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Hormone-Induced Modification of EGF Receptor Proteolysis in the Induction of EGF Action

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A proposal that EGF action is mediated through enhanced internalization of EGF receptors is modified to account for more recent evidence. EGF receptors turn over at a rapid rate, and the maintenance of a steady state of EGF receptors on the cell surface is provided through a rapid synthesis of EGF receptors, balancing their removal. This rapid turnover of unoccupied receptors may arise through their internalization and proteolysis in the lysosomes, in much the same way as receptors are internalized and degraded when exposed to EGF, which enhances internalization. This provides a dilemma for the endocytic activation concept, since slight enhancement of receptor internalization gives rise to a strong hormone response. This problem may be solved by the observation that EGF induces a change in its receptor, exposing an otherwise unavailable site for proteolytic cleavage. This hormone-dependent modification of receptors may be the critical step in the induction of responses to EGF and other hormones that are internalized with their receptors. Both platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) are shown to down-regulate EGF receptors, though transiently, placing still more stringent requirements on the specificity by which hormones might act through endocytic activation of their receptors.

Key words: **direct labeling of EGF receptors, transient down-regulation of EGF receptors, platelet derived growth factor, receptor proteolysis**

Three properties of murine EGF [1] have led several laboratories to select it for studies on the biochemical mechanism of polypeptide hormone-induced stimulation of cell proliferation: 1) EGF can be readily purified by a simple procedure [2]; 2) EGF can be radioiodinated to high specific activity and retain its biological activity [3]; and 3) many cultured cell lines elicit a mitogenic response to EGF (for review see [4]). The mitogenic activity of polypeptide hormones is usually studied in growth-arrested cell monolayers, maintained in culture with a minimal concentration of serum, which is itself rich in mitogens. Addition of nanomolar concentrations of EGF to a monolayer of responsive cells – eg, murine 3T3 cells – induces a sequence of events that ultimately leads to DNA replication. EGF gives rise to these events after binding specifically to high-affinity surface receptors

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[3–11]. Studies in which fluorescent and ferritin-labeled EGF derivatives were incubated with cells have shown that EGF enhances the patching of the ferritin or fluorescent labels, and thus presumably the patching of the receptor molecules on the cell surface [12–14].

Shortly after the addition of ferritin or fluorescent EGF probes, the probes begin to appear intracellularly in small vesicles, and their appearance in these vesicles is followed by their incorporation into the lysosomes [12–14]. When EGF and insulin probes, which can be distinguished one from the other by their specific fluorescence properties, are added together both are ultimately detected in the same lysosomal particles [15]. This shows that many hormones, and presumably their receptors as well, share common steps in internalization and/or ultimate deposition in the lysosomes [15]. Das and Fox used photoaffinity probes of EGF to specifically label receptors and showed directly that EGF receptors are internalized [10, 16]. Furthermore, receptor internalization correlates quantitatively with the loss of EGF binding activity that ensues when EGF is added to cells [17]. They also reported that EGF-induced stimulation of DNA synthesis in 3T3 cells is more closely correlated with EGF-induced receptor internalization than with saturation of the EGF receptor [18]. Both EGF receptor down-regulation, which in the cell line studied by Das and Fox is quantitatively equivalent to increased receptor internalization, and EGF-induced stimulation of DNA synthesis are half-saturated at the same level of EGF – a level at which no more than 10% of the EGF receptors are saturated at the steady state of binding. Das and Fox made the important distinction between a steady-state binding level of EGF and the dynamic process of enhanced receptor internalization that is induced by EGF, and they proposed that EGF might stimulate DNA synthesis by a process of "endocytic activation" [16–18]. Their data indicate that the limiting step in EGF-induced stimulation of DNA synthesis in these cells was not EGF binding itself, but rather, a step leading to the internalization and processing of the EGF receptor in the lysosomes, or possibly, internalization and processing itself.

Other recent findings are in essential agreement with these observations. Schechter et al have correlated the local aggregation of EGF:EGF-receptor complexes with the mitogenic activity of EGF [19]. They found that CNBr-treated EGF was antagonistic for EGF binding but biologically far less active. Biological activity of CNBr-treated EGF was restored when it was added to cells together with a carefully controlled concentration of anti-EGF IgG, which promoted aggregation and possibly enhanced the internalization of CNBr-EGF-occupied receptors.

In this review we deal primarily with recent studies from this laboratory which test for possible role(s) of EGF receptor internalization and processing in EGF-induced stimulation of DNA synthesis. We find that EGF receptor down-regulation* can occur in response to a variety of conditions other than EGF addition, and the known rapid turnover of this receptor poses additional constraints on the concept that EGF activity is elicited through a simple endocytic activation of receptor. Normal EGF receptor turnover may also proceed by internalization and proteolysis of EGF receptors in the lysosomes. The specificity problems suggested by these observations may be reconciled by recent observations reported here. We have observed that EGF induces changes in receptor that alter the specific sites that are initially susceptible to proteolytic nicking. These observations show that the susceptibility of the EGF receptor to possible proteolytic activation may occur only when it is occupied by EGF.

*In this paper the term "down-regulation" is used to refer to the phenomenon of ligand-induced decrease in ligand-binding activity. Receptor internalization is but one of many processes that could give rise to down-regulation.

MATERIALS AND METHODS

Swiss mouse 3T3 cells, clone 42 from G. Todaro, and human epithilioid carcinoma cells (strain A431 from G. Todaro) [12] were used in these studies. Cells were grown routinely in Dulbecco's modified Eagle's medium (DME) containing 5% (3T3) or 10% (A431) fetal calf serum (FCS) and used on or before the sixth passage from frozen stock. Most experiments were performed on serum-starved cells. These were prepared by growing cells to confluence, and by shifting the serum concentration in the growth medium to 0.5%. The cells were then incubated at 37°C for an additional 24 h to yield the serum-starved cells. The procedures for isolation of EGF from mouse submaxillary glands [1], radioiodination of EGF [10], assay for EGF binding, EGF receptor down-regulation [20, 21], DNA synthesis [16], protein synthesis [22], "direct" labeling of EGF receptors [23], surface-specific radioiodination, preparation of 3T3 cell membranes, and SDS-polyacrylamide gel electrophoresis [20] have been described.

