect on epithelial cell growth is reversible, the growth-inhibitory properties of TGF-β do not appear to result from cytotoxicity.

In KB cells, although TGF-β itself did not inhibit growth, it augmented the growth inhibition by EGF (26). Contrary to our data, others have reported stimulation of A431 vulvar squamous carcinoma cell growth by TGF-β (27). The differences in effects of TGF-β in A431 cells may evolve from different origins of the A431 cell lines. However, the inhibitory effect of TGF-β in our studies is very consistent and reproducible.

The effect of TGF-β on EGF receptor phosphorylation has been studied in hepatocytes (28). Although EGF receptors in hepatocytes are not affected by TGF-β to the same extent as by EGF, TGF-β has a considerable stimulatory effect on phosphorylation of membrane preparations of EGF receptors (28).

Recent studies showed that TGF-β and EGF can exert a cooperative effect at the transcriptional level (2, 29). Although it is felt that the biological responses of cells to TGF-β are exerted at a level distal to growth-activating factors (30), it is possible that the induction of phosphorylation of the EGF receptor by TGF-β could result from interactions with other regulatory factors (e.g., TGF-α), the expression of which might be enhanced after
treatment of A431 cells with TGF-β. However, when TGF-β effects on the steady-state levels of TGF-α message were studied in A431 cells, no change in the level of TGF-α message was observed (data not shown).

An important point is that the cellular response to TGF-β, as to other growth factors, depends on the context in which it is presented; the same growth factor can have quite different, even opposite effects on different cell lines or on the same cell line, depending on the cell density or the presence or absence of other factors (3). Presently, there are no data that can explain the variety of effects of TGF-β that have been observed with various cell lines.

Our study provides potential molecular insights into the mechanisms of action of TGF-β. TGF-β stabilizes the EGF receptor, slows down its degradation, and causes excess EGF receptor phosphorylation in A431 carcinoma cells. This is not reflected in a higher number of EGF binding sites on the cell surface. When binding of [125]I-EGF to its receptor was measured in A431 cells, contrary to endothelial cells (12) and to NRK fibroblasts (19), the abundance of exposed binding sites and the affinities for binding [125]I-EGF did not change in response to TGF-β (data not shown). This, together with the observation that the [15S]methionine-labeled protein has a longer half-life, suggests that the amount of receptor in intracellular pools is increased in TGF-β-treated cells.

As reported here, incubation of A431 cells with TGF-β induced an absolute and relative increase in the phosphorylation of EGF receptor tyrosines. Moreover, the kinase activity of the EGF receptor immunoprecipitated from A431 cell lysates was increased. The EGF receptor tyrosine kinase was activated as an early event, and autophosphorylating capacity was demonstrated to reach nearly maximal levels after 3 h of culture with TGF-β. A similar effect on EGF receptor kinase was recently reported to occur when MΦ-180 cervical carcinoma cells were treated with TNF (31). These studies demonstrated that TNF was able to rapidly (within 30 min) modulate tyrosine kinase activity of EGF receptors on tumor cell lines which were sensitive to the cytotoxic effects of TNF, but did not alter EGF receptor kinase activity in TNF-resistant tumor cells.

Several distinctions exist between the actions of EGF and TGF-β on the EGF receptor. The kinetics of kinase activation by TGF-β demonstrate that the onset of activation requires longer exposure to TGF-β than that required for activation by EGF. These observations are consistent with TGF-β activation of EGF receptor by an indirect mechanism. This is supported by the observation that TGF-β was unable to directly stimulate kinase activity in cell lysates (data not shown) in contrast with direct EGF action. This suggests that additional components of intact cells may be required for TGF-β activation of EGF receptor kinase activity. However, protein synthesis is not required for the effect of TGF-β upon EGF receptor phosphorylation, since cycloheximide pretreatment of the cultures did not change the response (data not shown).

