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Rapid Increases in the Transglutaminase Activity of A431 Cells following Treatment with Epidermal Growth Factor

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ABSTRACT: Transglutaminase activity was detected in lysates of A431 cells, a human epidermal carcinoma cell line. Enzyme activity was increased 1.5–2.5-fold in lysates prepared from cells pretreated with epidermal growth factor (EGF) relative to untreated control cells. Half-maximal activation of the transglutaminase activity occurred at 3–5 nM EGF, a concentration in good agreement with the K_d for EGF binding to its receptor in these cells. The increase in transglutaminase activity could be detected as early as 2 min after the addition of EGF, with the maximal response attained by 30 min. The activation was not blocked by pretreatment of the cells with cycloheximide, suggesting that the increased activity was not the result of an induction of transglutaminase synthesis. Fractionation of A431 cell lysates by centrifugation at 100000*g* for 30 min demonstrated that 90% of the transglutaminase activity was present in the soluble fraction and that this soluble transglutaminase activity was increased after treatment of the cells with EGF. The demonstration that EGF acutely increases the activity of a soluble, intracellular transglutaminase defines a novel pathway of growth factor action and provides a useful model system for identifying and comparing the mechanism(s) by which growth factors activate soluble enzymes.

Epidermal growth factor (EGF)[1] is a low molecular weight polypeptide that binds to a specific cell surface receptor and stimulates the phosphorylation of proteins on tyrosine residues (Carpenter & Cohen, 1979; Carpenter et al., 1978, 1979). While it seems likely that the intracellular effects of EGF are mediated via this increase in protein phosphorylation, little data are available to support this hypothesis. To develop a system in which to study the mechanism of signal transduction by EGF, we sought to identify an intracellular enzyme, the activity of which was regulated by EGF.

Transglutaminases are a family of Ca^{2+}-dependent enzymes that catalyze the formation of ϵ-(γ-glutamyl)lysine–protein cross-links and the incorporation of primary amines into protein-bound glutamine residues (Folk, 1980). These enzymes have been found in a wide range of species from sea urchins to mammals and in many different cell types (Folk, 1980; Cariello et al., 1984). Transglutaminase activity has been detected in both intracellular and extracellular locations. Formation of ϵ-(γ-glutamyl)lysine cross-links generally leads to a stabilization of protein structure. For example, factor XIIIa is a plasma transglutaminase responsible for the stabilization of fibrin clots. Several specific intracellular transglutaminases have been shown to be involved in the formation of cornified envelopes (Thacher & Rice, 1985) and the cross-linking of hair fiber protein (Chung & Folk, 1972; Harding & Rogers, 1972). While the biological role of a few specific transglutaminases has been defined, the function of the majority of intracellular transglutaminases remains unclear. Intracellular transglutaminases have been postulated to be involved in cell adhesion (Slife et al., 1986), cytoskeletal stabilization (Maccioni & Arechaga, 1986), cell growth and differentiation (Birckbichler & Patterson, 1978), and receptor-mediated endocytosis (Davies et al., 1980; Fesus et al., 1984).

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[1] Abbreviations: EGF, epidermal growth factor; HBSS, Hank's balanced salts solution; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PI, phosphatidylinositol.

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The possible involvement of a transglutaminase in cell growth led us to investigate the effect of EGF on the activity of this enzyme. We report here that treatment of A431 cells with EGF leads to a rapid increase in transglutaminase activity present in cell lysates that is independent of protein synthesis.

Experimental Procedures

Materials. EGF was purified as previously described (Savage & Cohen, 1972). N,N-Dimethylated casein was purchased from Sigma. [1,4(n)-^{3}H]Putrescine dihydrochloride and [1,4-^{14}C]putrescine dihydrochloride were purchased from Amersham.

