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Stabilized complexes of epidermal growth factor and its receptor on the cell surface stimulate RNA synthesis but not mitogenesis

(concanavalin A/internalization/processing/signal transduction/DNA synthesis)

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ABSTRACT **Treatment of mouse fibroblasts, prelabeled at 4°C with ^{125}I-labeled epidermal growth factor (EGF), with the lectin concanavalin A (Con A) stabilized the ^{125}I-labeled EGF–receptor complex to dissociation and prevented receptor-mediated endocytosis; after 5 hr at 37°C, approximately 50% of the ^{125}I-labeled EGF initially bound at 4°C remained cell associated, compared to less than 15% in control cells. The radioactivity lost from the Con A-treated cells was found as intact hormone in the medium, with almost no hormone degradation evident, whereas in control cells most of the medium radioactivity was in the form of low molecular weight degradation products. The trimolecular complex Con A–EGF–receptor was capable of stimulating RNA synthesis to levels greater than control (untreated) or EGF alone and maintained this stimulation for prolonged periods of time. However, there was no effect of Con A treatment on the stimulation of DNA synthesis induced by EGF prebound at 4°C. Thus, maintaining the EGF–receptor complex at the cell surface allows enhanced stimulation of an acute biological response to EGF (RNA synthesis) but not stimulation of DNA synthesis. These data support the idea that processing subsequent to receptor binding is necessary to produce the mitogenic signal.**

The binding of epidermal growth factor (EGF) to specific high-affinity receptors in responsive cells has been shown to induce both acute and delayed biological effects. The early events induced by hormone–receptor interactions include (*i*) phosphorylation of cellular proteins, including the EGF receptor (1–5); (*ii*) stimulation of Na^+/K^+ exchange (6); (*iii*) enhanced nutrient transport (7, 8); and (*iv*) enhanced RNA synthesis (9). The delayed biological response is, of course, increased DNA synthesis and mitogenesis (8–10). Unlike those hormones that act through activation or inhibition of adenylate cyclase, the nature of the second messenger produced by the binding of EGF to its receptor is currently unknown. Although it has been shown that continuous exposure of responsive cells to EGF for several hours is required for mitogenesis (11–15) and that both EGF (16, 17) and its receptor (18, 19) are proteolytically processed after endocytosis, the question of whether the EGF–receptor complex acts at the cell surface, requires internalization and further processing, or both is the subject of current investigations.

The biological responses to EGF can be mimicked by certain classes of lectins (7) and monoclonal anti-receptor antibodies (20, 21), suggesting that the receptor itself contains all the necessary information for signal transduction, requiring only the appropriate perturbation of its conformation by ligand binding to produce biological effects. It has been proposed that microaggregation of the receptor on the cell surface is sufficient for production of the mitogenic signal (21–23), a situation analogous to several other hormone–receptor systems (24–27). However, there is also evidence that intracellular processing of the EGF–receptor complex is required for stimulation of DNA synthesis (28–31), and the increased sensitivity of EGF after heterologous down-regulation of its receptor by platelet-derived growth factor (32) suggests a role for receptor internalization in inducing the mitogenic response.

Recently, it has been shown that incubation of cells prelabeled with ^{125}I-labeled EGF (^{125}I-EGF) at 4°C with Con A results in Con A–EGF–receptor complexes that are stable to dissociation and are not internalized at 37°C (33). We used this effect of Con A on prebound EGF to test whether prolonged occupation of EGF receptors in the absence of internalization could stimulate DNA synthesis. The results show that, although the Con A–EGF–receptor complex is functional in terms of stimulation of acute effects (stimulation of RNA synthesis), there is no stimulation of DNA synthesis. These data support the concept that internalization and processing of the hormone–receptor complex is important for inducing a mitogenic response.

MATERIALS AND METHODS

Cell Culture. Normal rat kidney (NRK) fibroblasts (a gift of H. Moses, Mayo Clinic) were used in these studies because of their demonstrated sensitivity to the mitogenic effects of EGF and their abrogated requirement for platelet-derived growth factor (34) and insulin (unpublished observations) for growth. The NRK cells were maintained in a complete medium consisting of α-minimum essential medium (α-MEM; GIBCO) and 10% fetal calf serum (Rehautin, Phoenix, AZ). Stock 100-mm tissue culture plates (Corning) were passaged every 3–4 days at 1:7 dilution. For experiments, cells were seeded at 25,000–50,000 cells per 35-mm dish and used after 4–7 days. Confluent cultures were fed α-MEM/0.1% fetal calf serum for 48 hr before initiation of the [^{3}H]uridine and [^{3}H]thymidine incorporation experiments.