Studies of EGF receptor metabolism demonstrate another potentially significant distinction between the actions of EGF and TGF-β. EGF stimulation of kinase activity was followed by an apparent down-regulation of both receptor protein and kinase activity, which may be due to internalization and degradation of the EGF-EGF receptor complex. In contrast, we were unable to demonstrate significant changes in EGF receptor internalization following 3 h of TGF-β treatment based upon [125]I-labeled EGF binding analysis and the half-life of metabolically labeled ([15S]methionine) EGF receptor, which was

### Table 2: Phosphoamino acid two-dimensional analysis of the EGF receptor

<table>
<thead>
<tr>
<th>Experiment</th>
<th>cpm* (±% in parentheses)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-Tyr</td>
</tr>
<tr>
<td>A. Control</td>
<td>4,900 (100)</td>
</tr>
<tr>
<td>B. TGF-β (30 pm)</td>
<td>7,900 (248)</td>
</tr>
<tr>
<td>C. EGF (20 nm)</td>
<td>11,000 (408)</td>
</tr>
<tr>
<td>D. TGF-β (30 pm) + EGF (20 nm)</td>
<td>19,000 (404)</td>
</tr>
</tbody>
</table>

*Rep Representative of seven experiments.

**1**P counts/min are normalized for a total yield of 22 ± 1%. Experimental details are given in the legend to Fig. 6.
The identification of the phosphorylation sites in the EGF receptor is important for an understanding of receptor function. Since EGF and TGF-β induce additive tyrosine phosphorylation of the EGF receptor, phosphopeptide maps of EGF receptors from cells stimulated with EGF and with TGF-β were compared through high performance liquid chromatography. These preliminary studies did not show any new phosphorylation sites of EGF receptor when cells were treated with TGF-β. It is presently being investigated whether this indicates that TGF-β activates the same tyrosine sites in an additional population of EGF receptor molecules.

There may be a quantitative relationship between EGF receptor kinase activity and growth response (23), and when an optimal amount of kinase activation is exceeded, growth inhibition may result. Although the TGF-β receptor was shown not to have an intrinsic kinase activity (32, 33), its effects on proliferation may potentially be transduced via other kinases, including the kinase activity of EGF receptor. It also is well established that TGF-β induces the expression of a variety of cellular proteins. It is therefore possible that TGF-β induces the expression of phosphatase-suppressing activity or that it reduces the expression of phosphatase activity (34–37), either of which could contribute to its effects upon the activity of the EGF receptor and other cellular kinases.

The present studies demonstrate that incubation with TGF-β stimulates EGF receptor tyrosine phosphorylation in intact A431 cells and modulates EGF receptor tyrosine kinase activity. However, the mechanism of TGF-β-induced modulation of EGF receptor and its precise role in the growth response remain unclear. These studies do not rule out a role for TGF-β as a modulator of other mechanisms involved in cellular growth, but they suggest that the capacity of TGF-β to alter A431 cell proliferation may partially reside in its ability to act as an indirect modulator of the phosphorylation and function of the EGF receptor. The identification of EGF receptor as a target of TGF-β action and the distinction between the actions of EGF and TGF-β on this receptor may supply important clues toward the understanding of mechanisms through which TGF-β interacts with regulatory signal transduction pathways.

### Materials and Methods

**Growth Factors.** Culture grade EGF was purchased from Collaborative Research, Bedford, MA, and TGF-β was purchased from R2D Systems, Inc., Minneapolis, MN.

**Cell Culture.** The A431 cell line (22) was grown in monolayer culture with Dulbecco's modified Eagle's medium:Ham's F-12 (1:1) containing 5% NCS.

**Cell Proliferation.** For direct cell counts, 5 × 10⁴ A431 cells were plated into individual wells of six-well plates.
Table 4  Time course analysis of tyrosine-specific phosphorylation of EGF receptor by exposure to TGF-β

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time of incubation (h)</th>
<th>PY69-1 (cpm [35S])</th>
<th>Fold of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.33</td>
<td>1,200</td>
<td>1</td>
</tr>
<tr>
<td>100 ng TGF-β</td>
<td>0.33</td>
<td>2,000</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3,500</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10,000</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>7,000</td>
<td>5.8</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>1,100</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1,200</td>
<td>1.0</td>
</tr>
<tr>
<td>20 ng EGF</td>
<td>0.33</td>
<td>14,000</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12,000</td>
<td>10</td>
</tr>
</tbody>
</table>

*PY69, antiphosphosyrosine mAb.

and incubated 24 h at 37°C before the addition of TGF-β or EGF to the culture media (1 ml/well). At different times after effector addition, wells were gently rinsed three times with PBS, and cells were released by trypsinization and pelleted by centrifugation (10 min at approximately 400 x g). Cells were resuspended in PBS and counted with a Coulter Counter (Coulter Electronics, Inc.). Each data point represents the average ± SE (5%) of four determinations.