Preparation of Cell Lysates. A431 cultures were grown overnight in 150-mm dishes to the desired confluency in Dulbecco's modified Eagle's medium supplemented with 7% newborn calf serum and 3% fetal calf serum. For EGF-treated cultures, the growth factor was added directly into the media for the desired incubation time. Incubations were stopped by removal of the media, followed by three washes with ice-cold HBSS. Cells were immediately scraped into 15 mL of cold HBSS and pelleted at 4 °C. Cell pellets were resuspended in 0.5 mL of ice-cold Ca^{2+}-free homogenization buffer containing 5 mM Tris, pH 7.4, 250 mM sucrose, 0.2 mM $MgSO_4$, 1 μg/mL leupeptin, 10 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, and 5 μg/mL α_2-macroglobulin. The cells were lysed with 40 strokes in a 1-mL ground glass Dounce homogenizer.

Transglutaminase Assay. Transglutaminase activity was assayed in a total volume of 100 μL containing (in final concentrations) 40 mM Tris, pH 8.3, 10 mM dithiothreitol, 5 mg/mL N,N-dimethylated casein, 5 mM $CaCl_2$, 0.12 mM [^{14}C]- or [^{3}H]putrescine (2 μCi/assay), and 50–100 μg of total lysate protein. Assays were begun by the addition of the labeled putrescine and Ca^{2+} and were incubated at 37 °C for 20 min (Birckbichler et al., 1976). The reactions were stopped by addition of 100 μL of cold 10% trichloroacetic acid. Precipitated protein was sedimented by centrifugation in a Savant high-speed microfuge. The supernatants were aspirated and the pellets redissolved in 40 μL of warm 0.4 N NaOH to remove trapped radioactive putrescine. The protein was reprecipitated with 0.3 mL of 10% trichloroacetic acid and pelleted, and the supernatant was removed. The pellets were dissolved in 0.5 mL of NaOH, the solution was added to 10 mL of Aquasol, and the resultant solution was counted in an LKB 1217 liquid scintillation counter. Nonspecific incorporation of radiolabel into the trichloroacetic acid precipitable material was determined in parallel assays in which the lysate was omitted or in which a zero time point was taken on reaction mixtures containing all components. Both methods yielded similar results. Transglutaminase activity was calculated as the difference between dpm incorporated into trichloroacetic acid precipitable protein in each assay and the dpm of nonspecific incorporation. Under these conditions, the incorporation of labeled putrescine was linear with time for 20 min and was also linear with respect to the amount of lysate protein added. Protein determinations were made by the method of Lowry et al. (1951).

Results

The characteristics of the transglutaminase activity in A431 cell lysates were first examined. Activity was measured as the ability of lysates to catalyze the incorporation of [^{14}C]- or [^{3}H]putrescine into the exogenous substrate N,N-dimethylated casein. As shown in Table I, incorporation of label into N,N-dimethylated casein was reduced to background levels upon addition of EGTA to the assay, verifying the characteristic

Table I: Characteristics of Transglutaminase Activity in A431 Cell Lysates[a]

		[^{3}H]putrescine incorpd (dpm/μg)
expt 1	control	229 ± 18
	+EGTA	0
expt 2	control	273 ± 18
	+100 μM dansylcadaverine	60 ± 19
expt 3	control	471 ± 5
	+500 μM cystamine	125 ± 13

[a] A431 cell lysates were prepared from 20% confluent cultures and assayed for transglutaminase activity as described under Experimental Procedures, except for the addition into the assay reaction of EGTA, monodansylcadaverine, or cystamine in the final concentrations given above. Values represent the mean ± standard deviation of triplicate determinations from a representative experiment.