^{125}I-EGF Binding. EGF was purified and iodinated as described (33). Cultures were incubated with ^{125}I-EGF (specific activity, 2–3 × 10^5 cpm/ng) in Hepes-buffered Hanks' salts/0.1% bovine serum albumin (Sigma), pH 7.4 (HBSA), at 3–4 × 10^5 cpm/ml per dish for 3 hr at 4°C. Cultures were rinsed with three 1-ml portions of HBSA at 4°C, incubated without or with various concentrations of Con A (Calbiochem) in HBSA for 20 min at 4°C, and then incubated at 37°C for up to 5 hr. At the appropriate times, cultures were cooled to 4°C and rinsed three times with ice-cold HBSA, and the cell-associated radioactivity was removed by solubilization in 1 ml of 0.2 M NaOH.

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Abbreviations: EGF, epidermal growth factor; ^{125}I-EGF, ^{125}I-labeled EGF; HBSA, Hepes-buffered Hanks' salts/0.1% bovine serum albumin.

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To determine the nature of the radioactivity lost from the cells in the presence and absence of Con A, conditioned medium was taken from cultures treated as described above and fractionated on a Bio-Gel P-4 (Bio-Rad) column as described (35). Intact ^{125}I-EGF elutes in the void volume, while degraded hormone elutes in the included volume.

[^{3}H]Uridine Incorporation. Quiescent cells were incubated with or without unlabeled EGF for 3.5 hr at 4°C in HBSA, rinsed with three 1-ml portions of HBSA, then incubated without or with Con A (200 μg/ml) for 20 min at 4°C. The Con A-containing medium was then replaced with HBSA without or with 50 mM methyl α-D-mannoside (Sigma) to displace the bound Con A, and the cells were incubated at 37°C. At the appropriate times, [^{3}H]uridine (ICN) at 1 μCi/ml was added to triplicate dishes of each treatment group and cells were incubated a further 15 min at 37°C. At the end of the incubation period, cultures were rinsed with 2 ml of phosphate-buffered saline, incubated with two 1-ml portions of 5% trichloroacetic acid at 4°C for 10 min, and then washed with three 1-ml portions of distilled H_2O. The acid-precipitable radioactivity was removed from the dishes with 1 ml of 0.2 M NaOH/0.1% sodium dodecyl sulfate, neutralized with 1 M HCl, added to 10 ml of liquid scintillation cocktail (Ready-Solv, Beckman), and counted in a Packard liquid scintillation counter.

[^{3}H]Thymidine Incorporation. Quiescent cells were incubated with or without unlabeled EGF (60 ng/ml) in HBSA for 3 hr at 4°C, rinsed, and then incubated, without or with Con A (200 μg/ml) for 20 min at 4°C. Cultures were then incubated for 16–18 hr in HBSA without or with 50 mM methyl α-D-mannoside. At the end of the incubation period, [^{3}H]thymidine (ICN) at 1 μCi/ml was added to each dish for 60 min at 37°C, and acid-precipitable radioactivity was determined as described above.

Cell numbers were determined, following trypsinization, with a Coulter Counter.

RESULTS

As reported earlier (33), when ^{125}I-EGF-labeled cells were pulsed briefly with the lectin Con A, dissociation of the hormone from the cells was inhibited (Fig. 1). This effect is concentration dependent; at the maximum concentration of Con A used (200 μg/ml), ≈50% of the prebound ^{125}I-EGF was retained by the cells after 5 hr at 37°C, whereas <15% was retained by control cells (Fig. 1). Incubation of Con A-treated cells with methyl α-D-mannoside reversed this effect (data not shown; see below). Since Con A has been shown to be a competitive inhibitor of EGF binding to its receptor (ref. 36; unpublished observations), the order of addition of hormone and lectin was a critical variable. Therefore, except where noted, EGF was allowed to bind to its receptor before the addition of Con A.