RESULTS

EGF-Dependent and EGF-Independent Turnover of EGF Receptors

The experiments described in this section show that a steady state of EGF binding activity is achieved when the rate of receptor synthesis and removal are equal. The experiments described in Figures 1–3 were designed to modify EGF binding activity by modulation of the rate of receptor synthesis or removal. This was accomplished by varying the serum concentration in the growth medium or by incubating cells with cycloheximide and/or EGF.

EGF receptor synthesis is serum dependent. When 3T3 cells are incubated at 37°C at high EGF concentration they lose more than 80% of their EGF binding activity within a few hours, and a new steady-state level of receptors is achieved (Fig. 1). We have studied the recovery of EGF binding activity after removal of EGF (Figs. 1 and 2). EGF binding is restored in a process that requires protein synthesis, and the rate of restoration correlates with the total cellular capacity for protein synthesis (Fig. 2). Both total protein and receptor synthesis are stimulated at high serum concentrations. It therefore appears that the cells constantly synthesize receptors at a rate dependent, among other things, on the serum concentration in the growth medium.

Turnover of unoccupied EGF receptors. If cells synthesize receptors continuously, there must be a mechanism for receptor destruction to account for maintenance of a constant number of receptors on the surface. Upon a shift from 5% to 0.5% serum, 3T3 cells lost 30% of their EGF binding activity within 4 h, and 40% in 12 h (Fig. 1). This loss of binding activity is correlated with a reduced capacity of serum-starved cells to synthesize protein (Fig. 2) or restore EGF binding activity after EGF-induced down-regulation of EGF receptors (Figs. 1 and 2). When serum-starved cells were incubated with cycloheximide to arrest protein synthesis maximally, EGF binding activity declined with a half-time of approximately 7 h (Fig. 3). This is similar to the half-time of receptor recovery after maximal down-regulation of EGF receptors in serum-starved cells (Fig. 2).

Hormone binding enhances internalization and lysosomal degradation of occupied receptors. The experiments treated in the preceding section, and presented in Figures 2 and 3, were done in parallel so that the rate measurements in the different experiments could be compared. In these experiments, the initial rate of loss of EGF binding activity when protein synthesis was arrested maximally by cycloheximide was equal within experi-

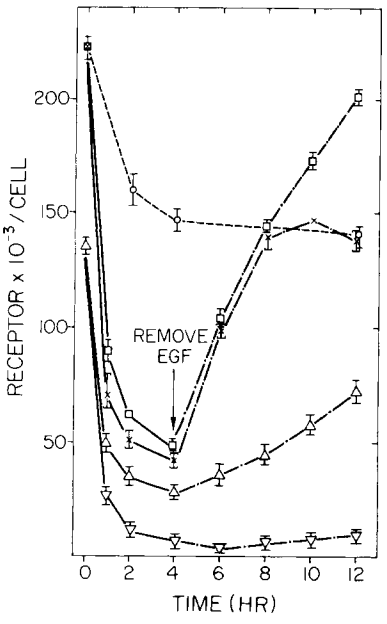


Fig. 1. Effect of serum concentration and cycloheximide on down-regulation of EGF receptors and on the recovery of EGF binding activity following EGF receptor down-regulation. Cells were grown to confluence in medium containing 5% fetal calf serum (FCS). Twenty-four hours prior to time 0, when EGF was added to down-regulate the EGF receptor, the FCS concentration in some dishes was changed to 0.5%. At time 0, the medium of all dishes was changed to fresh DME containing 5% or 0.5% FCS and 50 ng/ml of unlabeled EGF (□,x,△,▽) or no EGF (○). Cycloheximide (2 μg/ml) was added to some samples (▽). At time 4 h, EGF was removed, and the cells were incubated with DME plus FCS, as indicated below. ^{125}I-EGF binding was determined after incubation with 10 nM ^{125}I-EGF for 10 min at 22°C, as described in Materials and Methods. Receptors per cell was calculated directly from the specific binding data. (□ = cells grown and maintained in 5% FCS; ○,x = cells grown in 5% FCS and shifted to 0.5% FCS at time 0; △, ▽ = cells starved for 24 h in 0.5% FCS (serum-starved cells) and maintained in 0.5% FCS; - - - - - - = no EGF added; ——— = incubation with EGF added; — · — · — = incubation after EGF removal.)

mental error to the initial rate of restoration of EGF binding activity in cells treated with EGF maximally to down-regulate receptors. Assuming that the loss of EGF binding during cycloheximide treatment of cells is due only to the effects of cycloheximide on receptor synthesis, the rate of EGF receptor synthesis is equal in EGF-treated and EGF-untreated cells. This supports the contention that EGF gives rise to receptor down-regulation by altering the rate of receptor removal from the cell surface. This is obvious from the observations in Figure 3, which show that the rate of disappearance of EGF binding activity induced by 8 nM EGF is 10-fold greater than the maximal rate of loss of EGF binding in the presence of cycloheximide. We therefore propose that the addition of EGF shifts the removal of receptors from an unoccupied mechanism to a more efficient, occupied mechanism, perhaps by lowering the effective "K_m" of receptor for the same removal mechanism that functions in the absence of EGF. In the initial phase after EGF addition, enhanced internalization of EGF and EGF receptors is observed [6, 10, 11, 16]. The rate of enhanced receptor disappearance then declines as the number of receptors (which are considered to be the substrate in this case) declines. A new steady-state level of receptors is then reached when the rates of receptor removal and synthesis become equal.

