Transforming Growth Factor-β Modulates the High-affinity Receptors for Epidermal Growth Factor and Transforming Growth Factor-α

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ABSTRACT The epidermal growth factor (EGF) receptor mediates the induction of a transformed phenotype in normal rat kidney (NRK) cells by transforming growth factors (TGFs). The ability of EGF and its analogue TGF-α to induce the transformed phenotype in NRK cells is greatly potentiated by TGF-β, a polypeptide that does not interact directly with binding sites for EGF or TGF-α. Our evidence indicates that TGF-β purified from retrovirally transformed rat embryo cells and human platelets induces a rapid ($t_{1/2}$ = 0.3 h) decrease in the binding of EGF and TGF-α to high-affinity cell surface receptors in NRK cells. No change due to TGF-β was observed in the binding of EGF or TGF-α to lower affinity sites also present in NRK cells. The effect of TGF-β on EGF/TGF-α receptors was observed at concentrations (0.5–20 pM) similar to those at which TGF-β is active in promoting proliferation of NRK cells in monolayer culture and semisolid medium. Affinity labeling of NRK cells and membranes by cross-linking with receptor-bound ^{125}I-TGF-α and ^{125}I-EGF indicated that both factors interact with a common 170-kD receptor structure. Treatment of cells with TGF-β decreased the intensity of affinity-labeling of this receptor structure. These data suggest that the 170 kD high-affinity receptors for EGF and TGF-α in NRK cells are a target for rapid modulation by TGF-β.

Transforming growth factors (TGFs)[1] are hormonally active polypeptides that induce a transformed phenotype when added to normal cells (1). The transformed phenotype in normal rat kidney (NRK) cells, a known target cell line for TGFs (1), is induced by the synergistic interaction of at least two types of TGFs (2, 3). The first type, or TGF-α, is an analogue of epidermal growth factor (EGF) and is released by various neoplastic cell lines (1, 3, 4, 5). EGF and TGF-α are 6-kD polypeptides that exhibit significant amino acid sequence homology (3, 5, 6), and similar affinity and mode of interaction with a common receptor type (6, 7). The second type of TGF, or TGF-β, is a 23–25 kD molecule which consists of two disulfide-linked 11–12 kD polypeptide chains (8–11). TGF-β binds to specific receptors that do not recognize other growth factors (12–14). TGF-β has no transforming action on NRK cells when it acts alone but strongly potentiates the transforming action of TGF-α and EGF (8–11). TGF-β is found in normal tissues including human platelets, human placenta and bovine kidney (8–10), and in retrovirally transformed fibroblasts that also produce TGF-α (2, 3).

The biochemical events elicited by the interaction of TGF-β with its receptor are unknown at present. However, it has been shown that the EGF receptor is a target for regulation by agents that complement the proliferative action mediated by this receptor type. These include platelet-derived growth factor (15–18) and phorbol diester tumor promoters (19–23). The modulation of EGF receptors appears to be mediated by receptors specific for each of these agents. These receptor–receptor interactions are of interest because they can help identify primary biochemical events involved in the cellular actions mediated by growth factor receptors. Thus, direct phosphorylation of certain serine and threonine residues in

[1] *Abbreviations used in this paper:* C18 TGF-β, TGF-β purified through the C_{18} reverse phase chromatography step; EGF, epidermal growth factor; FeSV-Fre, Synder-Theilen feline sarcoma virus–transformed rat embryo (cells); NRK, normal rat kidney; TGF, transforming growth factor.

THE JOURNAL OF CELL BIOLOGY · VOLUME 100 MAY 1985 1508-1514

the EGF receptor by protein kinase C, the putative cellular receptor for tumor-promoting phorbol esters, has been recently implied as the biochemical basis for the modulatory action of phorbol esters on EGF receptors (22, 24). Because of the biological interaction between TGF-β and EGF analogues, we examined whether the action of TGF-β on target cells also involves modulation of EGF/TGF-α receptors. The results indicate that TGF-β from Snyder-Theilen feline sarcoma virus–transformed rat embryo (FeSV-Fre) cells and human platelets induces a rapid decrease in the binding of EGF and TGF-α to high-affinity receptors in NRK cells.

MATERIALS AND METHODS

Growth Factors: TGF-α and TGF-β from serum-free media conditioned by FeSV-Fre cells were purified to homogeneity as described previously (3, 11). TGF-β from human platelets (9) was purified to homogeneity by a modification (14) of a previously described method (11). No difference has been found in molecular structure or biological properties between TGF-β and FeSV-Fre cells and human platelets. When indicated, preparations of TGF-β from FeSV-Fre cells purified 2,500-fold through the C_{18} μBondapak reverse phase chromatography step (C18 TGF-β) (11) were used instead of homogeneous preparations of TGF-β. The purity of C18 TGF-β preparations estimated by the two bioassays described below ranged between 0.7 and 4%. Mouse submaxillary gland EGF was purified as described (25, 26). TGF-α and EGF were labeled with ^{125}I by the lactoperoxidase–glucose oxidase method as described previously (7) to a specific activity of 60–70 Ci/g and 80–100 Ci/g, respectively.

Cells and Membrane Preparations: NRK clone 49F cells (Dr. J. E. DeLarco, National Cancer Institute, and American Type Culture Collection, CRL 1570) were cultured in Dulbecco's modified Eagle's medium supplemented with 5% calf serum plus nonessential amino acids (Gibco Laboratories, Grand Island, NY) or with 10% calf serum alone, respectively. Membranes from NRK and A431 cells were isolated as described (7).

Treatment of Cells with TGF-β: NRK cells (6×10^4 cells/well) or A431 cells (2×10^4 cells/well) were plated on 35-mm wells and incubated for 2 d at 37°C with 2.0 ml of Dulbecco's modified Eagle's medium supplemented with 10% calf serum. The sparse cell monolayers were then washed with Waymouth's medium that contained 0.2% calf serum, and were incubated for 16–24 h at 37°C in 1.0 ml of this medium before additions of TGF-β were made. TGF-β lyophilized in the presence of 5–10 μg of bovine serum albumin (BSA) and reconstituted in 50 μl of Waymouth's medium was added to the wells to reach the desired final concentrations. Incubations continued for the indicated time at 37°C and were arrested as described below for binding measurements. In a control experiment described below, incubation of cells with TGF-β at 0–4°C was done in the presence of binding buffer instead of culture medium.