The amount of intact ^{125}I-EGF versus ^{125}I-labeled degradation products present in the incubation medium of control and Con A-treated cells was determined. As shown in Fig. 2, medium from control cells contained progressively larger amounts of degraded hormone with time at 37°C while the amount of intact ^{125}I-EGF decreased as the hormone that dissociated directly from surface receptors on warming to 37°C was rebound (37). In contrast, Con A-treated cells released only intact hormone into the medium, with only a small amount of low molecular weight degradation product apparent at the longest time point. This suggests that ^{125}I-EGF is not processed in Con A-treated cells. This result is consistent with data derived from subcellular fractionation experiments showing that Con A inhibits internalization of surface EGF–receptor complex in human astrocytoma cells and murine fibroblasts (33) as well as in NRK cells (data not shown). When Con A-treated cells were subsequently incubated with methyl α-D-mannoside, the inhibition of cellular ^{125}I-EGF processing was reversed (Fig. 2).

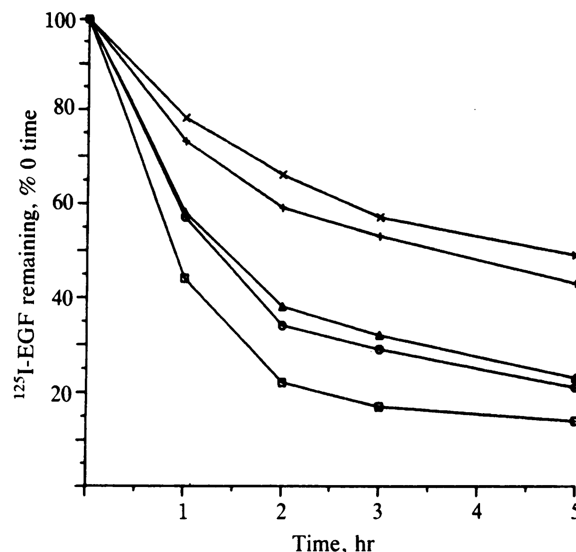

FIG. 1. Effect of Con A on dissociation of ^{125}I-EGF from NRK cells. Cells were labeled with ^{125}I-EGF (3.5×10^5 cpm/ml per dish) for 3 hr at 4°C, rinsed with three 1-ml portions of HBSA, and then incubated with HBSA containing Con A at various concentrations (in μg/ml: □, 0; ○, 5; △, 10; +, 100; ×, 200) for 20 min at 4°C. Cultures were then shifted to 37°C and, at the appropriate time, cooled to 4°C, rinsed with 1 ml of HBSA, and solubilized. The amount of ^{125}I-EGF bound at 0 time (= 100%) was 2782 cpm (SD = 238 cpm).

To assess the functional state of Con A–EGF-receptor complexes, we measured the stimulation of [^{3}H]uridine incorporation into acid-precipitable material by EGF in the presence and absence of the lectin. Cells were incubated without or with unlabeled EGF (30 ng/ml) at 4°C, rinsed, incubated without or with Con A, then treated without or with methyl α-D-mannoside and incubated at 37°C for up to 5 hr. As shown in Fig. 3, in the absence of EGF or Con A, there was a slight increase in [^{3}H]uridine incorporation, which declined after 1 hr. Con A, which has been shown to mimic some of the effects of EGF and insulin (7), maintained the slight stimulation of incorporation produced by the incubation medium. Prior incubation with EGF enhanced the increased RNA synthesis seen at early times, although levels declined after 1 hr. When cells that had been incubated with EGF were treated with Con A before warming, there was a continued increase in RNA synthesis over the 5-hr incubation period. When EGF/Con A-treated cells were incubated with methyl α-D-mannoside, essentially control levels of [^{3}H]uridine incorporation were seen. Methyl α-D-mannoside alone had no effect (data not shown). These data suggest that the Con A–EGF–receptor complexes, which were maintained at the cell surface, were functional in terms of their ability to acutely stimulate RNA synthesis.

The ability of prebound EGF to stimulate mitogenesis was tested, and the effect of Con A on this process was determined. Cells incubated for 3 hr at 4°C in HBSA alone showed a 2.5-fold increase in DNA synthesis over untreated controls. This stimulation was abolished when the cells were pulsed with Con A, and methyl α-D-mannoside reversed this inhibition (Fig. 4). When EGF at 60 ng/ml was present in the 4°C preincubation, DNA synthesis was 3.5-fold greater than control. Again, Con A abolished this stimulation, an effect reversed by methyl α-D-mannoside (Fig. 4). Thus, Con A inhibits the mitogenic response to prebound EGF but enhances the stimulation of RNA synthesis (Fig. 3). When either EGF (30 ng/ml) or fresh medium containing serum was added to quiescent cells at 37°C for 16–18 hr, DNA synthesis was stimulated 6- to 8-fold (Table 1). A 20-min pulse of Con A at 4°C before addition of fresh medium