Cell Cycle Analysis. Log-phase populations were harvested by trypsinization, counted, and replated in 100-mm culture dishes at 5 x 10⁵ cells/dish in DMEM/F-12 with 5% NCS and no growth factor supplements. Cells were incubated 24 h for attachment, the medium was removed, and fresh DMEM/F-12 with 1% NCS was added. Control plates received medium with 1 or 5% NCS and no further treatment. Two plates were treated with 100 ng EGF, two with 30 pm TGF-β, and two with 10 nm EGF plus 30 pm TGF-β. The cells were incubated further 24 or 48 h before they were harvested by trypsinization, pelleted, and gently resuspended in PBS. These cells were held on ice until they could be lysed and the nuclei could be stained.

The percentage of cells in different phases of the cell cycle was determined by flow cytometry (38-41). Briefly, cells were stained with propidium iodide (Sigma) and passed through the beam of an argon ion laser turned to 514 (FAC/Scan, Becton Dickinson). The resulting fluorescent signal was amplified, recorded in the memory, and analyzed in the form of a DNA histogram by using a computer program interfaced with the integrator.

EGF Receptor Phosphorylation. A431 cells grown in 35-mm culture dishes were equilibrated in phosphate-free medium containing 5% FBS and 1 mCi/ml [123P] phosphate for 12-16 h. Cells were treated with effector as noted. Monolayers were rinsed twice in ice-cold PBS, and cells were solubilized in 0.5 ml of lysis buffer containing 50 mM HEPES, (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 2 mM NaVO₃, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF. The lysates were incubated on ice for 5 min and then centrifuged at maximal speed in an Eppendorf microcentrifuge for 5 min. The supernatant was collected and stored at -70°C. After immunoprecipitation, the EGF receptor was released into sample buffer and separated on a 7% SDS-PAGE, as described below.

[35S]Methionine Labeling and Receptor Degradation. A431 cells were plated at 10⁵ cells/35-mm culture dish in complete medium. After a 12-h period to allow attachment, the medium was replaced by 1 ml fresh labeling medium containing 100 μCi of [35S] methionine/ml and

Time(min):  0  30  60  90  120  180

Fig. 8. EGF receptor tyrosine kinase activity in response to treatment of A431 cells with TGF-β. EGF receptor kinase activity was measured at different time points by the immune complex kinase assay after incubation of A431 cell cultures with TGF-β as described in 'Materials and Methods.' Cerenkov counts (cpm) of the presented bands in the autoradiography were as follows: 0 min = 66,000; 30 min = 80,000; 60 min = 92,000; 90 min = 99,000; 120 min = 111,000; an 180 min = 155,000.
1% dialyzed FBS. The cells were metabolically labeled for 14 h and then chased with medium containing 1% dialyzed serum, unlabeled methionine, and different concentrations of either TGF-β or EGF. At different time points, the cells were solubilized in 0.3 ml lysis buffer with no Na3VO4 as described above, and then immunoprecipitated and separated on 7% SDS-PAGE.