Binding Assays: At the end of the treatment with TGF-β, the medium was replaced with ice cold binding buffer (125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 2 mg/ml of BSA, and 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonate, pH 7.4). Cell monolayers were then incubated at 0–4°C with 1.5 ml of binding buffer that contained ^{125}I-TGF-α or ^{125}I-EGF at 0.3 nM final concentration unless otherwise indicated. Incubations proceeded for 3.5 h on a platform oscillating at 2 cycles/s. The medium was then aspirated, and the monolayers were washed four times with ice cold binding medium. The cell-associated radioactivity was determined after solubilization of cells with two consecutive 0.5-ml portions of 1% Triton X-100 solution in phosphate-buffered saline (PBS). Nonspecific binding, defined as the binding of ^{125}I-labeled ligands measured in the presence of 0.2 μM EGF, was routinely 8–15% of the total binding and was subtracted in all cases. Nonspecific binding was not changed by treatment of cells with TGF-β. Total binding was in all cases no more than 0.5% (NRK cells) on 7% (A431 cells) of the input radioactivity when ^{125}I-TGF-α or ^{125}I-EGF were present at 0.3 nM concentration in the assay. At least 90% of the ^{125}I-TGF-α and ^{125}I-EGF bound to cells after 3.5 h of incubation at 0–4°C was bound to cell surface receptors as determined by its ability to dissociate from the cells after a 3-min incubation in the cold with acetate-buffered solution at pH 4.5 (27). All binding determinations were done in triplicate. Cell numbers were determined with a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) after cells were detached from the culture vessels by a short exposure to an isotonic solution that contained 0.2% trypsin and 0.02% EDTA.

Binding of ^{125}I-TGF-α and ^{125}I-EGF to isolated membranes was determined by incubation of membranes with ligands in 250 μl of binding medium at 22°C. After 60 min, samples were placed on ice, diluted with 4.0 ml of ice cold binding medium, and filtered under negative pressure through 0.2 μM cellulose ester microporous filters (Amicon Corp., Danvers, MA). Filters were washed twice with 4.0-ml portions of cold medium and counted in a gamma counter.

Receptor down-regulation experiments were done with A431 cells plated in 16-mm wells (8×10^3 cells/well). 48 h after seeding, the spent medium was removed, and 1.0 ml of fresh medium with the indicated concentrations of TGF-β, TGF-α, or EGF was added. Incubations continued at 37°C for 16 h and were terminated by aspiration of the media. Cells were prepared for determination of ^{125}I-TGF-α binding as described in detail previously (7). Degradation of TGF-α and EGF during incubation for 16 h at 37°C with A431 cells in receptor down-regulation experiments was significant (8–15% of hormone initially added) only at the lowest (0.1–0.5 nM) concentrations of TGF-α and EGF tested in these experiments, as determined by a standard radioreceptor assay (7). The concentration values in the experiment shown in Fig. 3 have been corrected to account for this limited decrease in the concentrations of active hormone during experimentation.

Receptor Affinity-labeling: Sparse (2.5×10^6 cells/dish) NRK cell monolayers in 150-mm cell culture dishes were incubated in the cold with 15 ml of binding medium that contained 0.33 nM ^{125}I-TGF-α or 0.33 nM ^{125}I-EGF and unlabeled ligands as indicated. After 3.5 h, this medium was aspirated and cells were washed four times with ice cold binding medium. 25 ml of binding medium plus 320 μl of 27 mM disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL) in dimethyl sulfoxide (28) were immediately added to the cells, and incubation proceeded for 10 min at 0–4°C. Cells were then washed twice with 0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.0, detached from the plates by gentle scraping in the presence of this buffer, and centrifuged at 1,000 *g* for 5 min. The cell pellet was resuspended with 10 mM Tris, 1 mM EDTA, pH 7.0, and solubilized by heating for 3 min at 100°C with 100 μl of twice-concentrated electrophoresis sample buffer (29). Insoluble material was removed by centrifugation at 12,000 *g* for 10 min. Samples were electrophoresed on dodecyl sulfate–polyacrylamide slab gels as described (29). Electrophoresis gels were fixed, stained for protein, dried, and subjected to autoradiography as described (30). Molecular weight protein standards were carbonic anhydrase (30 kD), ovalbumin (45 kD), BSA (68 kD), phosphorylase b (94 kD), β-galactosidase (116 kD), and myosin (200 kD).

Affinity labeling of receptors in isolated membrane preparations was done essentially as described before (31) using 0.20 mM disuccinimidyl suberate. The efficiency of affinity labeling of cells and membranes using this methodology is ~7 and 8%, respectively, with ^{125}I-TGF-α, and ~6 and 4%, respectively, with ^{125}I-EGF (reference 7; and Massagué, J., unpublished results).

Bioassays for TGF-β: For the assay of [^{3}H]thymidine incorporation into DNA, sparse NRK cell monolayers were treated with TGF-β as described above. After 16 h of incubation with TGF-β, 0.5 μCi of [methyl-^{3}H]thymidine (New England Nuclear, Boston, MA) in 25 μl of Waymouth's medium was added to the cells. 8 h later, wells were washed twice with PBS, and cells were solubilized with 0.5 ml of 10 mM Tris, 5 mM EDTA, 0.5% sodium dodecyl sulfate, pH 7.4. Lysates were transferred to tubes which contained 1.5 ml of ice cold 10% trichloroacetic acid. Acid-precipitable material was recovered by filtration on 0.2 μm microporous cellulose filters. Filters were washed twice with 10% trichloroacetic acid and counted for radioactivity in the presence of 4.0 ml Econofluor (New England Nuclear). Control experiments in this laboratory have demonstrated that under the conditions of our assay, a strictly linear relationship exists between [^{3}H]thymidine incorporation into DNA and the index of nuclear labeling by [^{3}H]thymidine as determined by autoradiography of fixed cell monolayers. Thus, basal incorporation of [^{3}H]thymidine into growth-arrested NRK-49F monolayers is obtained under conditions in which 2–4% of the nuclei become labeled; maximal [^{3}H]thymidine incorporation elicited by full serum supplementation is paralleled by > 95% nuclear labeling; and a fractional stimulation of [^{3}H]thymidine incorporation by TGF-β or other mitogens is closely met by the corresponding increase in the percent of labeled nuclei.

The assays for induction of anchorage-independent growth of NRK cells was performed in the presence of 0.3 nM mouse submaxillary EGF, and read as previously described (2).