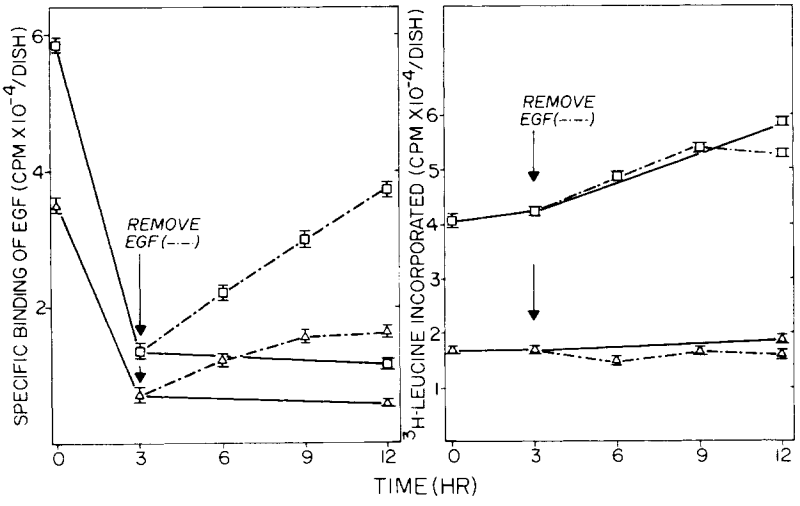


Fig. 2. Comparison of rates of EGF receptor recovery after down-regulation with rates of protein synthesis in serum-starved and unstarved cells. Cells were grown to confluence in medium containing 5% FCS. Twenty-four hours prior to time 0, the FCS concentration in some dishes was changed to 0.5%. At time 0, the medium of all dishes was changed to fresh DME containing 5% or 0.5% FCS and 50 ng/ml of EGF. After a 3 h incubation, EGF was removed from some samples, and DME containing 5% or 0.5% FCS was added to these dishes. ^{125}I-EGF binding (left) and protein synthesis (right) indicated by ^{3}H-leucine uptake into acid-insoluble material were determined as described in Materials and Methods. Cells were incubated in leucine-free minimal essential medium (LF-MEM, Gibco) containing 0.01 mM L-4,5-^{3}H-leucine (New England Nuclear, 5 Ci/mmole) for 60 min at 37°C prior to their being processed for determination of labeled leucine uptake into acid insoluble material. (□ = cells grown and maintained in 5% FCS; △ = cells starved for 24 h in 0.5% FCS and maintained in 0.5% FCS; —— = incubation in the presence of 50 ng/ml of EGF; —·—·— = incubation after EGF removal.)

Identification of the EGF Receptor by Surface-Specific Radioiodination and by "Direct Labeling" with ^{125}I-EGF.

The EGF receptors on murine cells were initially identified by Das et al [10, 16], who observed a high molecular weight ^{125}I-labeled band by SDS-PAGE after cross-linking a specifically bound ^{125}I-EGF photoaffinity probe to the receptor protein. More recent observations show that a small portion of cell-bound ^{125}I-EGF becomes directly attached to its surface receptor on murine 3T3 cells to form a high molecular weight, radioactive band resistant to dissociation in SDS-PAGE [23, 24]. The formation of this direct-labeled hormone-receptor complex is dependent on specific binding of EGF, and the complex can be precipitated with anti-EGF IgG from a Triton X-100 extract of cells incubated with hormone [23]. Upon further incubation at 37°C, this complex is degraded to the same lower molecular weight products described previously for the cross-linking studies [16]. These experiments identify only a small portion of the EGF receptors; direct and photoaffinity labeling with ^{125}I-EGF proceeds to a maximal yield of only a few percent. Since "direct" and photoaffinity-labeling procedures might therefore detect a small, but uncharacteristic fraction of the total receptor population, we turned to surface-specific iodination to label a more sizable, and thus possibly a more representative, fraction of the EGF receptors. Untreated murine 3T3 cells and cells treated with EGF to down-regulate the EGF receptors were labeled by surface-specific iodination. Gel electrophoresis revealed the presence of the murine EGF receptor in the untreated cells as a 170,000 dalton single

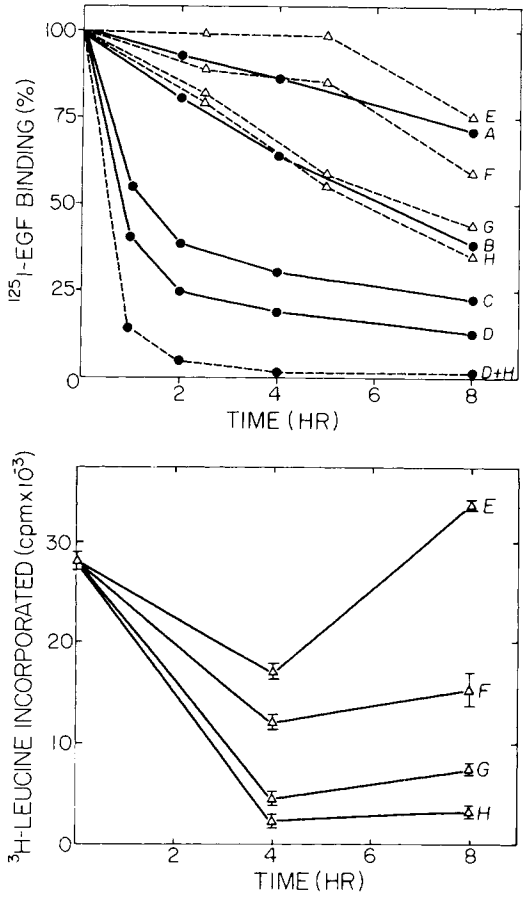


Fig. 3. (Upper) Effect of EGF and/or cycloheximide treatment on EGF binding activity. (Lower) Effect of cycloheximide on protein synthesis. Cells were grown to confluence in DME containing 5% FCS. These cells are from the same batch used for the studies in Figure 2. The FCS concentration was changed to 0.5% and the incubations continued for 24 h. At time 0, 0.4 (A), 2 (B), 10 (C), and 50 (D) ng/ml of unlabeled EGF; 0.04 (E), 0.2 (F), 1.0 (G), and 5.0 (H) μg/ml of cycloheximide; or 50 ng/ml of EGF and 5 μg/ml of cycloheximide (D + H) were added. ^{125}I-EGF binding (upper) and protein synthesis (lower) were determined at the indicated times after the cells had been washed thoroughly to remove EGF and/or cycloheximide.

polypeptide chain [20]. Since the EGF receptor is a minor surface component of these cells, its visualization was possible only when trypsin treatment of cells was used to enrich the cell surface for proteins with a relatively high turnover rate during a brief period of additional growth. In the experiment described in Figures 4 and 5, a surface-labeled band with the electrophoretic mobility of the EFG receptor (M_r = 170,000) was readily detected after a brief period of growth of the trypsin-treated 3T3 cells. This 170,000 dalton band was missing in lane B where EGF had been added to the cells to induce EGF receptor internalization during the brief period of receptor synthesis. Lanes C and D of the same figure show the affinity-labeled EGF: EGF receptor complex produced by direct labeling. The directly labeled receptor migrated more slowly than radiolabeled, unoccupied