**Immunoprecipitation.** Immunoprecipitating mAb 528 to the EGF receptor was utilized as adapted from Sunada et al. (42) and as described by Honegger et al. (43). Sixty μg mAb 528 were first complexed to 40 mg PAS (Sigma) by coincubation in 20 mM HEPES (pH 7.5) for 1 h at 22°C. The PAS-mAb complex was washed three times with HNTG buffer [20 mM HEPES-150 mM NaCl-0.1% Triton X-100-10% glycerol (pH 7.5)] and incubated. Three mg sample (the equivalent of 4.5 μg mAb 528) was incubated for 90 min at 4°C with 0.25-ml portions of cell lysates containing equal quantities of TCA-prepitable radioactivity. The PAS-mAb complex containing bound EGF receptor was washed three times with HNTG. EGF receptor was released into sample buffer [10% glycerol-0.7 M β-mercaptoethanol-3% SDS-62.5 mM Tris-HCl buffer (pH 6.8)] containing bromophenol blue by boiling at 100°C for 5 min and then separated on a 7% SDS-PAGE. Labeled EGF receptor was visualized by autoradiography and quantified by counting of the excised bands.

**Phosphoamino Acid Two-Dimensional Analysis of the EGF Receptor.** Phosphoamino acid analysis of immunoprecipitated EGF receptor separated on the SDS-PAGE was performed by partial acid hydrolysis (1 h at 110°C in 6 M HCl) and thin layer electrophoresis by the method of Cooper et al. (44). After removal of the acid by drying under vacuum, hydrolysates were resuspended in 250 μl of H2O and applied to a Dowex AGI-X8 column (Bio-Rad). The absorbed 32P-labeled materials were eluted with 0.5 M HCl and lyophilized. The recovery of radioactivity through the procedure was about 65%. 32P-labeled phosphoamino acids were analyzed by thin layer electrophoresis of an aliquot of each digest, as described (44). Individual phosphoamino acids were detected by ninhydrin staining of carrier phosphoamino acids, and radioactivity within each phosphoamino acid was measured by liquid scintillation chromatography.

**Tyrosine Phosphorylation of the EGF Receptor in Intact Cells by Western Blot Analysis.** Cells, treated with either TGF-β or EGF as described above, were solubilized in Western solubilization buffer (20 mM HEPES-1% Triton X-100-5 mM MgCl2-120 mM KCl-10% glycerol-2 mM Na3VO4-1 mM PMSF-10 μg/ml aprotinin-10 μg/ml leupeptin). Lysates were mixed with sample buffer, and equal amounts of total protein, predetermined by Bio-Rad assay, were loaded onto SDS-PAGE. Proteins were transferred to nitrocellulose at 100 mA utilizing a Trans-Blot apparatus (Bio-Rad). The state of tyrosine phosphorylation of the EGF receptors was investigated by using a monoclonal antiphosphotyrosine antibody (PY69; ICN) and 125I-protein A as described (45). Total EGF receptor was detected with RKII polyclonal antibodies as described (46).

**Measurement of EGF Receptor Tyrosine Kinase Activity.** Cells grown in culture dishes were treated with effector for various intervals as noted. Monolayers were rinsed three times with ice-cold PBS, and cells were lysed and immunoprecipitated as described before. Immune complexes were washed three times with 0.1% Triton X-100 in PBS (pH 7.4) and resuspended in 25 μl of 20 mM HEPES (pH 7.4)-100 μM Na3VO4-0.1% Triton X-100. EGF receptor kinase activity assay was initiated as described (31) by the addition of 25 μl of 20 mM HEPES-0.1% Triton X-100-6 mM MnCl2 buffer containing 10 μCi of [32P]ATP (in a total of 5 μM ATP) to each sample. The reaction was incubated 10 min at 4°C and terminated by the addition of 1 ml of ice-cold lysis buffer with 2 mM Na3VO4 and with the addition 0.1% SDS and 5 mM sodium pyrophosphate. Immune complexes were washed two additional times. Pellets were heated in sample buffer and subjected to 7% SDS-PAGE. Gels were dried and subjected to autoradiography, and EGF receptor autophosphorylated bands were excised and quantified by Cerenkov counting.

**Acknowledgments.**

We wish to thank Dr. F. Traganos for assistance in the fluorescence-activated cell sorter analysis. Dr. A. Goldenberg for initial assistance in EGF binding experiments, and F. Paul for expert typing of this manuscript. The RKII polyclonal antibody against the COOH-terminal portion of the EGF receptor was kindly given to us by Dr. J. Schlessinger.

**References.**

15. Igniot, R. A., Heino, J., Hemler, M., and Massague, J. Regulation of