RESULTS

TGF-β Decreases the Binding of TGF-α and EGF to Mitogenically Responsive NRK Cells

Sparse monolayers of NRK cells incubated for 24 h in medium that contained 0.2% calf serum were used in this study because under these conditions, NRK cells respond mitogenically to TGF-β as determined by [^{3}H]thymidine in-

corporation into DNA, nuclear labeling determinations, and cell number measurements (reference 11; and Massagué, J., unpublished results). TGF-β from FeSV-Fre cells and human platelets markedly stimulated the incorporation of [^{3}H]thymidine into acid-precipitable material at concentrations (0.5–20 pM) which were also effective in inducing anchorage-independent proliferation of NRK cells in the presence of EGF analogues (Fig. 1 *B* and reference 11). We examined the ability of TGF-β–treated NRK cells to bind ^{125}I-TGF-α and ^{125}I-EGF. The binding of ^{125}I-TGF-α and ^{125}I-EGF to NRK cells was determined at 0–4°C to minimize the contribution of post-binding events, i.e., internalization, intracellular degradation and release, or retention of ^{125}I-ligand, that rapidly occur at higher temperatures (27, 32, 33) and might lead to erroneous quantitations of cell surface receptors. Incubation at 37°C with TGF-β from FeSV-Fre cells or human platelets decreased the ability of NRK cells to bind ^{125}I-TGF-α or ^{125}I-EGF when radioligands were present in the binding assay at a subsaturating (0.3 nM) concentration (Fig. 1*A*). The effect of TGF-β on TGF-α and EGF binding to NRK cells was

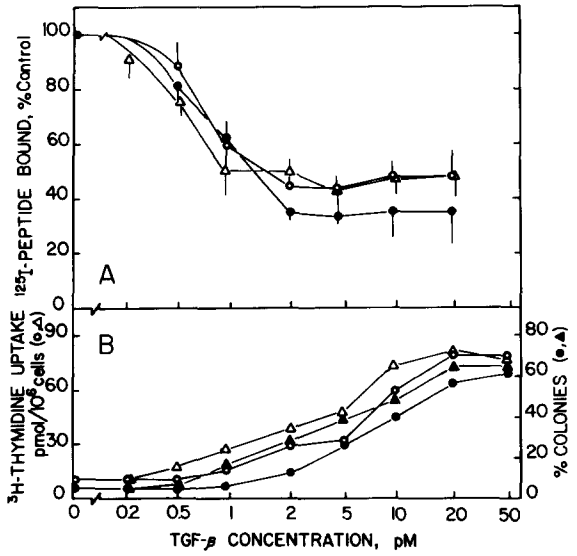

FIGURE 1 Dose dependent inhibition of TGF-α and EGF binding to NRK cells by TGF-β. (*A*) Sparse NRK monolayers were incubated for 24 h at 37°C with medium that contained 0.2% calf serum. The indicated concentrations of TGF-β from FeSV-Fre cells (○, ●) or human platelets (△) were then added to the cells, and incubations continued for 2 h at 37°C. The medium was then aspirated, and monolayers were incubated for 3.5 h at 0–4°C with binding buffer that contained 0.3 nM ^{125}I-TGF-α (●) or 0.3 nM ^{125}I-EGF (○, △). The specifically bound radioactivity was determined after the unbound ligands were washed out. Data are the mean (±SD, *bars*) of triplicate determinations and are expressed as the percentage of the specific binding of ^{125}I-TGF-α (810 cpm/well) or ^{125}I-EGF (390 cpm/well, ○; 405 cpm/well, △) respectively, to cells not treated with TGF-β. The data corresponding to FeSV-Fre cell-derived TGF-β and platelet-derived TGF-β were obtained in separate experiments. Five repeated experiments with TGF-β from either source yielded similar results. (*B*) Induction of [^{3}H]thymidine uptake by various concentrations of TGF-β from FeSV-Fre cells (○) or human platelets (△) added to NRK monolayers cultured as described above, and proportion of NRK cells that developed large colonies in semisolid medium in the presence of 0.3 nM EGF plus the indicated concentrations of TGF-β from FeSV-Fre cells (●) or human platelets (▲). Data are the mean of duplicate determinations. The incorporation of [^{3}H]thymidine by NRK cell monolayers treated with 10% calf serum was 185–215 pmol/10^6 cells.

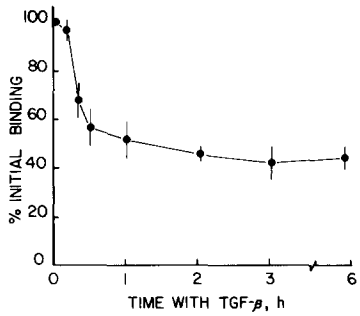

FIGURE 2 Time-dependent inhibition of ^{125}I-EGF binding to NRK cells by TGF-β. Sparse NRK monolayers were incubated for 30 h at 37°C in the presence of 0.2% calf serum-supplemented medium. At the indicated times before the end of this incubation, 100 pM TGF-β from human platelets was added to the cell cultures. After this incubation, the medium was aspirated, and monolayers were incubated for 3.5 h at 0–4°C in the presence of ice cold binding buffer that contained 0.3 nM ^{125}I-EGF. The specifically bound radioactivity was then determined, and is plotted as the percentage of ^{125}I-EGF binding (785 cpm/well) to monolayers not treated with TGF-β. Data are the mean (±SD, *bars*) of triplicate determinations. This experiment was repeated twice with similar results.

TABLE I

Competition of EGF, TGF-α, and TGF-β with ^{125}I-TGF-α for Binding to Isolated Membranes

Membrane source	Competing ligand	^{125}I-TGF-α bound
		fmol/mg protein
NRK cells	None	5.4
	66 nM TGF-α	1.2
	66 nM EGF	1.6
	66 nM TGF-β	5.6
	330 nM TGF-β	5.6
A431 cells	None	2220
	66 nM TGF-α	140
	66 nM EGF	140
	66 nM TGF-β	2130
	330 nM TGF-β	2170

Membranes were incubated for 60 min at 22°C in the presence of 0.25 nM ^{125}I-TGF-α alone or with the indicated concentrations of TGF-α, EGF, or platelet-derived TGF-β. Total binding was then determined as described in Materials and Methods. Data are the mean of duplicate determinations.

concentration-dependent and was half-maximal at ~0.5–1 pM TGF-β (Fig. 1*A*). This effect was also rapid and time-dependent, being half-maximal after ~20 min, and was completed after ~2 h of cell exposure to TGF-β (Fig. 2). It persisted for at least 6 h after addition of TGF-β (Fig. 2). The modulation of ^{125}I-TGF-α and ^{125}I-EGF binding to NRK cells by TGF-β was temperature-dependent. No effect on ^{125}I-TGF-α or ^{125}I-EGF binding was observed in NRK cells exposed to TGF-β at 0–4°C instead of 37°C (not shown).

