Journal of Cellular Biochemistry 27:23–30 (1985)
Membrane Receptors and Cellular Regulation 109–116

Modulation of Type α Transforming Growth Factor Receptors by a Phorbol Ester Tumor Promoter

Roger Davis, Betsy Like, and Joan Massagué

Department of Biochemistry, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Epidermal growth factor (EGF) and an EGF-like transforming growth factor (eTGF) from retrovirally transformed cells bind to a common receptor type in A431 cells. We have investigated the effects of the tumor promoter phorbol myristate acetate [PMA] on EGF/eTGF receptors in intact A431 cells. Treatment with PMA at 37°C induces a complete loss of high-affinity (K_d = 35–50 pM) binding sites for eTGF and EGF on the cell surface of A431 cells. This effect is half-maximal at 0.1 nM PMA, exhibits rapid kinetics, and persists for at least 4 hr in the presence of PMA. eTGF and PMA added to intact A431 cells induce the phosphorylation of immunoprecipitable 170kd EGF /eTGF receptors. The EGF/eTGF receptor isolated from control cells was found to contain phosphoserine and phosphothreonine. PMA and eTGF caused a marked increase in the level of these two phosphoamino acids. In addition, eTGF but not PMA caused the appearance of phosphotyrosine in the EGF/eTGF receptor in vivo. We conclude that the tumor-promoting phorbol diester regulates both the affinity and phosphorylation state of the A431 cell receptor for the type α transforming growth factors, eTGF and EGF.

Key words: receptor, epidermal growth factor, transforming growth factors, receptor regulation, tumor-promoting phorbol esters

Transforming growth factors (TGFs) are bioactive polypeptides that induce a transformed phenotype and anchorage-independent proliferation when added to normal cells in culture [1]. The transformed phenotype in NRK cells, a known target for TGFs, is induced by the coordinate action of at least two types of TGFs, αTGFs and βTGFs [2,3]. βTGFs are 23–25kd molecules consisting of two disulfide-linked 11–12kd polypeptide chains [4–6]. They are potent mitogens [6] that do not induce phenotypic transformation when added alone to NRK cells but strongly potentiate the transforming action of αTGFs [3–6]. αTGFs include epidermal growth factor (EGF) and an EGF-like transforming growth factor (eTGF) which is released by certain neoplastic cells [1,3,7,8]. EGF and eTGF are structurally and functionally related 6–7kd polypeptides [3,8]. They exhibit limited amino acid sequence homology, interact with a

Received April 24, 1984; revised and accepted August 21, 1984.

common receptor type, and induce slow, anchorage-independent proliferation of NRK cells.

The receptor for EGF and eTGF presumably plays a central role in the induction of the transformed phenotype by TGFs in NRK cells. Studies have indicated that the receptor for EGF is a target for modulation by factors that regulate cell growth, including polypeptide growth factors and tumor-promoting agents [9–13]. The effects of these factors may involve a structural modification of the EGF receptor. For example, tumor-promoting esters of phorbol that acutely modify the activity of EGF receptors induce the phosphorylation of unique sites in this receptor molecule [14,15]. This information is extended by the present studies which show that phorbol myristate acetate acutely modifies the binding of eTGF to its receptor concomitantly with a marked change in the phosphorylation state of this receptor species in intact A431 cells.

EXPERIMENTAL PROCEDURES

Growth Factors

eTGF from serum-free media conditioned by Snyder-Theilen feline sarcoma virus-transformed rat embryo cells was purified to homogeneity as described before [3]. EGF was purified from mouse submaxillary glands [16]. eTGF and EGF were labeled with ^{125}I using the lactoperoxidase-glucose oxidase method as described before [3] to a specific activity of 60–70 Ci/g and 80–100 Ci/g, respectively.