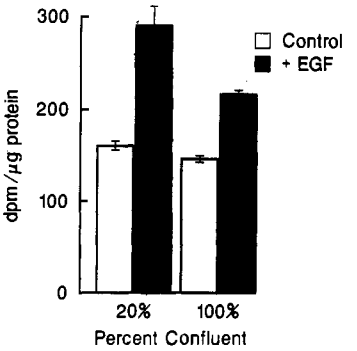

FIGURE 1: Effect of confluency on EGF stimulation of transglutaminase. A431 cultures were grown overnight to either 20% or 100% confluency. Cells were preincubated in the presence or absence of 67 nM EGF for 15 min at 37 °C. Lysates were then prepared and assayed for transglutaminase activity as described under Experimental Procedures. Values represent the mean ± standard deviation of triplicate determinations from a representative experiment.

Ca^{2+} dependency of the enzyme. Addition of the transglutaminase inhibitors monodansylcadaverine and cystamine to the assay also reduced the incorporation of labeled putrescine by 70% and 75%, respectively. These findings demonstrate that the characteristics of the transglutaminase activity present in A431 cells are consistent with the properties of transglutaminases in other systems (Birckbichler & Patterson, 1978; Lorand & Conrad, 1984).

To examine the effect of EGF on transglutaminase activity in A431 cells, confluent and subconfluent cultures were treated for 15 min with 30 nM EGF prior to preparation and assay of cell lysates. The results are shown in Figure 1. The basal levels of transglutaminase activity are approximately the same in lysates prepared from subconfluent or confluent cultures. Incubation of the subconfluent cultures with EGF resulted in an 80% increase in lysate transglutaminase activity relative to that in controls. Treatment of confluent cultures with EGF also caused an increase in transglutaminase activity. However, the effect was only about half that seen in subconfluent cultures. Thus, the potential for stimulation of the enzyme is greater in subconfluent cells, reflecting either a condition of the enzyme itself or a greater sensitivity of the culture to EGF. All further experiments were conducted with subconfluent cultures. Using subconfluent cultures, activation of the transglutaminase by EGF ranged from 1.5- to 2.5-fold in different experiments.

The time course for the activation of the transglutaminase by EGF is shown in Figure 2. An increase in enzyme activity was seen as early as 2 min after the addition of EGF. Maximal activation was observed by 30 min, and no further increase

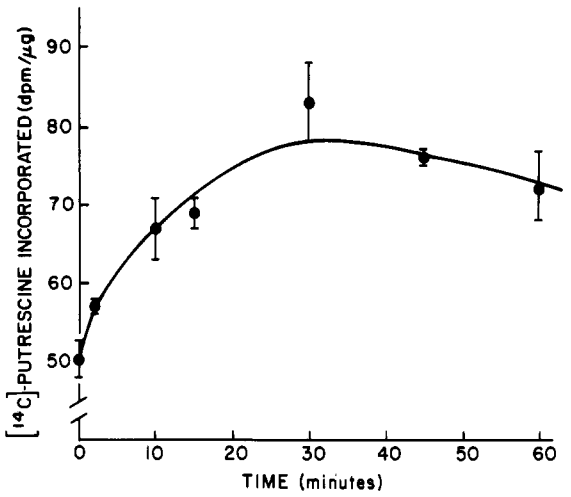

FIGURE 2: Time course of EGF stimulation of transglutaminase activity in A431 cells. A431 cells were incubated with 30 nM EGF for the indicated times. Cells were then harvested and lysed, and the cell lysates were assayed in triplicate for the ability to catalyze the incorporation of [^{14}C]putrescine into N,N-dimethylated casein. Values represent the mean ± standard deviation from a representative experiment.

Table II: Effect of Cycloheximide on the Ability of EGF To Stimulate Transglutaminase Activity[a]

	[^{3}H]putrescine incorpd (dpm/μg of protein)	
	untreated	+cycloheximide
control	256 ± 17	210 ± 4
+EGF	379 ± 4 (148)	420 ± 8 (200)

[a] A431 cells were preincubated with 10 μg/mL cycloheximide for 90 min at 37 °C. The cultures were then incubated in the absence or presence of 30 nM EGF for 15 min at 37 °C. Lysates were prepared and assayed for transglutaminase activity. Values represent the average ± standard deviation of triplicate determinations from a representative experiment. Numbers in parentheses indicate the stimulation by EGF as the percent of control activity. The ability of cycloheximide to inhibit protein synthesis in A431 cells was determined by measuring the in vivo incorporation of [^{35}S]methionine into trichloroacetic acid precipitable material. In cycloheximide-treated A431 cells, [^{35}S]-methionine incorporation was reduced by 90% to 94% relative to controls, verifying an inhibition of protein synthesis.

was noted following incubation with EGF for periods of up to 1 h.