TGF-β did not appear to interact directly with EGF/TGF-α binding sites. Thus, biologically saturating concentrations of TGF-β did not compete with ^{125}I-TGF-α for binding to membrane preparations from NRK or A431 cells (Table I). No detectable decrease in the binding of 50 pM ^{125}I-TGF-α or 50 pM ^{125}I-EGF to A431 cells was observed when cells were exposed for 2 h at 37°C to concentrations of TGF-β that were active on NRK cells (not shown). Exposure of A431 cells to TGF-α or EGF decreases the number of cell surface binding sites for these two ligands in parallel (7). This receptor down-regulation phenomenon is a function of the persistent occupancy of binding sites by the corresponding ligand (33,

34). However, biologically active concentrations of TGF-β did not cause down-regulation of EGF/TGF-α receptors in A431 cells under conditions in which TGF-α and EGF did (Fig. 3). These data suggest that the modulation of TGF-α and EGF binding to NRK cells by TGF-β involves an indirect mechanism that is operative in NRK cells but is apparently missing in A431 cells.

TGF-β Decreases Binding of EGF and TGF-α to High-affinity Cell Surface Receptors

To gain more information on the nature of the change in EGF/TGF-α receptors induced by TGF-β on NRK cells, we analyzed the binding of various concentrations of ^{125}I-TGF-α to control and TGF-β–treated cells. Scatchard analysis of the binding of ^{125}I-TGF-α to control NRK monolayers at 0–4°C yielded a curvilinear isotherm (Fig. 4). The curvilinearity of Scatchard plots in other hormone receptor systems has been explained by either negative cooperativity among binding sites whereby increasing occupancy of receptors by the ligand leads to a decreasing affinity, or the simultaneous presence of binding sites with different affinities for the ligand. However, it has been shown (21) that EGF receptors in rat fibroblasts are not subject to negative cooperativity. Therefore, the simplest interpretation for the complex binding of EGF and TGF-α to receptors in NRK cells is the presence of two classes of binding sites with high and low affinity for the ligand, respectively. According to this interpretation, control NRK cells exhibit on their surface a small (~6×10^3 sites/cell) population of binding sites with high (K_d = 0.2–0.3 nM) affinity for ^{125}I-TGF-α, and a larger (~1.8×10^4 sites/cell) population of binding sites with low (K_d = 2.7–2.9 nM) affinity for this ligand, as estimated graphically from the slope and abscissa intercept of the two rectilinear components of the Scatchard plot (Fig. 4). Treatment of NRK cells for 2 h at 37°C with platelet-derived TGF-β induced a marked decrease in high-affinity binding of ^{125}I-TGF-α to the cells, whereas little or no decrease was observed in binding to the low-affinity sites (Fig. 4). Similar results were obtained when the experiment was repeated with TGF-β from FeSV-Fre cells (not illustrated). Thus, TGF-β appears to affect selectively the high-affinity binding sites for ^{125}I-TGF-α in NRK cells.

A 170-kD Receptor Species is the Target for Modulation by TGF-β

We sought to define structurally the EGF/TGF-α receptor species that was the target for modulation by TGF-β in NRK cells. Intact NRK cells and isolated NRK membranes were cross-linked with bound ^{125}I-TGF-α and ^{125}I-EGF using disuccinimidyl suberate, and were displayed by electrophoresis on dodecyl sulfate–polyacrylamide gels and autoradiography (Fig. 5). NRK membrane preparations affinity-labeled with ^{125}I-TGF-α or ^{125}I-EGF exhibited a 170-kD labeled component (Fig. 5, lanes *a* and *d*) whose labeling could be inhibited by an excess native TGF-α or EGF added during incubation of membranes with ^{125}I-labeled ligands (Fig. 5, lanes *b*, *c*, *e*, and *f*). We identify this 170-kD membrane component as a common receptor species for TGF-α and EGF similar in molecular size and binding properties to EGF/TGF-α receptors in other cell types (7, 33–36). No other specifically labeled components could be identified in cross-linked NRK membranes even at the relatively high (10 nM) concentrations of ^{125}I-TGF-α and ^{125}I-EGF used in these experiments. At these ligand concentrations, various membrane components were labeled in addition to the 170-kD species, but none of them was sensitive to the presence of an excess of native TGF-α or EGF (Fig. 5, lanes *a–f*).

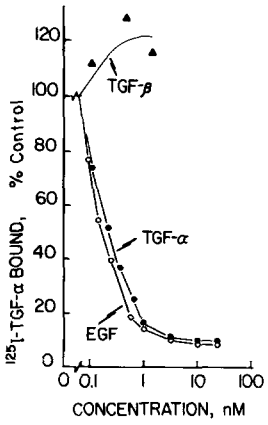

FIGURE 3 TGF-β does not down-regulate EGF/TGF-α receptors in A431 cells. Sparse monolayers of A431 cells were incubated for 16 h at 37°C with fresh culture medium that contained C18 TGF-β (▲), TGF-α (●), or EGF (○). The concentrations of TGF-β present in the assays were estimated by two different bioassays of the partially purified C18 TGF-β preparation (see Materials and Methods). At the end of this incubation, cell surface–bound ligands were dissociated by a brief exposure to medium at acidic pH (27), and the specific binding of 1 nM ^{125}I-TGF-α to the cells was determined. Results are expressed as the percentage of ^{125}I-TGF-α binding (23,500 cpm/well) to monolayers not treated with bioactive peptides. Data are the average of duplicate determinations.

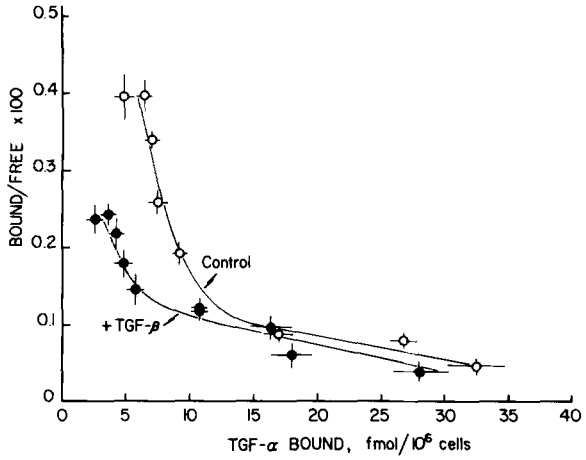

FIGURE 4 TGF-β decreases the high-affinity binding of ^{125}I-TGF-α to cell surface receptors. Sparse NRK monolayers incubated for 24 h in medium that contained 0.2% calf serum received 100 pM TGF-β (●) or no additions (○). Incubations continued for 2 h at 37°C. This medium was then aspirated, and monolayers were incubated at 0–4°C for 3.5 h in the presence of various (0.1–5.0 nM) concentrations of ^{125}I-TGF-α in binding buffer. A Scatchard plot with the amount of ligand specifically bound under these conditions plotted against the ratio of bound over free ligand is shown. Data are the mean (±SD) of triplicate determinations.