Treatment of Cells With Phorbol Myristate Acetate

A431 cells (Dr G.J. Todaro, National Cancer Institute) were plated at low density (5–10 $\times$ 10^3 cells/well) in 16-mm tissue culture plastic wells, and incubated at 37°C for about 48 hr with Dulbecco's modified Eagle medium supplemented with 10% calf serum (Gibco). The medium was then aspirated and the monolayers were washed once at 22°C with binding medium (128 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate, 1 mg/ml of bovine serum albumin, pH 7.5). Monolayers were then incubated at 37°C with 0.5 ml of binding medium plus 4β-phorbol-12-myristate 13-acetate (PMA) (Sigma) dissolved in 5 μl of dimethyl sulfoxide to achieve the desired final concentration. Five microliters of dimethyl sulfoxide without PMA was added to control cell monolayers. After the time indicated in each experiment, the media were aspirated, and wells were washed twice with ice-cold binding buffer for binding measurements.

Binding Assays

At the end of the treatment with PMA, the washed monolayers were incubated at 0–4°C with 0.3 ml of binding buffer containing ^{125}I-eTGF or ^{125}I-EGF at 50 pM final concentration unless otherwise indicated. After 4.5 hr the medium was aspirated and the monolayers were washed three times with ice-cold binding buffer. The cell-associated radioactivity was determined after solubilization of cells with two consecutive 0.5-ml aliquots of 0.1% sodium dodecyl sulfate solution. Nonspecific binding defined as the binding of ^{125}I-labeled ligands determined in the presence of 50 nM native EGF was 6% or less of total binding when 50 pM ^{125}I-labeled ligands were used, and was subtracted in all cases. Total binding was in all cases less than 18% of the input radioactivity. Ninety-five percent or more of the ^{125}I-eTGF or ^{125}I-EGF bound to A431 cells after 4.5 hr of incubation at 0–4°C was bound to cell surface

receptors as determined in control experiments by its ability to dissociate from the cells after a short treatment at pH 4.5 performed as described before [17]. All binding determinations were done in triplicate. Cell numbers were determined in a Coulter counter after detachment of cells from the culture vessels by a brief exposure to trypsin-containing buffer.

Immunoprecipitation of eTGF/EGF Receptors

Anti-EGF/eTGF receptor serum was obtained from a rabbit that had been injected with 10 μg of affinity-purified EGF/eTGF receptor from A431 cells [18] mixed with Freund's complete adjuvant and injected with a further 10 μg of receptor mixed with incomplete adjuvant after 4 weeks.

A431 cells (35-mm dish) were incubated at 37°C for 4 hr with 1 mCi [^{32}P]orthophosphate (carrier-free, New England Nuclear) in 1 ml of a buffer containing 120 mM NaCl, 6 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose, and 25 mM 4-(2-hydroxyethyl)-2-piperazine-ethansulfonic acid (pH 7.4). The cells were then treated with PMA and/or eTGF before solubilizing with 1.5 ml of 25 mM 4-(2-hydroxyethyl)-2-piperazine-ethansulfonic acid (pH 8.0), 0.15M NaCl, 1 mM ethylenediamine tetraacetic acid, 1% Triton X-100, 0.1% $NaDodSO_4$, 50 mM NaF, 50 μM Na_3VO_5, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. The solubilized cells were then centrifuged at 100,000g for 30 min. Ten microliters of antireceptor serum (diluted 1:20) was then added to 1 ml of the A431 cell extract for 17 hr at 4°C. This immunoprecipitation was performed in antibody excess. Immune complexes were then recovered by incubating at 22°C for 60 min with protein A–Sepharose CL-4B (Pharmacia). The protein A–Sepharose was then extensively washed and the EGF receptors solubilized by boiling with 5% $NaDodSO_4$, 50 mM dithiothreitol. The solubilized immunoprecipitate was then analyzed by electrophoresis on a 7% polyacrylamide gel [19] and autoradiography.

Phospoamino Acid Analysis

Analysis of the phosphoamino acid content of bands excised from polyacrylamide gels was done as described in the legend to Figure 4.