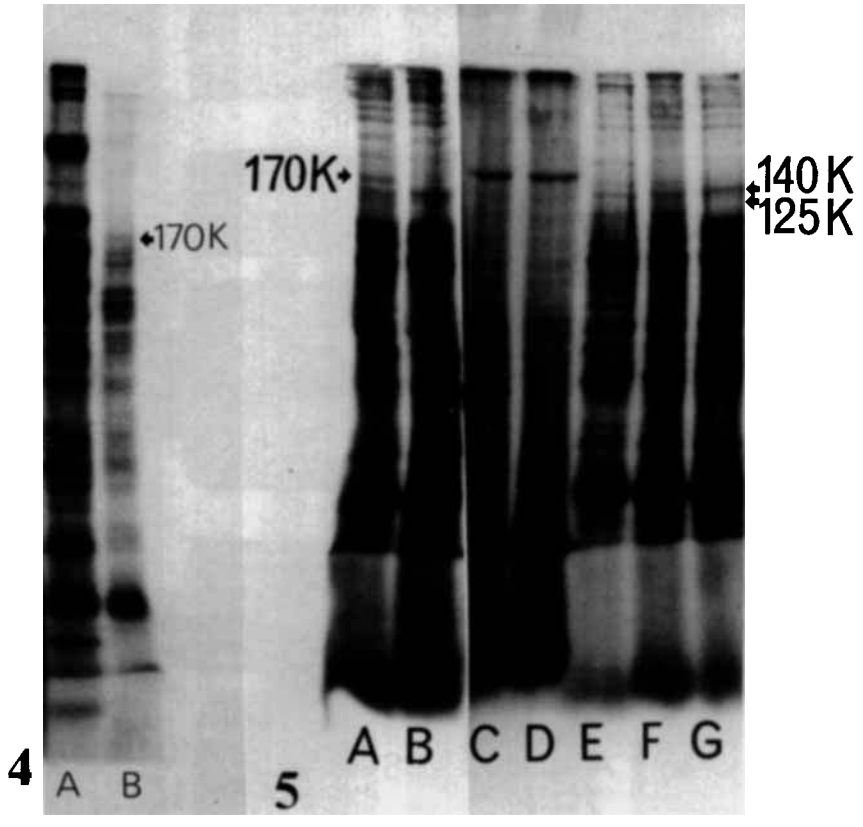


Fig. 4. A,B (left). Effect of trypsin on radioiodinated proteins displayed on surface labeled 3T3 cells. Confluent 3T3 monolayers were radiolabeled by surface-specific iodination [20]. After radiolabeling sample B was incubated with 40 μg/ml of trypsin for 10 min as described in Materials and Methods; sample A received no trypsin treatment. The monolayers were dissolved in buffer containing SDS and processed for SDS polyacrylamide gel electrophoresis on a 5–10% gradient slab gel [20]. Proteins were visualized by autoradiography. The arrow indicates the position of the EGF receptor determined as described for Figure 5.

Fig. 5. A–G, (right). A,B: Effect of EGF on the appearance of proteins subject to surface-specific iodination during the resynthesis of trypsin-sensitive surface proteins. 3T3 monolayers were treated with 40 μg/ml of trypsin, incubated in binding medium containing 50 ng/ml of unlabeled EGF (B) or no EGF (A) for 2 h at 37°C, labeled by surface-specific iodination and processed for gel electrophoresis on a 5–10% gradient slab gel, and visualized by autoradiography.

C,D: Direct labeling of EGF receptors. The 3T3 cell monolayers were incubated for 30 min at 37°C in medium containing 50 ng/ml of ^{125}I-EGF. After unbound ^{125}I-EGF was removed, the cells were processed for gel electrophoresis and autoradiography. The relatively high background in these samples is caused by unreacted ^{125}I, which was not removed after the chloroglycoluril-mediated iodination of

E,F,G: Effect of EGF-induced EGF receptor down-regulation on the appearance of a 140,000 dalton band produced by trypsin treatment of surface-radioiodinated cells. 3T3 monolayers were labeled by surface-specific iodination, incubated for 0 (E) or 60 (F,G) min in medium containing 50 ng/ml of unlabeled EGF (G) or no EGF (F), treated with 40 μg/ml of trypsin, and processed for gel electrophoresis in SDS solution. Proteins were visualized by autoradiography. Samples A–G were resolved on the same 5–12.5% polyacrylamide gradient slab gel, but samples A and B were phtographed from a different autoradiograph than were samples C-G. From Wrann et al [20].

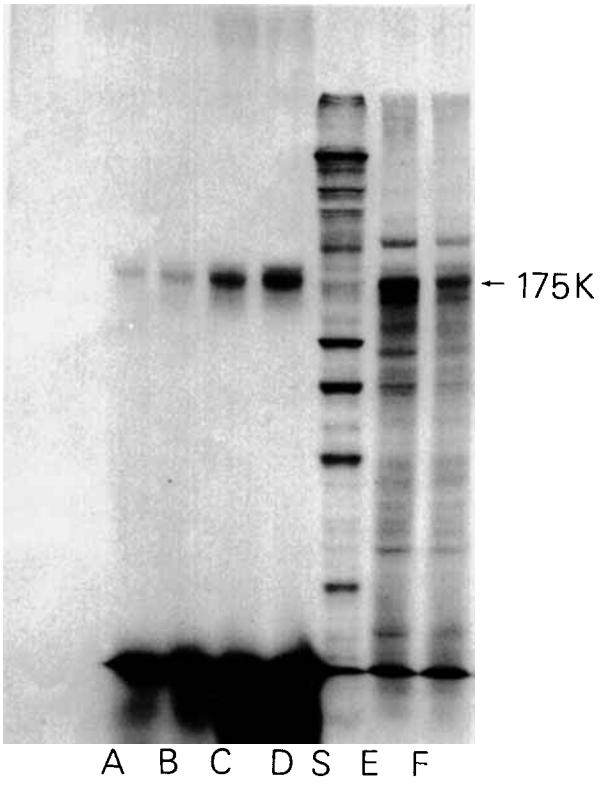


Fig. 6. Identification of the EGF receptor on A431 cells by surface-specific iodination and direct labeling with ^{125}I-EGF. A431 cells or mouse 3T3 cells were grown to confluence in 35 mm culture dishes with DME containing 10% FCS. Direct labeling of the EGF receptor. A431 monolayers were washed twice with medium and incubated in medium containing 100 ng/ml of ^{125}I-EGF prepared by the chloramine-T procedure (lanes A and B) or the chloroglycoluril procedure (lanes C and D) for 30 (lanes A and C) and 90 min (lanes B and D). Unbound EGF was removed, and the cells were dissolved in SDS solution and processed for gel electrophoresis as described under Experimental procedures. Surface labeling. A431 (lanes E and F) cell monolayers were washed twice with binding medium and incubated with medium containing unlabeled EGF 1000 ng/ml (lane F) for 4 h at 37°C to allow for EGF receptor down-regulation. The cell sample for lane E received a similar treatment but was incubated in the absence of EGF. All the monolayers were washed thoroughly, surface iodinated, and processed for gel electrophoresis. Radiolabeled proteins were visualized by autoradiography prior to sampling for direct determination of radioactivity in excised regions of the gels. Lane S, molecular weight standards. From Wrann and Fox [21].