A 170-kD EGF/TGF-α receptor species could also be identified after electrophoresis and autoradiography of intact NRK cells affinity-labeled with 0.3 nM ^{125}I-TGF-α or 0.3 nM ^{125}I-EGF (Fig. 5, lanes *g* and *i*). In addition to this 170-kD species, NRK cell preparations also exhibited trace amounts of a 140-kD component specifically labeled with ^{125}I-TGF-α and ^{125}I-EGF (Fig. 5, lanes *g* and *i*; and Fig. 6). This lower M_r species may have originated by limited proteolysis of the affinity-labeled 170-kD receptor species during scraping and homogenization of the affinity-labeled cells, as has been previously documented (37, 38). No other components specifically labeled by ^{125}I-TGF-α or ^{125}I-EGF could be detected in intact NRK cells (Figs. 5 and 6).

When intact NRK cell monolayers were affinity-labeled

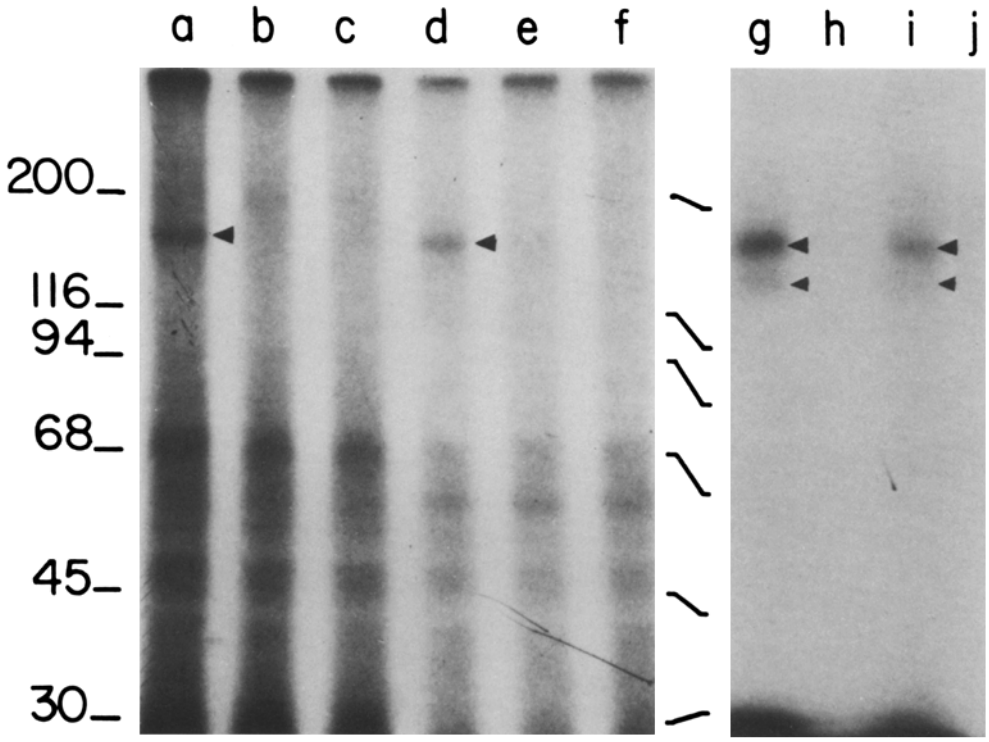

FIGURE 5 Affinity-labeling of EGF/TGF-α receptor species in NRK membranes and intact cells. Alilquots (0.3 mg of membrane protein) of NRK membranes suspended in 0.3 ml of binding medium were incubated for 60 min at 23°C in the presence of 10 nM ^{125}I-TGF-α (lanes *a–c*) or 10 nM ^{125}I-EGF (lanes *d–f*). An excess (1 μM) of unlabeled TGF-α (lanes *b* and *e*) or EGF (lanes *c* and *f*) was also present in the indicated samples. Monolayers of NRK cells were incubated for 3.5 h at 0–4°C in the presence of 0.33 nM ^{125}I-TGF-α (lanes *g* and *h*) or 0.33 nM ^{125}I-EGF (lanes *i* and *j*), alone (lanes *g* and *i*) or with 100 nM EGF (lanes *h* and *j*). At the end of incubations, membranes and cells were washed free of unbound ligand and cross-linked at 0°C with bound ligands using disuccinimidyl suberate as described in Materials and Methods. Electrophoresis of the affinity-labeled samples was performed on a 6–10% polyacrylamide gradient gel (lanes *a–f*) or on a 5–9% polyacrylamide gel (lanes *g–j*). Shown are the autoradiograms of the fixed, dried gels. Numbers indicate the molecular weight in kilodaltons and the position of protein standards run on parallel tracks. Arrowheads point at the 170-kD and the 140-kD labeled species.