RESULTS

We compared the ability of PMA to affect the binding of tracer (50 pM) concentrations of ^{125}I-eTGF and ^{125}I-EGF to sparse A431 cells. Treatment of cells with PMA was conducted at 37°C, and binding of ^{125}I-eTGF and ^{125}I-EGF was measured at 0–4°C to minimize the contribution of postbinding events, ie, internalization, retention, and later release of ligands, that occur at higher temperatures [17]. At least 95% of the cell-associated radioactivity was bound to cell surface receptors in binding measurements done at 0–4°C, as determined in control experiments (see Experimental Procedures). PMA markedly decreased the binding of ^{125}I-eTGF and ^{125}I-EGF to A431 cells in a concentration- and time-dependent manner (Fig. 1). The effect on ^{125}I-eTGF and ^{125}I-EGF binding was half-maximal at 0.1 nM PMA, and maximal at 3 nM PMA in A431 cells treated with PMA for 60 min at 37°C (Fig. 1A). Inhibition of ^{125}I-eTGF and ^{125}I-EGF binding by PMA was rapid and exhibited parallel kinetics. Maximal inhibition of the binding of both ligands occurred after 30 min with PMA and remained constant for at least 4 hr under our experimental conditions (Fig. 1B). A partial inhibition of ^{125}I-eTGF and ^{125}I-EGF binding was

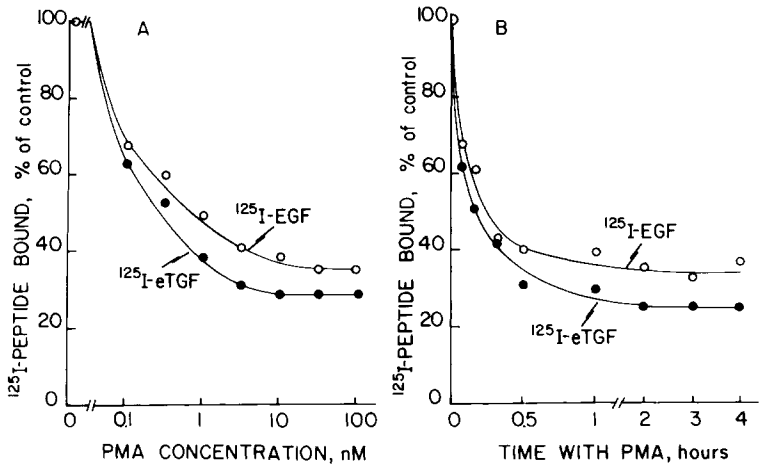

Fig. 1. Concentration- and time-dependent inhibition of ^{125}I-eTGF and ^{125}I-EGF binding to A431 cells by PMA. A) A431 cells were incubated for 60 min at 37°C with the indicated concentrations of PMA. B) A431 cells were incubated at 37°C for 240 min and received 1 nM PMA at the indicated times before the end of this incubation. At the end of incubations, cell monolayers were washed twice with ice-cold binding buffer, and the specific binding of 50 pM ^{125}I-eTGF (●) or 50 pM ^{125}I-EGF (○) was determined at 0–4°C as described in Experimental Procedures. Results are expressed as percent of specific binding of ^{125}I-eTGF or ^{125}I-EGF to control cells not treated with PMA. In each experiment determinations were done in triplicate. Data are the average of duplicate experiments.

observed in A431 cells treated with PMA at 4°C instead of 37°C. After 4 hr of incubation at 4°C, the effect of PMA on ^{125}I-eTGF and ^{125}I-EGF binding was only about half of the effect observed at 37°C (not illustrated).