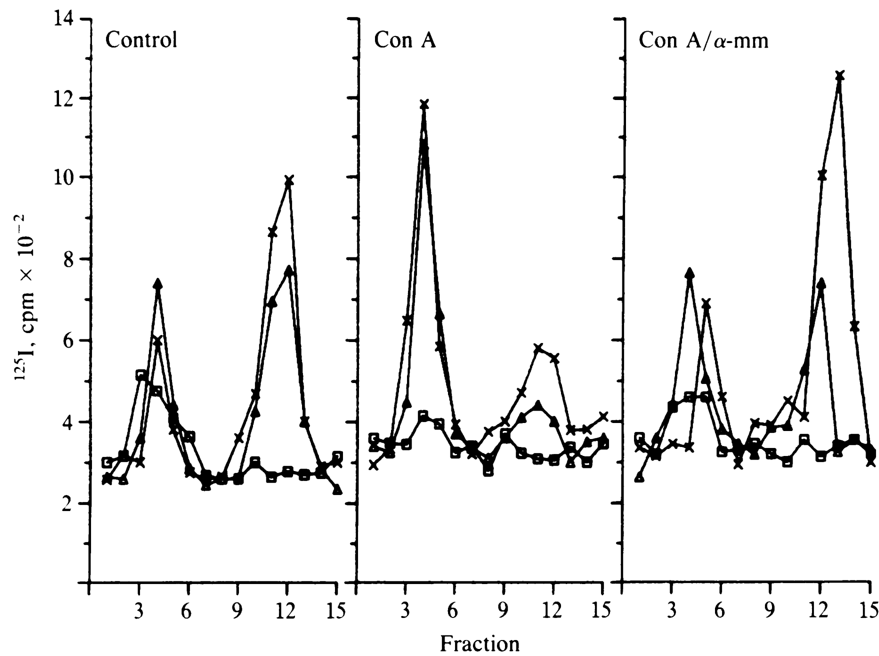

FIG. 2. Effect of Con A on ^{125}I-EGF degradation. Cells were labeled with ^{125}I-EGF (4.5×10^5 cpm/ml per dish), rinsed, and incubated without or with Con A (200 μg/ml) for 20 min at 4°C. Then, the medium was replaced with HBSA without or with 50 mM methyl α-D-mannoside (α-mm), and the cells were incubated at 37°C for 0–5 hr. The medium was removed and fractionated on a Bio-Gel P-4 column to separate intact (fractions 3–5) from degraded (fractions 10–13) ^{125}I-EGF. □, 0 hr; ▲, 2 hr; ×, 5 hr.

did not inhibit the stimulation of DNA synthesis and, in fact, slightly enhanced it (Table 1, experiment 1). Continuous exposure of cells to fresh medium plus Con A produced similar results (Table 1, experiment 2). Continuous exposure of cells to HBSA containing EGF and Con A completely inhibited the stimulation of DNA synthesis by EGF (Table 1, experiment 2), probably by interfering with EGF binding to its receptor (36). Thus, the Con A-mediated inhibition is restricted to EGF-stimulated DNA synthesis and does not affect the stimulation produced by serum.