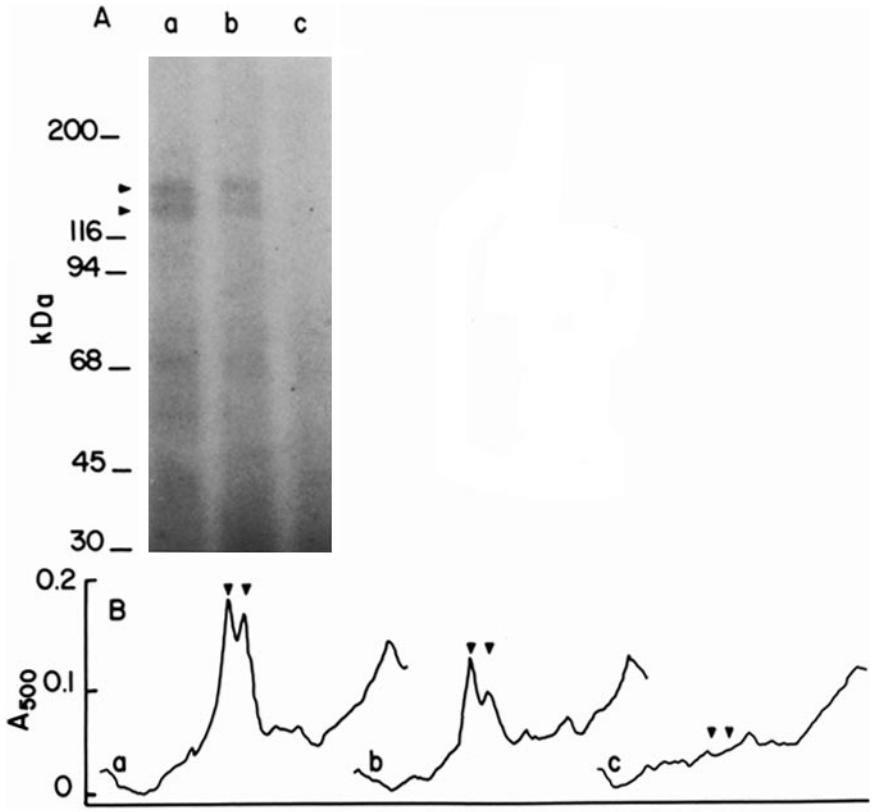

FIGURE 6 TGF-β induces a decrease in the labeling of the 140–170 kD EGF/TGF-α receptor in NRK cells. (*A*) Sparse NRK monolayers cultured for 24 h in medium that contained 0.2% calf serum received 0.2 μg/ml of C18 TGF-β (equivalent to 100 pM TGF-β) (lane *b*) or no additions (lanes *a* and *c*). After 2 h at 37°C, monolayers were washed with binding medium and incubated for 3.5 h at 0–4°C with binding medium that contained 0.3 nM ^{125}I-TGF-α alone (lanes *a* and *b*) or in the presence of 0.2 μM native EGF (lane *c*). Monolayers were then washed, cross-linked to bound ligands, solubilized, and electrophoresed on a 6–10% polyacrylamide gradient gel. An autoradiogram from the fixed, dried gel is shown. (*B*) Densitometry of gel lanes *a*, *b* and *c*, between lane origin and the 68-kD position. Arrowheads point at specifically labeled bands.

with 0.3 nM ^{125}I-TGF-α after preincubation with TGF-β, the intensity of labeling of the 140–170 kD receptor species was only about one half of that in control cells (Fig. 6). This decrease in labeling is consistent with a lower occupancy of the 170-kD receptor species in TGF-β–treated cells. These data suggest that the 170 kD EGF/TGF-α receptor species is the target for modulation of EGF and TGF-α binding to NRK cells by TGF-β.

DISCUSSION

The results reported here indicate that exposure of NRK cells to biologically active concentrations of TGF-β leads to a change in EGF/TGF-α cell surface receptors. In confirmation of previous results (39), we find that EGF/TGF-α receptors in NRK cells that are mitogenically responsive to TGF-β exhibit a complex ligand binding pattern (Fig. 4). Complex binding of ^{125}I-EGF that yields curvilinear Scatchard plots has also been observed in other cell types (7, 16, 20, 21, 32). The simplest interpretation of these binding properties is the presence of a mixed cell surface receptor population minimally consisting of two classes of binding sites with high (K_d = 0.2–0.3 nM) affinity and low (K_d = 2.7–2.9 nM) affinity for the ligand, respectively. According to this model, our data suggest that TGF-β induces a rapid ($t_{1/2}$ = 0.3 h) and persistent inhibition of ligand binding to high-affinity sites on the surface of NRK cells without significantly altering the binding to lower affinity sites. This effect of TGF-β is temperature- and concentration-dependent. The effect on ^{125}I-TGF-α and ^{125}I-EGF binding to NRK cells was half-maximal at 0.5–1.0 pM TGF-β, a concentration lower than the concentration (2–10 pM) of TGF-β required for half-maximal stimulation of [^{3}H]thymidine incorporation into DNA or anchorage-independent proliferation of NRK cells. The apparent discrepancy in the range of TGF-β concentrations required for these two types of effects is likely due to substantial degradation of TGF-β during prolonged (1–10 d) incubation with cells in the mitogenic activity assays.

EGF and TGF-α share a common 170-kD receptor type in NRK cells as determined by receptor-affinity labeling methodology (Figs. 5 and 6). The sharing of a common receptor species by EGF and TGF-α has been documented in other cell types as well (1, 7, 39, 40). The decreased affinity labeling of this receptor species in TGF-β–treated NRK cells as compared with untreated cells is consistent with it being the target for TGF-β regulation of TGF-α and EGF binding.

No other receptor species for TGF-α or EGF could be identified in affinity-labeling experiments performed in this laboratory with five different NRK cell membrane preparations or with intact NRK cells obtained from two different sources. Similarly, only 140–170 kD receptor species common for EGF and TGF-α were detected by affinity labeling of membranes from other tissues and cell types including rat and human liver, rat and human placenta, rat adipocytes, A431 human epitheloid carcinoma, T24 human bladder carcinoma, T85 human osteosarcoma, WI38 human lung fibroblasts, FeSV-Fre cells, and Swiss 3T3 mouse fibroblasts (reference 7; and Massagué, J., manuscript submitted for publication). The results from ligand binding experiments (Table I and reference 7) are also inconsistent with the hypothesis of separate receptor types existing for EGF and TGF-α. These results are in contrast to the previously reported presence of a 60-kD membrane component in NRK cells that can interact with "sarcoma growth factor" (partially purified mouse TGF-α that also contains TGF-β, see reference 2) but not with mouse EGF (40). This difference could be due to the use in previous studies of crude preparations of "sarcoma growth factor" derivatized with [^{125}I]iodo *N*-succinimidyl-3-(4-hydroxyphenyl) propionate and purified by binding and elution from A431 cells (40) instead of homogenous preparations of chemically and biologically intact ^{125}I-TGF-α (7) as used in the present studies. The 60-kD labeled component could be a minor TGF-β receptor species (14) affinity labeled by ^{125}I-TGF-β present in the radiolabeled sarcoma growth factor preparations.

The modulation of EGF/TGF-α receptors by TGF-β does not appear to result from a direct interaction of this factor with EGF or TGF-α binding sites for various reasons. First, TGF-β does not compete with ^{125}I-TGF-α (Table I) or ^{125}I-EGF (not shown) for binding to receptors in isolated membrane preparations. Other studies (12–14) show that ^{125}I-TGF-β binds with high affinity to membrane receptors in various cell lines including NRK cells, and binding is competed for by TGF-β but not by EGF or TGF-α. Furthermore, receptor affinity–labeling studies indicate that the receptor for ^{125}I-TGF-β in rat fibroblasts is a glycosylated, disulfide-linked complex that contains a 280-kD ligand binding subunit, and is therefore distinct from EGF/TGF-α receptor structures (14, 41, 42). Secondly, active concentrations of TGF-β do not cause down-regulation of EGF/TGF-α receptors in A431 cells under conditions in which mitogenically equivalent concentrations of EGF or TGF-α do. Third, we did not observe any decrease in the binding of low concentrations of ^{125}I-TGF-α or ^{125}I-EGF to A431 cells treated with TGF-β under conditions in which TGF-β elicited a marked decrease in the binding of these ligands to NRK cells. These observations indicate that the effect of TGF-β on EGF/TGF-α receptors in NRK cells is mediated by a mechanism that may not be operative in other cell types (such as A431 cells), or under other cell culture conditions.