receptor, with an apparent molecular weight of 175,000 daltons. This difference in M_r can be accounted for by the attachment of a single EGF molecule to the 170,000 dalton receptor. Still more convincing evidence for EGF receptor identification by surface-specific iodination was obtained with the human epithelioid carcinoma cell line A431, which has approximately 20 times more surface receptors for EGF than normal fibroblasts [25]. Following surface-specific radioiodination of cells, a 175,000 dalton protein was the most heavily labeled protein band detected after SDS-PAGE and autoradiography of the slab gel (Fig. 6). During EGF-induced EGF receptor down-regulation, the radioactivity in this

band decreased in parallel to the loss of surface receptors as determined by ^{125}I-EGF binding. A similar decrease (~ 50%) was observed in the affinity-labeled EGF receptor band of these cells, which migrated with an apparent M_r = 180,000.

EGF alters a proteolysis-susceptible site on the EGF receptor. The addition of extracellular trypsin in the surface-labeling experiments with 3T3 cells led to an unexpected observation. When the order of reactions in the experiment described in Figure 5 (A, B) was changed so that cells were surface-iodinated first, incubated next in the absence of EGF, and finally treated with trypsin prior to gel electrophoresis, a 140,000 dalton protein was detected. This band was clearly missing in the EGF-treated samples, where an increased amount of a 125,000 dalton protein was found instead (Fig. 5, lanes E–G). This indicates that EGF exposes a new trypsin cleavage site on receptors, giving rise to the 125,000 dalton cleavage product rather than the 140,000 dalton product observed when EGF was not present during trypsin treatment. The observation of the 125,000 dalton protein was also made in studies where ^{125}I-EGF was incubated with isolated membranes to label the receptor directly. As observed with intact cells, a small portion of the membrane-bound ^{125}I-EGF attached to receptor to form a 175,000 dalton component (Fig. 7, lane A). When trypsin was added to the ^{125}I-EGF-treated membranes, the 175,000 dalton complex was degraded to a 125,000 dalton complex, and these data indicate a precursor-product relationship between the 175,000 and 125,000 dalton components. A small quantity of this 125,000 band was also present in the sample not incubated with trypsin. This probably is produced by degradation of EGF receptors by an endogenous membrane protease. Das and Fox [16] had reported earlier that incubation of cells in the presence of chloroquine resulted in some degradation of the 175,000 dalton ^{125}I-EGF:EGF receptor photoaffinity complex to a component of approximately 125,000 daltons. This may be the same component described here.

The observations described in this section support the following scheme of EGF receptor turnover. Receptor removal and degradation in the absence of EGF (unoccupied mode) is accomplished by proteolysis resulting in degradation products that do not give rise to the EGF response(s). The addition of EGF changes the conformation of receptor, shifting receptor removal to the occupied mode and exposing different protease-cleavage sites. Cleavage at these sites may convert receptor to an "activated" form.

Platelet-Derived and Fibroblast Growth Factors Transiently Down-Regulate EGF Receptors and Modify Their Susceptibility to Down-Regulation by EGF.

Transient down-regulation of EGF receptors by PDGF and FGF. Mitogenic concentrations of PDGF (platelet-derived growth factor [26, 27]) or FGF (fibroblast growth factor [28]) modulate EGF binding activity in murine 3T3 cells [29, 30]. In the studies treated in this report, platelet extract (PE) was used as the source of PDGF, and purified bovine pituitary FGF as the source of FGF. Both these hormones stimulated ^{3}H-thymidine uptake by 3T3 cells to a greater extent than did EGF (Fig. 8). Since sensitive radioreceptor assays have not been developed for FGF or PDGF, the influence of these hormones on their own receptors cannot be determined effectively. In fact, we know of no report of a binding assay for PDGF. Though high concentrations of FGF or PDGF do not compete with EGF for binding to the EGF receptor (data not shown), both hormones effectively down-regulate EGF binding activity (Figs. 9 and 10). However, the properties of the down-regulation phenomenon induced by FGF and PDGF differ from the EGF-induced phenomenon. When EGF is added to cells, EGF binding activity is reduced to a new steady-state level and is then maintained at that level. With PDGF or FGF addition, EGF binding