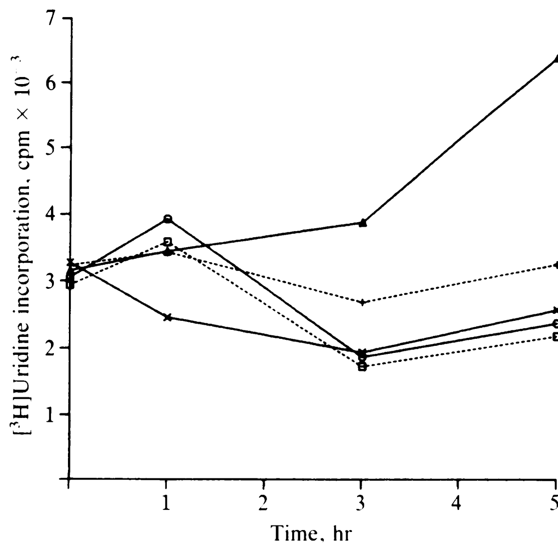

FIG. 3. Effects of EGF, Con A, and methyl α-D-mannoside on stimulation of [^{3}H]uridine incorporation. Cells were grown in 0.1% serum 48 hr before the experiment was initiated. Cultures were incubated without or with EGF (30 ng/ml) at 4°C for 3.5 hr, rinsed with three 1-ml portions of HBSA, and incubated first without or with Con A (200 μg/ml) for 20 min at 4°C and then without or with 50 mM methyl α-D-mannoside for various times at 37°C. Cultures were then pulsed with [^{3}H]uridine at 1 μCi/ml for 15 min at 37°C and the amount of acid-precipitable radioactivity was determined. □, −EGF/Con A/methyl α-D-mannoside; ○, +EGF, −Con A/methyl α-D-mannoside; △, +EGF/Con A, −methyl α-D-mannoside; ×, +EGF/Con A/methyl α-D-mannoside; +, −EGF, +Con A, −methyl α-D-mannoside. The average SD for the points was 12% (not shown).

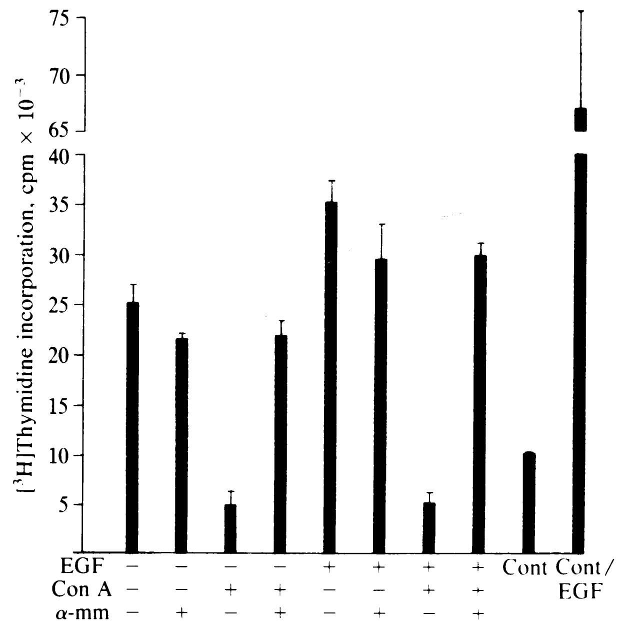

FIG. 4. Effects of EGF, Con A, and methyl α-D-mannoside on [^{3}H]thymidine incorporation. Cells were grown in 0.1% serum for 48 hr before initiation of the experiment. Cultures were incubated without or with EGF (60 ng/ml) for 3 hr at 4°C, rinsed, and then incubated without or with Con A (200 μg/ml) for 20 min at 4°C and for 16 hr in α-MEM/0.1% bovine serum albumin without or with 50 mM methyl α-D-mannoside (α-mm). At the end of the incubation, the amount of [^{3}H]thymidine incorporation into acid-precipitable material was determined. Control cultures (Cont) remained undisturbed until addition of [^{3}H]thymidine; Cont/EGF cultures had EGF at 30 ng/ml added directly to the low-serum medium with [^{3}H]thymidine incorporation determined 16 hr later.

Table 1. Effect of Con A on serum- and EGF-stimulated DNA synthesis

Addition	[^{3}H]Thymidine incorporation, cpm
Experiment 1	
None (control)	15,180
EGF	127,296
10% serum (no Con A)	118,870
10% serum/Con A (20 min, 4°C)	157,420
Experiment 2	
None (control)	1,392
10% serum (no Con A)	127,914
10% serum/Con A	117,542
HBSA (no Con A)	6,778
HBSA/Con A	2,444
EGF (no Con A)	61,048
EGF/Con A	2,047

In experiment 1, EGF (30 ng/ml) was added directly to unchanged complete medium for 16 hr before [^{3}H]thymidine incorporation. Con A (200 μg/ml) was added to cells for 20 min at 4°C, after which cultures were rinsed with two 1-ml portions of HBSA and 1 ml of complete medium and then incubated 16 hr in complete medium. In experiment 2, all additions were continuous for the 18-hr incubation before [^{3}H]thymidine incorporation. All Con A additions were at 200 μg/ml; EGF was in HBSA at 30 ng/ml.

DISCUSSION

It has previously been shown that Con A treatment of cells labeled with ^{125}I-EGF prevents dissociation of the EGF–receptor complex and also inhibits internalization (33). In the present study we have confirmed this finding in a different cell line and have used the phenomenon to differentiate between the biological actions of EGF that occur at the cell surface versus actions requiring further processing of the hormone–receptor complex. The data show that Con A stabilizes the EGF–receptor complex to spontaneous dissociation. Loss of ^{125}I-EGF from control cells involves internalization and hormone degradation, while loss from Con A-treated cells is directly from surface receptors into the medium as intact hormone. Con A does not interfere with the degradation of ^{125}I-EGF if the hormone has been internalized before treatment with the lectin (ref. 37; unpublished observations).