The results reported here are in contrast to the observation (43) of a small and transient decrease of EGF binding to NRK cells after addition of TGF-β, then a two-fold increase in binding at later (6 h) time points. The reason for this discrepancy is unclear, but it is interesting to note that the different effects of TGF-β on EGF binding observed in these two studies are correlated with differences in the ability of NRK cell cultures to respond mitogenically to TGF-β. Thus, whereas other studies (43) were carried out with confluent monolayers of NRK cells reported to be mitogenically unresponsive to TGF-β, the results reported here were performed with sparse, serum-deprived cultures of NRK cells that respond mitogenically to TGF-β. It is possible that TGF-β has a dual action on EGF/TGF-α receptors, and that the inhibitory effect of TGF-β on EGF/TGF-α binding predominates in situations in which cells evolve from a growth-arrested state to a mitogenically active one.

The effect of TGF-β on EGF/TGF-α receptors described here is similar, and may be related to the modulation of the affinity of this receptor type by platelet-derived growth factor (14–17), fibroblast-derived growth factor (18), and tumor promoters including phorbol diterpene esters, certain indole alkaloids, and polyacetates (20, 21, 23). These agents have been reported to potentiate or complement the cellular actions of EGF analogues, yet they acutely decrease the binding of ^{125}I-EGF to high-affinity receptors in cultured cells. It is possible as proposed before (21) that potentiation of long-term actions of EGF by these agents is a consequence of a decreased degradation of EGF in the medium due to its decreased binding and internalization by cellular receptors. However, the exact significance of these paradoxical observations may not be clear until the role and cellular dynamics of EGF/TGF-α receptors with high and low apparent affinity for the ligand are established.

The observations reported here may help identify primary

molecular events involved in the action of TGF-β. Thus, the effect of TGF-β on EGF/TGF-α receptors may be accomplished by the induction of a molecular change in this receptor molecule that could convert it into a lower affinity state or target it for rapid removal from the cell surface. In an analogous situation, a molecular change of the EGF receptor, namely its phosphorylation on discrete sites by protein kinase C, has been proposed as the basis for modulation of EGF receptors by phorbol ester tumor promoters (22, 24, 44). Alternatively, TGF-β may induce a redistribution between the cell surface and an intracellular location(s) of preexisting EGF/TGF-α binding sites with different affinity for the ligands. Efforts to address the molecular basis for the modulation of EGF/TGF-α receptors by TGF-β are underway.

I thank Betsy Like and Brenda Kelly for excellent technical assistance, and Katherine Alvarez, Denise Bassett, and Mary Taubert for preparation of the manuscript.

This work was supported by Grant CA34610 from the National Cancer Institute. The author is the recipient of a Career Development Award from the Juvenile Diabetes Foundation International.

Received for publication 21 November 1984, and in revised form 21 January 1985.