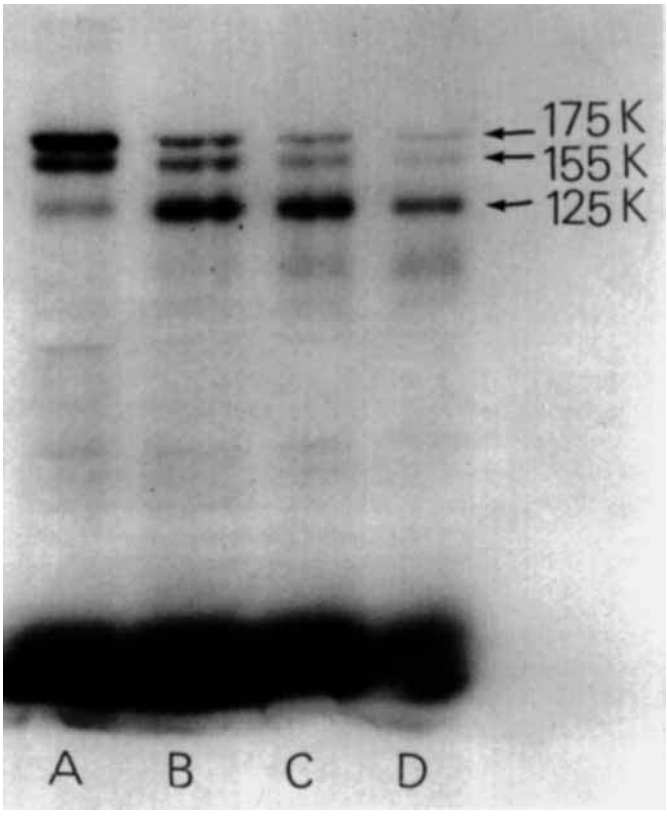


Fig. 7. Tryptic degradation of direct labeled EGF receptors on isolated 3T3 membranes. Isolated surface membranes from 3T3 cells were incubated for 60 min at 23°C in medium containing 50 ng/ml of ^{125}I-EGF to produce direct-labeled EGF receptor [23]. The samples were layered above a 1 ml cushion of 10% (w:w) sucrose in medium and sedimented for 2 min in a Brinkman microfuge to remove unbound EGF. The pellets were washed with medium and incubated for 30 min at 0°C with 0 (A), 2.5 (B), 25 (C), or 250 μg/ml (D) of trypsin for 30 min. Trypsin was removed, and the samples were washed twice with medium, dissolved in SDS solution, and processed for SDS-polyacrylamide gel electrophoresis on a 10% slab gel. The labeled proteins were visualized by autoradiography. From Wrann et al [20].

activity first decreases but then rebounds to normal or near-normal levels (Figs. 9 and 10). We call this phenomenon "transient down-regulation." In some experiments, and especially where cells were treated and maintained with EGF for an extended time to achieve a down-regulation steady state for the EGF receptor, the EGF binding activity achieved after the rebound was higher than that encountered prior to addition of FGF (Fig 10). This behavior may be related to inhibition of EGF-induced EGF receptor down-regulation by FGF and PDGF (Figs. 9 and 10), a phenomenon treated in the next section. With FGF, the threshold for transient down-regulation of EGF binding was decreased when cells were previously treated with EGF to achieve a lower steady state of EGF binding activity. We have not performed tests to determine if the PDGF threshold for PDGF-mediated transient down-regulation of EGF binding is also decreased by EGF.

Transient down-regulation of EGF binding by FGF or PDGF is dose-dependent for both these hormones (Fig. 10 and data not shown). Comparison of mitogenesis (Fig. 8)

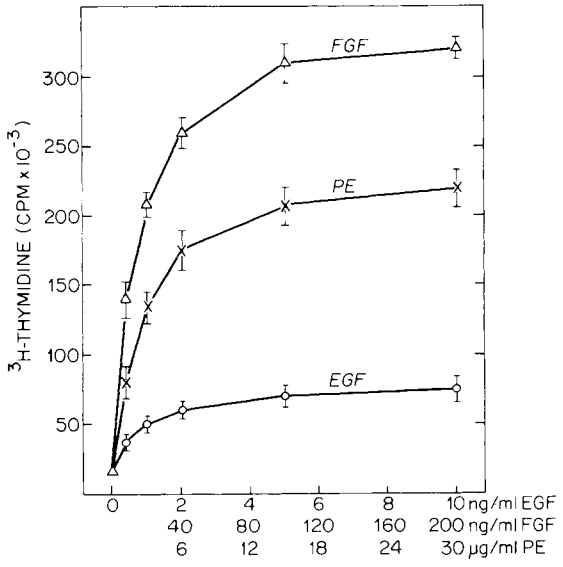


Fig. 8. Stimulation of DNA synthesis of serum-starved 3T3 cells by EGF, FGF, and PE. The indicated amounts of EGF, FGF, and PE were added to cultures of serum-starved 3T3 cells. Uptake of ^{3}H-thymidine into DNA was determined 18 h later.

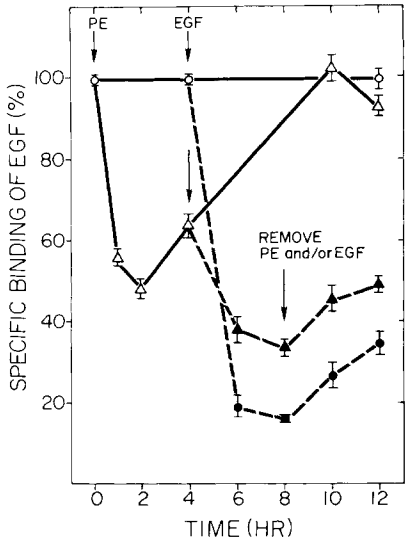


Fig. 9. Effect of PDGF on EGF-induced EGF receptor down-regulation and resynthesis. Serum-starved 3T3 cells were treated with 10 μl/ml of PE (triangles) or nor-PE (circles) at time 0. Unlabeled EGF was added to a final concentration of 50 ng/ml at time 4 h. PE and/or EGF was removed at time 8 h where indicated, and the cells were incubated with 0.5% FCS/DME. ^{125}I-EGF binding was measured on samples processed for assay at the indicated times.

and EGF receptor transient down-regulation induced by FGF or PDGF indicate similar dose-response curves for both these responses. Similarities in the dose-response curves for a membrane perturbation (EGF receptor transient down-regulation) and mitogenesis may indicate that the ability of FGF and PDGF to induce them is closely linked to the abilities of FGF and PDGF to induce obligatory alterations in the target cell membranes, including, but not limited to, internalization of FGF and PDGF receptors. However, we think it is unlikely that transient down-regulation of EGF receptors is linked in any direct, causal way to the abilities of PDGF or FGF to induce DNA synthesis in responsive cells.

Platelet-derived growth factor and fibroblast growth factor inhibit EGF-induced down-regulation of the EGF receptor. The experiments described in Figures 9 and 10 show that the addition of FGF or PDGF leads to transient down-regulation of the EGF receptor, but that EGF binding sometimes rebounds to a level that excedes that observed prior to FGF or PDGF addition. This phenomenon was especially pronounced in cases in which the cells had been maintained at a steady state of partial EGF-induced down-regulation of the EGF receptor, suggesting that incubation of cells with FGF or PDGF might cause inhibition of EGF-induced down-regulation of its receptor. An example of an experiment designed to test this hypothesis directly is given in Figure 9. The experiment in Figure 9 describes the typical pattern of PE-induced transient down-regulation and EGF-induced down-regulation of EGF binding. When EGF was added to the PE- treated (PE is platelet extract, a source of PDGF) cells during the period of EGF receptor rebound, EGF-induced down-regulation of EGF binding was observed, but to a lesser extent than observed with EGF alone. The decreased effectiveness of EGF on down-regulation of EGF binding in PE-treated cells was not due to an effect of PE on EGF receptor turnover. The rate of reappearance of EGF binding activity after EGF-induced down-regulation was the same in PE-treated and untreated cells.