An increase in transglutaminase activity following treatment of mouse peritoneal macrophages and HL60 cells with retinoids has been reported (Murtaugh et al., 1983; Maddox & Haddox, 1985). In those systems, the increased activity was the result of an induction of the transglutaminase. Since the time course of transglutaminase activation by EGF was sufficiently long to allow for induction of the enzyme, we examined the effect of protein synthesis inhibitors on this response. A431 cells were incubated in the absence or presence of 10 μg/mL cycloheximide for 90 min prior to challenge with 30 nM EGF. As shown in Table II, EGF was able to stimulate an increase in transglutaminase activity in both control and cycloheximide-treated cultures. This suggests that the increased transglutaminase activity in lysates prepared from cells pretreated with EGF is due to an activation of preexisting enzyme and not to the synthesis of new enzyme.

As can be seen from Table II, the stimulation by EGF was actually greater in the cycloheximide-treated cultures than in the control cultures. This result was reproduced in 10 separate experiments and was not consistently associated with a decrease in basal activity. Statistical analysis of the data from all 10 experiments by paired t test indicated that the difference

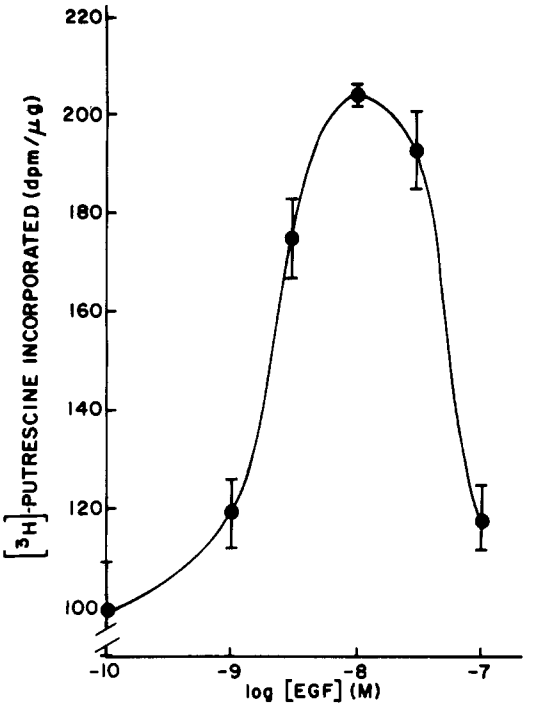

FIGURE 3: Dose–response curve for the stimulation of transglutaminase activity by EGF. Dilutions of EGF were prepared in a buffer containing 10 mM Tris, pH 7.0, and 0.1% bovine serum albumin as carrier protein. Aliquots of these dilutions were added directly to the growth medium. A431 cells were incubated with the appropriate concentration of EGF for 15 min at 37 °C prior to harvesting and preparation of the lysates. Values represent the mean ± standard deviation of triplicate determinations.

between the control and cycloheximide-treated cultures was significant at the $p < 0.01$ level. One possible explanation for this finding is that cycloheximide inhibits the synthesis of a labile repressor of either the transglutaminase or some other enzyme in the signal transduction pathway. Mixing experiments were performed to test for the presence of inhibitors in control cultures. When lysates from control and EGF-treated cultures were mixed and assayed, the resulting activity was additive. This suggests that, if present, the inhibitor does not function at the level of the transglutaminase. These experiments do not rule out the possibility that an inhibitor of an enzyme necessary for signal transduction is present in the control cultures.