REFERENCES

1. De Larco, J. E., and G. J. Todaro. 1978. Growth factors from murine sarcoma virus-transformed cells. *Proc. Natl. Acad. Sci. USA.* 75:4001–4005.
2. Anzano, M. A., A. B. Roberts, J. M. Smith, M. B. Sporn, and J. E. De Larco. 1983. Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type α and β transforming growth factors. *Proc. Natl. Acad. Sci. USA.* 80:6264–6268.
3. Massagué, J. 1983. Epidermal growth factor-like transforming growth factor. I. Isolation, chemical characterization, and potentiation by other transforming factors from feline sarcoma virus-transformed rat cells. *J. Biol. Chem.* 258:13606–13613.
4. Marquardt, H., and G. J. Todaro. 1982. Human transforming growth factor: production by a melanoma cell line purification, and initial characterization. *J. Biol. Chem.* 257:5220–5225.
5. Marquardt, H., M. W. Hunkapiller, L. E. Hood, D. R. Twardzik, J. E. De Larco, J. R. Stephenson, and G. J. Todaro. 1983. Transforming growth factors produced by retrovirus-transformed rodent fibroblasts and human melanoma cells: amino acid sequence homology with epidermal growth factor. *Proc. Natl. Acad. Sci. USA.* 80:4684–4688.
6. Marquardt, H., M. W. Hunkapiller, L. E. Hood, and G. J. Todaro. 1984. Rat transforming growth factor type I: structure and relation to epidermal growth factor. *Science (Wash. DC).* 223:1079–1082.
7. Massagué, J. 1983. Epidermal growth factor-like transforming growth factor. II. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. *J. Biol. Chem.* 258:13614–13620.
8. Roberts, A. B., M. A. Anzano, L. C. Lamb, J. M. Smith, and M. B. Sporn. 1981. New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. *Proc. Natl. Acad. Sci. USA.* 78:5339–5343.
9. Assoian, R. K., A. Komoriya, C. A. Meyers, D. M. Miller, and M. B. Sporn. 1983. Transforming growth factor-β in human platelets: identification of a major storage site, purification and characterization. *J. Biol. Chem.* 258:7155–7160.
10. Roberts, A., M. A. Anzano, C. A. Meyers, J. Wideman, R. Blacker, Y.-C. E. Pan, S. Stein, S. R. Lehrman, J. M. Smith, L. C. Lamb, and M. B. Sporn. 1983. Purification and properties of type β transforming growth factor from bovine kidney. *Biochemistry.* 22:5692–5698.
11. Massagué, J. 1984. Type β transforming growth factor from feline sarcoma virus-transformed rat cells. Isolation and biological properties. *J. Biol. Chem.* 259:9756–9761.
12. Frolik, C. A., L. M. Wakefield, D. M. Smith, and M. B. Sporn. 1984. Characterization of a membrane receptor for transforming growth factor-β in normal rat kidney fibroblasts. *J. Biol. Chem.* 259:10995–11000.
13. Tucker, R. F., E. L. Branum, G. D. Shipley, R. J. Ryan, and H. L. Moses. 1983. Specific binding to cultured cells of ^{125}I-labeled type β transforming growth factor from human platelets. *Proc. Natl. Acad. Sci. USA.* 81:6757–6761.
14. Massagué, J., and B. Like. 1985. Cellular receptors for type β transforming growth factor. Ligand binding and a affinity-labeling in human and rodent cell lines. *J. Biol. Chem.* 260:2636–2645.
15. Wrann, M. M., C. F. Fox, and R. Ross. 1980. Modulation of epidermal growth factor receptor on 3T3 cells by platelet-derived growth factor. *Science (Wash. DC).* 210:1363–1365.
16. Collins, M. K. L., J. W. Sinnett-Smith, and E. Rozengurt. 1983. Platelet-derived growth factor treatment decreases the affinity of the epidermal growth factor receptors of Swiss 3T3 cells. *J. Biol. Chem.* 258:11689–11693.
17. Bowen-Pope, D. F., P. E. Dicorleto, and R. Ross. 1983. Interactions between the receptors for platelet-derived growth factor and epidermal growth factor. *J. Cell Biol.* 96:679–683.
18. Rozengurt, E., M. Collins, K. D. Brown, and P. Pettican. 1982. Inhibition of epidermal growth factor binding to mouse cultured cells by fibroblast-derived growth factor: evidence for an indirect mechanism. *J. Biol. Chem.* 257:3680–3686.
19. Lee, L-S., and I. B. Weinstein. 1978. Tumor-promoting phorbol esters inhibit binding of epidermal growth factor to cellular receptors. *Science (Wash. DC).* 202:313–315.
20. Shoyab, M., J. E. De Larco, and G. J. Todaro. 1979. Biologically active phorbol esters specifically alter affinity of epidermal growth factor membrane receptors. *Nature (Lond.).* 279:387–391.
21. Magun, B. E., L. M. Matrisian, and G. T. Bowden. 1980. Epidermal growth factor. Ability of tumor promoters to alter its degradation, receptor affinity and receptor number. *J. Biol. Chem.* 255:6373–6381.
22. Iwashita, S., and C. F. Fox. 1984. Epidermal growth factor and potent phorbol tumor promoters induce epidermal growth factor receptor phosphorylation in a similar but distinctively different manner in human epidermoid carcinoma A431 cells. *J. Biol. Chem.* 259:2559–2567.
23. Friedman, B., A. R. Frackelton, A. H. Ross, J. M. Connors, H. Fujiki, T. Sugimura, and M. R. Rosner. 1984. Tumor promoters block tyrosine specific phosphorylation of the epidermal growth factor receptor. *Proc. Natl. Acad. Sci. USA.* 81:3034–3038.
24. Cochet, C., G. N. Gill, J. Meisenhelder, J. A. Cooper, and T. Hunter. 1984. C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity. *J. Biol. Chem.* 259:2553–2558.
25. Savage, C. R., and S. Cohen. 1972. Epidermal growth factor and a new derivative: rapid isolation procedures and biological and chemical characterization. *J. Biol. Chem.* 247:7609–7611.
26. Matrisian, L. M., B. R. Larsen, J. S. Finch, and B. E. Magun. 1982. Further purification of epidermal growth factor by high-performance liquid chromatography. *Anal. Biochem.* 125:339–351.
27. Haigler, H. T., F. R. Maxfield, M. C. Willingham, and I. Pastan. 1980. Dansylcadaverine inhibits internalization of ^{125}I-epidermal growth factor in BALB 3T3 cells. *J. Biol. Chem.* 255:1239–1241.
28. Pilch, P. F., and M. P. Czech. 1979. Interaction of cross-linking agents with the insulin effector system of isolated fat cells: covalent linkage of ^{125}I-insulin to a plasma membrane receptor protein of 140,000 daltons. *J. Biol. Chem.* 254:3375–3381.
29. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
30. Massagué, J., B. J. Guillette, M. P. Czech, C. J. Morgan, and R. A. Bradshaw. 1981. Identification of a nerve growth factor receptor protein in sympathetic ganglia membranes by affinity labeling. *J. Biol. Chem.* 256:9419–9424.
31. Massagué, J., and M. P. Czech. 1982. The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor. *J. Biol. Chem.* 257:5038–5045.
32. Carpenter, G., L. King, and S. Cohen. 1979. Rapid enhancement of protein phosphorylation in A-431 cell membrane preparations by epidermal growth factor. *J. Biol. Chem.* 254:4884–4891.
33. Aharonov, A., R. M. Pruss, and H. R. Herschman. 1978. Epidermal growth factor: relationship between receptor regulation and mitogenesis in 3T3 cells. *J. Biol. Chem.* 253:3970–3977.
34. Wrann, M. M., and C. F. Fox. 1979. Identification of epidermal growth factor receptors in a hyperproducing human epidermoid carcinoma cell line. *J. Biol. Chem.* 254:8083–8086.
35. Hock, R. A., E. Nexo, and M. D. Hollenberg. 1980. Solubilization and isolation of the human placenta receptor for epidermal growth factor-urogastrone. *J. Biol. Chem.* 255:10737–10743.
36. Cohen, S., R. A. Fava, and S. T. Sawyer. 1982. Purification and characterizations of epidermal growth factor receptor/protein kinase from normal mouse liver. *Proc. Natl. Acad. Sci. USA.* 79:6237–6241.
37. Linsley, P. S., and C. F. Fox. 1980. Controlled proteolysis of EGF receptors: evidence for transmembrane distribution of the EGF binding and phosphate acceptor sites. *J. Supramol. Struct.* 14:461–471.
38. Cassel, D., and L. Glaser. 1982. Proteolytic cleavage of epidermal growth factor receptor: a Ca^{++}-dependent, sulfhydryl-sensitive proteolytic system in A431 cells. *J. Biol. Chem.* 257:9845–9848.
39. De Larco, J. E., R. Reynolds, K. Carlbery, E. Engle, and G. J. Todaro. 1980. Sarcoma growth factor from mouse sarcoma virus-transformed cells: purification by binding and elution from epidermal growth factor receptor-rich cells. *J. Biol. Chem.* 255:3685–3690.
40. Massagué, J., M. P. Czech, K. Iwata, J. E. De Larco, and G. J. Todaro. 1982. Affinity labeling of a transforming growth factor receptor that does not interact with epidermal growth factor. *Proc. Natl. Acad. Sci. USA.* 79:6822–6826.
41. Massagué, J. 1985. Type-β transforming growth factor receptors in cells chronically exposed to the ligand. *In* Cancer Cells 3/Growth Factors and Transformation. J. Feramisco, B. Ozanne, and C. Stiles, editors. Cold Spring Harbor Laboratory, New York. 73–78.
42. Massagué, J. 1985. Subunit structure of the high affinity receptor for type β transforming growth factor. Evidence for a disulfide-linked, glycosylated receptor complex. *J. Biol. Chem.* In press.
43. Assoian, R. K., C. A. Frolik, A. B. Roberts, D. M. Miller, and M. B. Sporn. 1984. Transforming growth factor-β controls receptor levels for epidermal growth factor in NRK fibroblasts. *Cell.* 36:35–41.
44. Hunter, T., N. Ling, and J. A. Cooper. 1984. Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. *Nature (Lond.).* 311:480–483.