To determine the nature of the change in eTGF and EGF binding sites induced by PMA, we assayed the binding of various concentrations of ^{125}I-eTGF and ^{125}I-EGF to control and PMA-treated cells. Analysis of the binding data by the method of Scatchard yielded curvilinear plots for both ^{125}I-eTGF and ^{125}I-EGF (Fig. 2). These data can be interpreted as indicating the presence of two populations of binding sites for eTGF and EGF in the cell surface of intact A431 cells. According to this interpretation, A431 cells exhibit a relatively small (2×10^5 sites/cell) population of high-(K_d = 35–50 pM)- binding sites, and a larger ($1.7–2.1 \times 10^6$ sites/cell) population of lower-affinity (K_d = 0.35 nM) binding sites for eTGF and EGF. These values agree well with those previously reported for eTGF and EGF binding to isolated A431 membrane preparations [20]. Treatment of A431 cells with PMA completely abolished the high-affinity cell surface receptors for eTGF, yielding an apparently homogeneous population of low-affinity (K_d = 0.52 nM) binding sites. This effect was paralleled by a similar conversion of heterogeneous EGF binding sites into a population of low-affinity (K_d = 0.18–0.55 nM) binding sites.

We have investigated the effect of PMA and eTGF on the phosphorylation state of the EGF/eTGF receptor in intact A431 cells. This was achieved by labeling cells with [^{32}P]orthophosphate and isolating the EGF/eTGF receptors by immunoprecipitation with a polyclonal antibody. Addition of eTGF caused an increase in the phosphorylation state of the EGF/eTGF receptor (Fig. 3). PMA was also observed to enhance the phosphorylation state of the EGF/eTGF receptor in a dose-dependent manner.

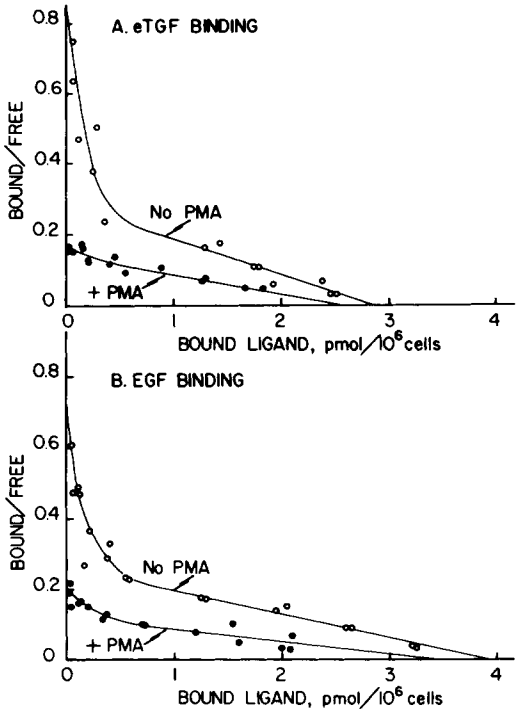

Fig. 2. PMA treatment abolishes high-affinity binding sites from A431 cell surfaces. A431 cells were incubated for 60 min at 37°C with 100 nM PMA (●) or without PMA (○). The specific binding of 10 pM, 20 pM, 50 pM, 100 pM, 500 pM, 1 nM, 2 nM, and 5 nM ^{125}I-eTGF (A) or ^{125}I-EGF (B) was then determined in control and PMA-treated cells. The binding data have been plotted according to Scatchard. The experiment was done twice with similar results.

The half-maximal effect of PMA occurred at a concentration of less than 1 nM. Incubation of A431 cells with PMA and eTGF resulted in a greater incorporation of phosphate into the EGF/eTGF receptor than was observed with either agent alone. However, the effects of eTGF and PMA were not additive, as the increase in phosphorylation caused by PMA was much greater in the absence of eTGF than in its presence.