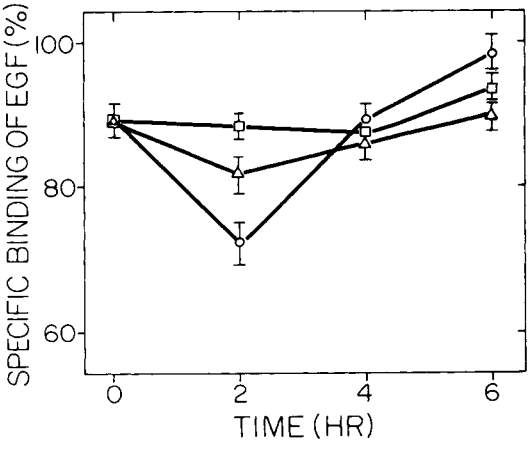


Fig. 10. Transient down-regulation of EGF receptors by FGF. Serum-starved 3T3 cells were treated with 1 ng/ml of EGF for 12 hr prior to time 0, and the EGF remained present in the medium during the subsequent incubations with FGF. At time 0, FGF was added to the cells at 25 (□), 50 (△), and 100 (○) ng/ml, and the cells were incubated for the times indicated and washed thoroughly to remove unlabeled EGF. ^{125}I-EGF binding was determined using the standard binding assay.

DISCUSSION

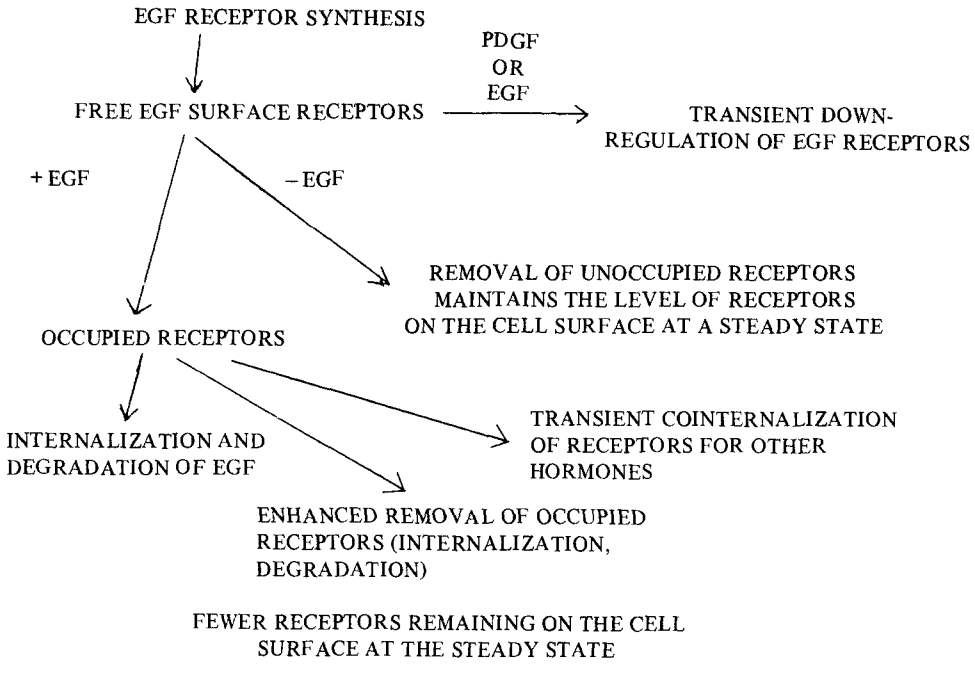
The ultimate goal in the study of any receptor system is the identification of the "second messenger" for hormone action and the delineation of the mechanism by which the second messenger functions. In the case of EGF, much is now known about both hormone and receptor, and the fates of the hormone and receptor molecules after their interaction [6, 12–21, 23, 24].

In fact, few hormones or receptors have been studied as extensively as EGF and its receptor. However, the quest for the second messenger still remains. In a recent review, Carpenter and Cohen have listed six possible mechanisms for second messenger generation in an anything but all-inclusive list of hypotheses [4]. Among these are the generation of a second messenger through ligand-induced patching of receptor, through phosphorylation of receptor or other proteins, or through internalization and proteolytic activation of receptor (and/or EGF).

We have selected as our working hypothesis the endocytic activation of receptor, and our reasons for making this selection are twofold: First, in Swiss 3T3 cells, the enhanced internalization and proteolytic processing of receptor induced by EGF has been correlated with the EGF-induced activation of DNA synthesis [18]. Second, a host of biological processes are activated by proteolytic post-translational processing of proenzymes or prohormones. Among these are the classical case of the conversion of chymotrypsinogen to chymotrypsin [31], the cleavage events that lead to the blood clotting [32, 33] and complement [34] cascade mechanisms, and the elegant studies of Steiner and his colleagues on the proinsulin to insulin conversion [35]. Additionally, Glenn and Cunningham have obtained evidence linking the stimulation of cell division by thrombin to the proteolysis of the thrombin receptor [36].

The endocytic activation hypothesis applied to EGF action encounters difficulty when examined in the light of evidence indicating that EGF receptors turn over rapidly [11, 37] (Figs. 1–3). Though the fate of receptors removed during normal turnover has not been demonstrated directly, it is conceivable that both normal turnover and EGF-induced loss of EGF binding activity occur by endocytosis and lysosomal processing. If this is the case, it becomes difficult to invision how a mere enhancement of receptor internalization could lead to a specific activation of EGF-induced processes leading to stimulation of DNA synthesis. One answer to this dilemma is the EGF-induced exposure of new protease-sensitive sites on receptor [20] (Fig. 5). This experiment indicates that EGF produces a conformational change in receptor, and this may expose new sites to the proteases existing in the lysosomes or to endogeneous protease(s) that reside on the cell surface and appear to mimic trypsin in their specificity (Figs. 5 and 7). On the basis of these results, we have modified our initially proposed model of endocytic activation to one in which the internalization and processing of receptor is necessary, but not sufficient, for activation of second messenger production. This activation step may also require EGF to alter the sites on receptor sensitive to protease action, wherein the cleavage of the essential protease-sensitive site occurs only when receptor is in the EGF-induced conformation. The observation that hormone-induced modification of receptor occurs in the EGF system (possibly as an obligatory step in the activation process) suggests that enhanced internalization of EGF receptors need not be obligatory for endocytic activation. The turnover of receptors at the rate characteristic of normal, unoccupied receptor turnover could prove adequate for induction of an EGF response in the absence of any change in steady-state receptor level if EGF is internalized with receptor.