The increased level of transglutaminase activity was dependent upon the dose of EGF. As shown in Figure 3, half-maximal stimulation of the activity occurred at 3–5 nM EGF, a value in good agreement with the K_d for EGF binding to its receptor in these cells (Kawamoto et al., 1983). Maximal response occurred at 30 nM EGF and declined at higher concentrations of the growth factor.

Transglutaminase activity has been reported to be present in both the particulate and soluble fractions of the cell. To determine the localization of the transglutaminase activated by EGF, lysates were separated into soluble and particulate fractions by centrifugation at 100000g for 30 min. The data in Figure 4 demonstrate that the majority of the transglutaminase activity in A431 cells was recovered in the soluble fraction. This soluble transglutaminase activity was increased by pretreatment of the cells with EGF.

Discussion

Several different forms of intracellular transglutaminase have been reported, including a soluble tissue or liver-type transglutaminase, a particulate transglutaminase, and an epidermal transglutaminase (Folk, 1980). In this paper we

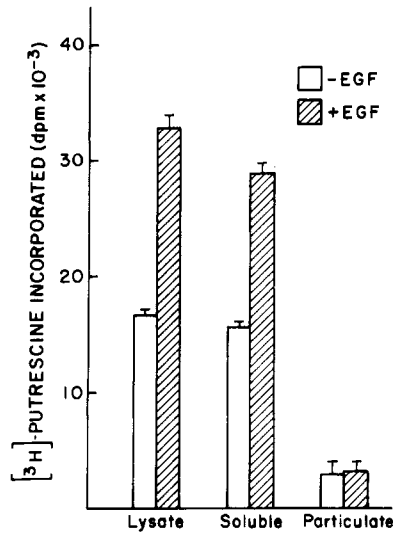

FIGURE 4: Localization of transglutaminase activity in A431 cells. A431 cells were incubated in the presence or absence of 30 nM EGF for 15 min prior to harvesting. Lysates were prepared as described under Experimental Procedures and centrifuged at 100000*g* for 30 min at 4 °C. The supernatants were collected and the pellets washed once by resuspension in homogenization buffer. The washed pellets were resuspended in the original volume of homogenization buffer. Equal volumes of the original lysate, supernatant, and washed pellet were then assayed in triplicate for transglutaminase activity. To correct for protein differences between the EGF-treated and control samples, values for the EGF-treated fractions were normalized with respect to the protein content of the equivalent fraction from control cells. All values represent the mean ± standard deviation from a representative experiment.

demonstrate that A431 cells possess a soluble transglutaminase activity that is increased in response to EGF. Activation of the transglutaminase occurs during treatment of whole cells with the growth factor and is retained following lysis of the cells. This is similar to the observed activation of S6 kinase by growth factors (Smith et al., 1980; Thomas et al., 1982).

Increases in transglutaminase activity were seen as early as 2 min after the addition of EGF, and the effect was maximal by 30 min. This time course is similar to that for the activation of S6 kinase activity by growth factors (Smith et al., 1980) but is somewhat slower than that seen for EGF effects on the stimulation of other biological responses. For example, stimulation by EGF of tyrosine kinase activity (Carpenter et al., 1978, 1979) and Ca^{2+} mobilization (Johnson et al., 1986; Moolenaar et al., 1986) in A431 cells is maximal by 2 min with no detectable lag. Similarly, EGF-stimulated phosphatidylinositol turnover in A431 cells is maximal by 5 min (Pike & Eakes, 1987). This suggests that one or more intermediate steps beyond those involved in these early responses may be required for the activation of the transglutaminase. Although the transglutaminase is a Ca^{2+}-dependent enzyme, it is unlikely that released Ca^{2+} is directly responsible for the activation of this enzyme because the assays are performed in the presence of saturating concentrations of this divalent metal ion and the time course of transglutaminase activation is much slower than that of Ca^{2+} mobilization. A more likely possibility is that the transglutaminase is covalently modified or interacts with a regulatory protein that increases its activity.