To further characterize the phosphorylation of the EGF/eTGF receptor induced in intact A431 cells by eTGF and PMA, we analyzed the level of phosphoserine, phosphothreonine, and phosphotyrosine in the EGF receptor. A431 cells were labeled with [^{32}P]phosphate and then treated with 10 nM eTGF or 10 nM PMA. The EGF/eTGF receptors were isolated by immunoprecipitation and $NaDodSO_4$ polyacrylamide gel electrophoresis. The EGF/eTGF receptors were then extracted from the gel and partially hydrolyzed for 1 hr at 110°C under vacuum as described [21]. Phosphoamino acids were separated by thin-layer electrophoresis at pH 3.5 [21]. Autoradiography (Fig. 4) indicated the presence of [^{32}P]phosphoserine and [^{32}P]phosphothreonine in the EGF/eTGF receptors derived from control cells. PMA caused a marked increase in the level of [^{32}P]phosphoserine and [^{32}P]phosphothreonine. eTGF, however, caused the appearance of [^{32}P]phosphotyrosine in addition to increasing the level of [^{32}P]phosphoserine

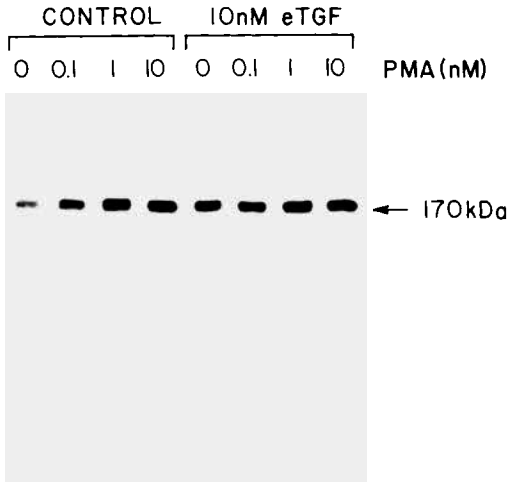

Fig. 3. Effect of eTGF and PMA on the phosphorylation state of the eTGF/EGF receptors of A431 cells. A431 cells were incubated for 4 hr with 1 mCi/ml [^{32}P]orthophosphate and then treated with different concentrations of PMA for 30 min. eTGF was then added to some incubations and after a further 30 min the incubations were terminated by washing the cell monolayers and extracting the EGF/eTGF receptors with a buffer containing 1% Triton X-100 and 0.1% $NaDodSO_4$. The receptors were then immunoprecipitated with a polyclonal antibody raised in a rabbit injected with purified A431 cell EGF/eTGF receptor. The immunoprecipitate was then solubilized and analyzed by $NaDodSO_4$ gel electrophoresis. The figure is an autoradiogram (4-hr exposure) of the Coomassie Blue -stained, dried gel. Similar results were obtained with three separate experiments.

and [^{32}P]phosphothreonine. The effect of eTGF on the phosphorylation state of the EGF/eTGF receptor is therefore in marked contrast to the effect of PMA.

DISCUSSION

In contrast to a recent report which indicates that phorbol esters have no effect on the binding of ^{125}I-EGF to A431 cells [22], we have observed a marked inhibition of both ^{125}I-eTGF and ^{125}I-EGF binding to the high-affinity EGF/eTGF receptors of A431 cells in the presence of the potent tumor promoter PMA (Figs. 1,2). This discrepancy could be due to the use in previous studies [22] of concentrations of labeled EGF and binding sites above the K_d for binding of EGF to high-affinity receptors. Under those experimental conditions high-affinity EGF binding sites are likely undetectable. In our experiments the effect of PMA was rapid and occurred at low concentrations of this agent (ID_{50} = 0.1 nM). According to our data PMA could cause a rapid removal of high-affinity binding sites from the surface of A431 cells. Alternatively, the effect of PMA could involve an allosteric change in the high-affinity binding sites, converting them into low-affinity sites. The relatively small size of the high-affinity binding site population in A431 cells, and the complexity of Scatchard analysis applied to intact cell systems do not allow the distinction between these two possibilities. However, the data demonstrate that the effect of PMA on ligand binding to the EGF/eTGF receptor is similar for ^{125}I-eTGF and ^{125}I-EGF, further stressing the analogy between the interaction of eTGF and EGF with a common receptor type [1,8,20].