Schema 1



New phenomena that bear on this concept are also described here: the transient down-regulation of receptors induced by PDGF and FGF and the ability of these hormones to inhibit EGF-induced down-regulation of its own receptors. Presuming that the transient down-regulation of EGF receptors observed in these studies (Figs. 9 and 10) constitutes EGF receptor internalization, these data indicate that PDGF and FGF both induce internalization of their own receptors and that, during this process, internalization of EGF receptors in the unoccupied mode (Schema 1) is enhanced. As the receptors for FGF and PDGF achieve a down-regulated steady state, the rates of their internalization decrease, and EGF receptor synthesis restores the level of EGF receptors to the initial or a or a higher level, giving rise to a transiency in EGF receptor down-regulation. The inhibition of EGF-induced EGF receptor down-regulation by FGF and PDGF can be explained by a similar model, in which some common factor (or factors) is involved in the internalization of all these receptors and is limiting for internalization. Since it appears that EGF receptor down-regulation involves clathrin and internalization in coated vesicles [38], other receptors – eg, those for FGF and PDGF – may also be internalized in a process that involves their inclusion into coated vesicles.

Recent ultrastructural observations show that a portion of the EGF receptors resides in a patched configuration prior to EGF addition [13]. In addition, we have observed that in all cell lines tested so far, a considerable portion of the EGF receptors remains in a very high molecular weight form after extraction of membranes with solutions of Triton X-100 [39]. It appears that many of the EGF receptors are already included in organized regions of the membrane. If these regions consist of mixtures of receptors capable of ligand-induced internalization, then the addition of any one of a host of hormones may trigger the transient

internalization of a portion of all receptors that share a common organized membrane region in their internalization. If endocytic activation of receptors is a process that functions in an obligatory fashion in activation of second messenger production, this same process may be shared by a wide variety of hormones, which could vary considerably in their effects on cells.

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REFERENCES

1. Cohen S: J Biol Chem 237:1555–1562, 1962.
2. Savage CF Jr, Cohen S: J Biol Chem 247:7609–7611, 1972.
3. Carpenter G, Lembach KJ, Morrison MM, Cohen S: J Biol Chem 250:4297–4304, 1975.
4. Carpenter G, Cohen S: Annu Rev Biochem 48:193–216, 1979.
5. Hollenberg MD, Cuatrecasas P: J Biol Chem 250:3845–3853, 1975.
6. Carpenter G, Cohen S: J Cell Biol 71:159–171, 1976.
7. Savage CR Jr, Inagami T, Cohen S: J Biol Chem 247:7612–7621, 1972.
8. Holladay LA, Savage RC Jr, Cohen S, Puett D: Biochemistry 15:2624–2633, 1976.
9. Pruss RM, Herschman HR: Proc Natl Acad Sci USA 74:3918–3921, 1977.
10. Das M, Miyakawa T, Fox CF, Pruss RM, Aharanov A, Herschman HR: Proc Natl Acad Sci USA 74:2790–2794, 1977.
11. Aharonov A, Pruss RM, Herschman HR: J Biol Chem 253:3970–3977, 1978.
12. Haigler H, Ash JF, Singer SJ, Cohen S: Proc Natl Acad Sci USA 75:3317–3321, 1978.
13. Haigler HT, McKanna JA, Cohen S: J Cell Biol 81:382–395, 1979.
14. Schlessinger J, Schechter Y, Willingham MC, Pastan I: Proc Natl Acad Sci USA 75:2659–2663, 1978.
15. Maxfield FR, Schlessinger J, Schechter Y, Pastan I, Willingham MC: Cell 14:805–810, 1978.
16. Das M, Fox CF: Proc Natl Acad Sci USA 75:2644–2648, 1978.
17. Fox CF, Das M: J Supramol Struct 10:199–214, 1979.
18. Fox CF, Vale R, Peterson SW, Das M: Cold Spring Harbor Conferences on Cell Proliferation series, Vol 6, Hormones and Cell Culture, pp. 143–157, 1979.
19. Schechter Y, Hernaez L, Schlessinger J, Cuatrecasas P: Nature 278:835–838, 1979.
20. Wrann M, Linsley P, Fox CF: FEBS Lett 104:415–419, 1979.
21. Wrann M, Fox CF: J Biol Chem 254:8083–8086, 1979.
22. Samson ACR, Fox CF: J Virol 12:579–587, 1973.
23. Linsley PS, Blifield C, Wrann M, Fox CF: Nature 278:745–748, 1979.
24. Baker JB, Simmer RL, Glenn KC, Cunningham DD: Nature 278:743–745, 1979.
25. Fabricant RN, De Larco JE, Todaro GJ: Proc Natl Acad Sci USA 74:565–569, 1977.
26. Pledger WJ, Stiles CD, Antoniades HN, Scher CD: Proc Natl Acad Sci USA 74:4481–4485, 1977.
27. Ross R, Vogel A: Cell 14:203–210, 1978.
28. Gospodarowicz D, Moran JS: Annu Rev Biochem 45:531–558, 1976.
29. Fox CF, Wrann M, Vale R: J Supramol Struct 9 (Suppl 3):176, 1979.
30. Wrann M, Fox CF: Fed Proc 38:301, 1979.
31. Dryer WJ, Neurath H: J Biol Chem 217:527–539, 1955.
32. Rovery M, Poilroux M, Curnier A, Desnuelle P: Biochim Biophys Acta 17:565–578, 1955.
33. Bennet B, Tatnoff OD: Med Clin North Am 56:95–104, 1972.
34. Muller-Eberhard HJ: Annu Rev Biochem 44:697–724, 1975.
35. Steiner DF: Diabetes 26:322–340, 1977.
36. Glenn KC, Cunningham D: Nature 278:711–714, 1979.
37. Carpenter G: J Cell Physiol 99:101–110, 1979.
38. Keen JH, Willingham MC, Pastan IH: Cell 16:303–312, 1979.
39. Linsley P, Fox CF, Iwata K: J Supramol Struct (Suppl 4):113, 1980.