The activation clearly is not the result of an EGF-dependent induction of the transglutaminase gene since the effect occurs in cycloheximide-treated cells. The fact that cycloheximide-treated cells responded more strongly to EGF than control cultures is of interest. We found no evidence of a labile repressor of transglutaminase but cannot rule out this possibility. Similar findings were reported by Zettergren et al. (1984), who noted that treatment with cycloheximide enhanced the ability of erythrogenic toxin and toxic shock syndrome toxin to increase transglutaminase activity in human mononuclear leukocytes. These investigators ascribed the effect to a cycloheximide-induced increase in toxin binding but showed no supporting data. This mechanism is not likely to account for our results since cycloheximide is not known to increase EGF binding. Further work will be required to define the basis of this effect.

The dose–response curve for the stimulation of transglutaminase activity by EGF was biphasic, with increasing activity seen between 10^{-10} and 10^{-8} M EGF and a sharp decline occurring at concentrations above 10^{-8} M. A similar finding has been reported for the stimulation of PI kinase activity by EGF in A431 cells (Walker & Pike, 1987). The physiological significance of this observation is not known.

Stimulation of the transglutaminase was modest in the cell lysates. However, preliminary fractionation of the lysates has indicated that the stimulation by EGF is retained in the partially purified material and is increased to nearly 4-fold.[2] Thus, the presence of inhibitory contaminants or other transglutaminase activities may mask the full stimulation of the relevant transglutaminase when assayed in crude cell lysates.

The present results demonstrate a short-term stimulation of transglutaminase activity by EGF; however, changes in transglutaminase activity have also been noted following long-term treatment with various agents. Prolonged incubation of cells with serum (Murtaugh et al., 1983) or retinoic acid (Maddox & Haddox, 1985) has been shown to increase cellular transglutaminase activity by increasing synthesis of the enzyme. Long-term culture of A431 cells in the presence of EGF may also increase transglutaminase activity, but this effect occurs at a time when the cells are dying as a result of the culture conditions (Rosdy et al., 1986). The relationship of these findings to our observations is unclear since our EGF effect is acute and is apparently not the result of gene induction. Identification of the transglutaminase(s) activated by acute and chronic treatments will be necessary to clarify this issue.

The physiological function of most intracellular transglutaminases is unknown. These enzymes have been implicated in a variety of processes related to growth and differentiation (Slife et al., 1986; Maccioni & Arechaga, 1986; Birckbichler & Patterson, 1978; Davies et al., 1980; Fesus et al., 1984), but the role of protein cross-linking in these processes has not been elucidated. Microtubules, but not tubulin, have been shown to be substrates for intracellular transglutaminases (Maccioni & Arechaga, 1986; Maccioni & Seeds, 1986). If cytoskeletal proteins represent a physiological target for cross-linking enzymes, it is possible that the changes in cell shape that occur in response to growth factors may be mediated in part by a transglutaminase. Novogrodsky et al. (1978) have observed that treatment of human lymphocytes with phytomitogens leads to an increase in transglutaminase activity in 10–30 min. Taken together with the present data, this suggests that activation of a transglutaminase may be a general short-term response to mitogens and implies that this enzyme may be important in mediating the early effects of growth factors.

The present findings document the stimulation of a soluble, intracellular enzyme by EGF. The acute activation of a transglutaminase by EGF in A431 cells defines a novel

[2] Dadabay and Pike, unpublished observations.

pathway of growth factor action and provides a useful model system for identifying and comparing the mechanism(s) by which growth factors activate different soluble enzymes.

Registry No. EGF, 62229-50-9; transglutaminase, 80146-85-6; putrescine, 110-60-1.

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