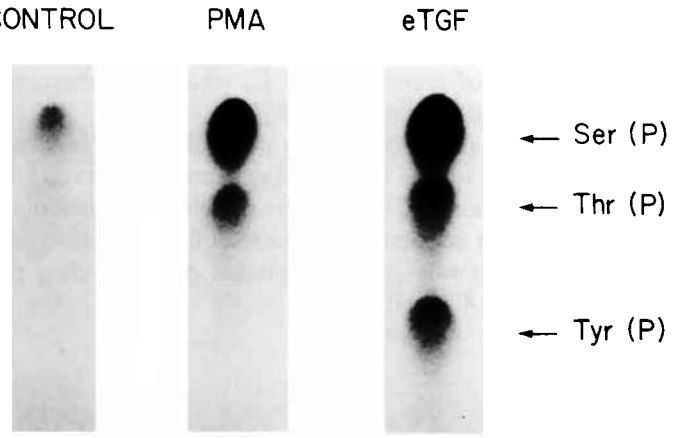

Fig. 4. Phosphoamino acid analysis of the eTGF/EGF receptor isolated from A431 cells. A431 cells were labeled for 4 hr with 1 mCi/ml [^{32}P]orthophosphate and then treated with and without 10 nM PMA for 30 min. eTGF (10 nM) was then added to some incubations for 10 min. The EGF/eTGF receptors were isolated by immunoprecipitation and NaDodSO$_4$ polyacrylamide gel electrophoresis. The receptor was located in the dried gel by autoradiography and the gel slice containing the receptor was excised. The EGF/eTGF receptors were then extracted from the gel slice, precipitated with trichloroacetic acid, washed with ether:ethanol (1:1) at −20°C, and partially hydrolyzed in 6 M HCl for 1 hr at 110°C under vacuum as described [21]. Phosphoamino acids were resolved by thin-layer electrophoresis at pH 3.5 [21] on 100-μm cellulose-coated plastic sheets (Machery-Nagel). Phosphoamino acid standards (Sigma) mixed with each sample were identified with ninhydrin. [^{32}P]phosphoamino acids were located by autoradiography. The figure is a 24 hr exposure by Kodak X-OMAT AR film using a Dupont Cronex Lightning Plus enhancing screen at −70°C. Radioactivity associated with each phosphoamino acid was quantitated by measuring the Cerenkov radiation with a β counter. The radioactivity (cpm) detected was phosphoserine (123, 432, 457), phosphothreonine (34, 131, 158), and phosphotyrosine (1, 0, 49) for samples dervied from control, PMA-treated, and eTGF-treated cells, respectively.

The EGF/eTGF receptor in vitro is associated with a tyrosine kinase activity which is stimulated by EGF [23] and eTGF [24]. This kinase activity is responsible for the autophosphorylation of the EGF receptor in vitro [23,24] and the phosphorylation of exogenous substrates on tyrosine residues [25,26]. In intact cells the EGF/eTGF receptor is a phosphoprotein whose phosphorylation state is increased by the addition of EGF [27]. However, in contrast to the autophosphorylation of the receptor in vitro, phosphoserine and phosphothreonine account for the majority of the phosphate content of the EGF/eTGF receptor in intact cells [27]. We investigated the effect of PMA and eTGF on the phosphorylation state of the EGF/eTGF receptor of A431 cells (Fig. 3). It was observed that both PMA and eTGF increased the phosphorylation state of the receptor. Phosphoamino acid analysis of the EGF/eTGF receptors of A431 cells treated with PMA or eTGF indicates that these agents contrast in their effect on the phosphorylation state of the receptor (Fig. 4). PMA increases the level of phosphoserine and phosphothreonine. eTGF also induces phosphorylation of receptor serine and threonine residues, but in addition causes the appearance of phosphotyrosine. The effect of eTGF to stimulate the tyrosine phosphorylation of the EGF/eTGF receptor in intact A431 cells is consistent with the effect of eTGF to stimulate the tyrosine kinase activity of the EGF/eTGF receptor in vitro [24]. The stimulation of serine and threonine phosphorylation of the EGF/eTGF receptor by

eTGF is similar to that reported for EGF [27]. The effect of PMA to stimulate threonine phosphorylation of the EGF/eTGF receptor [14,15] may be because PMA can stimulate the activity of protein kinase C, which can phosphorylate the EGF/eTGF receptor on threonine residues in vitro [14].