The results from the [^{3}H]uridine incorporation experiments were important in showing that the Con A–EGF–receptor complex was maintained on the cell surface in a configuration which was functional, at least in terms of stimulating RNA synthesis. It would be of interest to measure the effect of Con A on some of the other acute actions of EGF, such as stimulation of tyrosine kinase activity (38).

The stimulation of DNA synthesis by EGF was prevented by treatment with Con A both when the lectin was present during a continuous exposure and in a pulse–chase exposure to both agents. Given the data supporting the inhibition of loss and internalization of the EGF–receptor complex following incubation with Con A, this result suggests that stimulation of DNA synthesis by EGF requires processing steps that occur subsequent to binding. Other reports have appeared suggesting that some aspects of intracellular processing of EGF–receptor complex are required for stimulation of DNA synthesis (28–31). This effect of Con A on the prevention of EGF-stimulated DNA synthesis is not a generalized phenomenon, since no inhibition was seen when serum (i.e., complete medium) was given to the cells. Thus, serum contains enough "other" factors to overcome the need for EGF in inducing mitogenesis in fibroblasts. Alternatively, there might be sufficient serum glycoproteins to completely bind the added Con A. However, the cells pulsed with Con A in serum-free medium also showed no inhibition of DNA synthesis when subsequently fed complete medium. We cannot at this time rule out the possibility that even though the Con A–EGF–receptor complex can achieve a conformation capable of stimulating RNA synthesis, it cannot achieve a conformation that can produce the mitogenic signal. Further investigation of the known responses to EGF that might be affected by Con A should help resolve this issue.

The continuous presence of EGF is required for at least 6 hr to produce a mitogenic response (11, 12, 14, 15), and recent data have shown that the degree of mitogenic stimulation is linearly related to the steady-state level of receptor occupancy (39). Furthermore, Leof *et al.* (15) have shown that EGF is required for the first half of the G_0/G_1 phase (6 hr) but not for the traverse of the late G_1 phase of the cell cycle. Taken together, these data suggest that the continuous generation of a second messenger(s) by persistent occupation of EGF receptors is required to carry cells through the first half of G_0/G_1. Such a model has been proposed by others (14, 15). However, the nature of the molecular event(s) triggered by EGF–receptor interactions that are required for the G_1 traverse are not known.

The effect of persistent occupation of receptors by their agonists on biological responses has been studied in several hormone–receptor systems. The use of photoreactive hormone analogs to covalently link hormones to their receptors has been shown to prolong signal production in these systems (40–43). We have taken advantage of the Con A stabilization of the EGF–receptor complex on the plasma membrane to show that a persistently occupied cell surface EGF receptor will produce prolonged stimulation of RNA synthesis but will not enhance DNA synthesis. We therefore conclude that occupation of surface receptors *per se* is insufficient to produce the mitogenic signal and that further processing within the cell is required. It also seems likely that the EGF–receptor complex acts through several different effectors, both at the cell surface and within the cell, to produce its various biological responses.

These results bear on the more general models proposed to explain both the immediate and the long-term biological responses produced by peptide growth factors (for reviews, see refs. 44 and 45). It has been suggested that interaction of the growth factor (e.g., EGF, insulin, somatomedin) with its receptor at the cell surface triggers rapid events such as tyrosine kinase activity, metabolite transport, and ion fluxes, thus preparing the cell for further metabolic activation. Stimulation of the later responses (mitogenesis) then requires internalization and the production of a secondary signal. The cytosolic orientation of the tyrosine kinase catalytic site associated with virtually all the growth factor receptors makes it an attractive candidate for this function. By analogy with the lysosomal enzyme–phosphomannose receptor system, it also seems possible that one of the roles of receptor amino acid (tyrosine, serine, or threonine) phosphorylation might include targeting of the internalized receptor to the appropriate organelle. At present, firm data are lacking in support of these hypotheses.

In summary, we have found that Con A stabilizes the cell surface EGF–receptor complex to dissociation and internalization and that the Con A–EGF–receptor complex is capable of stimulating RNA synthesis but that the induction of DNA synthesis by EGF is prevented. These data support the idea that the mitogenic signal produced by the EGF–receptor complex requires intracellular processing steps for its expression.

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