We conclude that the tumor-promoting phorbol ester, PMA, rapidly inhibit the high-affinity binding of the type α transforming growth factors eTGF and EGF. This effect is associated with a marked increase in the phosphorylation state of the EGF/eTGF receptor. The results suggest the hypothesis that phosphorylation of the EGF/eTGF receptor may play a role in the regulation of the binding properties of this receptor type by PMA in A431 cells.

ACKNOWLEDGMENTS

This work was supported by National Cancer Institute Grant RO1 CA 34610. R.J.D. was the recipient of a postdoctoral fellowship from the Damon Runyon–Walter Winchell Cancer Fund.

REFERENCES

1. De Larco J E, Todaro G J: Proc Natl Acad Sci USA 75:4001, 1978.
2. Anzano M A, Roberts A B, Smith J M, Sporn M B, De Larco J E: Proc Natl Acad Sci USA 80:6264, 1983.
3. Massagué J: J Biol Chem 258:13606, 1983
4. Assoian R K, Komoriya A, Meyers C A, Miller D M, Sporn M B: J Biol Chem 258:7155, 1983.
5. Frolik C A, Dart L L, Meyers C A, Smith D M, Sporn M B: Proc Natl Acad Sci USA 80:3676, 1983.
6. Massagué J: J Biol Chem 259:9756, 1984.
7. Marquardt H, Todaro G J: J Biol Chem 257:5220, 1982.
8. Marquardt H, Hunkapiller MW, Hood L E, Todaro G J: Science 223:1079, 1984.
9. Wrann M M, Fox C F, Ross R: Science 210:1363, 1980.
10. Collins M K L, Sinnett-Smith J W, Rozengurt E: J Biol Chem 258:11689, 1983.
11. Rozengurt E, Collins M, Brown K D, Pettican P: J Biol Chem 257:3680, 1982.
12. Shoyab M, De Larco J E, Todaro G J: Nature 279:387, 1979.
13. Magun B E, Matrisian L M, Bowden G T: J Biol Chem 255:6373,1980.
14. Cochet C, Gill G N, Meisenhelder J, Cooper J A, Hunter T: J Biol Chem 259:2553, 1984.
15. Iwashita S, Fox C F: J Biol Chem 259:2559, 1984.
16. Savage C R, Cohen S: J Biol Chem 247:7609, 1972.
17. Haigler H T, Maxfield F R, Willingham M C, Pastan I: J Biol Chem 255:1239, 1980.
18. Cohen S, Carpenter G, King L Jr: J Biol Chem 255:4834, 1980.
19. Laemmli, U K: Nature 277:680, 1970.
20. Massagué J: J Biol Chem 258:13614, 1983.
21. Hunter T, Sefton B M: Proc Natl Acad Sci USA 77:1311, 1980.
22. Froscia M, Tapley P M, Guy G R, Pappalardo S, Jones M J, Murray A N: Carcinogenesis 3:837, 1982.
23. Carpenter G, King L Jr, Cohen S: J Biol Chem 254:4884, 1979.
24. Reynolds F M Jr, Todaro G J, Fryling L, Stephenson J R: Nature 292:259, 1981.
25. Pike L, Gallis B, Casnellie J E, Bornstein P, Krebs E G: Proc Natl Acad Sci USA 79:1443, 1982.
26. Pike L, Marquardt H, Todaro G J, Gallis B, Casnellie J E, Bornstein P, Krebs E G: J Biol Chem 257:14628, 1982.
27. Hunter T, Cooper J A: Cell 24:741, 1981.