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Role of Epidermal Growth Factor-Stimulated Protein Kinase in Control of Proliferation of A431 Cells

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Epidermal growth factor (EGF), which stimulates tyrosine-specific protein kinase activity both in vivo and in vitro, inhibits proliferation of A431 human epidermoid carcinoma cells. After mutagenesis clonal cell lines that were resistant to the growth inhibitory effects of EGF were selected. All six variants examined contained decreased EGF-stimulated protein kinase. The number of EGF receptors in variant cells decreased in parallel with EGF-stimulated protein kinase activity so that the specific activity of EGF-stimulated protein kinase per EGF receptor remained constant in variant cell lines with up to tenfold reductions in both activities. This result suggests that both EGF binding and kinase activities reside in the same or closely coupled molecules. The effect of EGF on growth of two resistant variants was examined in detail. Clone 29 contains ~50% and clone 4 contains ~20% of the EGF-stimulated protein kinase activity of the parental A431 cell line. In serum-supplemented medium, EGF stimulated proliferation of clone 29 but did not affect growth of clone 4. In a 1:1 mixture of DME and F-12 medium without serum, EGF caused both clone 29 and clone 4 to grow as well as in 10% serum. These variants, which were selected for resistance to the growth inhibitory effects of EGF, thus exhibit a strong mitogenic response to EGF. This result suggests that resistance to the growth inhibitory effect of EGF may involve both a decrease in EGF-stimulated protein kinase and an alteration in the response pathway.

Key words: epidermal growth factor, protein kinase, epidermoid cancer cells

The discovery that protein kinase activity which specifically catalyzes phosphorylation at tyrosine residues is an intrinsic property of the protein product of the transforming gene of a number of retroviruses pointed to the importance of this previously unrecognized enzyme activity in control of cell growth and transformation [1–11]. Although present in significantly lower concentrations than the viral gene products, similar proteins that are thought to be the cellular progenitors of the retroviral transforming proteins exist in untransformed cells [12–17]. The polypeptide

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growth factors epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) stimulate distinct tyrosine-specific protein kinase activities in vivo and in isolated membranes in vitro [18–24], suggesting that these protein kinase activities may also mediate the biological effects of these peptides.

Because EGF stimulates growth of several types of cells in culture [25,26], it has been proposed that activation of tyrosine kinase mediates this proliferative effect. The recent observations that EGF inhibits proliferation of GH_4C_1 rat pituitary tumor cells [27], A431 human epidermoid carcinoma cells [23,28], and certain human breast cancer cells [29] indicate that the relationship between biological effects of EGF and tyrosine kinase activity is complex. In A431 cells EGF-dependent inhibition of cell proliferation is associated with activation of tyrosine kinase activity [23,30] and phosphorylation of at least one cellular substrate which is also phosphorylated by a viral transforming protein, $p60^{v\text{-}src}$ [31].

To determine the role of tyrosine kinase activity in the biological effects of EGF, variant cell lines that are resistant to the growth inhibitory effects of EGF have been selected. These variants include clones whose growth is stimulated by EGF. EGF receptor and EGF-stimulated tyrosine-specific protein kinase activities have been compared in A431 cells whose growth is inhibited, stimulated, or unaffected by EGF.

RESULTS

Effect of EGF on Growth of Variant A431 Cells

After exposure to the mutagen N-methyl-N′-nitro-N-nitroso-guanidine, A431 cells were maintained and subcultured in the presence of 0.1 μM EGF[32]. Colonies were cloned, grown to confluence in the presence of EGF, and then grown for more than 20 population doublings in the absence of EGF. Because a separate selection was not carried out in the absence of mutagen, it is possible that resistant cells were already present in the uncloned A431 cell population. Resistant clones are therefore referred to as variants. The phenotype of these clones has been stable for more than 50 generations. Parental A431 and the cell variants possess approximately 69 human chromosomes.

As shown in Figure 1, in serum containing medium, EGF severely inhibited growth of A431-8, a cloned, unselected A431 cell, at a half-maximally effective concentration of $\sim$0.07 nM. In contrast, EGF stimulated proliferation of clone 29, a variant selected for EGF resistance, over the same concentration range (half-maximally effective concentration of EGF of $\sim$0.1 nM). EGF had no effect on the growth of clone 4, a variant also selected for EGF resistance. Similar results were obtained by measuring the ability of A431, clone 29, or clone 4 cells to form colonies from single cells in plastic dishes (data not shown).

The growth-stimulating effects of EGF on clone 29 were more readily seen when the serum concentration in the medium was reduced. As shown in Table I, addition of EGF to medium without serum caused clone 29 cells to grow as well as cells grown in medium that contained serum or a defined growth medium. As also shown in Table I, a growth stimulatory effect of EGF on clone 4 was evident in serum-free medium. In the presence of serum or a defined medium that effectively supports proliferation, EGF exerted minimal effects on growth of clone 4. The inhibitory effect on A431-8 cells was evident in serum-free as well as in serum-containing medium.

TABLE I. Effect of EGF on Growth of Clone 29, Clone 4, and $A431_8$ Cells

Culture conditions[a]	Clone 29 cell number ($\times 10^{-4}$/plate)[b]	% Change	Clone 4 cell number ($\times 10^{-4}$/plate)	% Change	$A431_8$ cell number ($\times 10^{-4}$/plate)	% Change
Minus serum	19.3 ± 0.20		12.8 ± 0.05		62.4 ± 0.19	
+ EGF	60.8 ± 0.16	+215	20.6 ± 0.02	+61	10.1 ± 0.03	−84
Plus serum	39.6 ± 0.08		28.0 ± 0.03		66.2 ± 0.04	
+ EGF	64.4 ± 0.12	+63	31.0 ± 0.10	+11	14.2 ± 0.05	−79
Defined medium	34.6 ± 0.01		21.8 ± 0.03		62.6 ± 0.11	
+ EGF	55.0 ± 0.20	+60	25.2 ± 0.09	+16	8.2 ± 0.04	−87

[a]$A431_8$, clone 29 cells, and clone 4 cells were subcultured in 3.5 cm tissue culture dishes in DME–F-12 (1:1) medium containing 5% calf serum and 5% fetal calf serum. After attachment of cells to the tissue culture dish, cells were washed with DME–F-12 without serum for 30 min at 37°C; medium was then replenished with fresh DME–F-12 without or with 5% calf serum and 5% fetal calf serum, or with defined medium [33]. EGF (0.1 μM) was added under each growth condition as indicated, and cell number was determined in triplicate dishes 72 hr later.
[b]Mean ± SD, n = 3.

EGF-Stimulated Protein Kinase Activity in Variant A431 Cells

To quantitate the effects of EGF on tyrosine-specific protein kinase activity in vivo, total cellular phosphotyrosine content was determined in cells that were labeled to equilibrium with ^{32}P [2,17]. As shown in Figure 2, EGF increased phosphotyrosine content in A431 cells approximately ten-fold, rising from 0.02 to 0.22% of the acid-stable phosphorylated amino acids of the cell [23,32]. In clone 29 the EGF-stimulated increase in phosphotyrosine content was significantly less, while in clone 4 an increase was barely detectable. These results indicate that EGF-stimulated tyrosine-specific protein kinase activity is reduced in variants that are resistant to the growth inhibitory effects of EGF.

Because phosphotyrosine is a rare phosphoamino acid even in EGF-stimulated cells, quantitation of total cell phosphotyrosine content has limited sensitivity. A more sensitive in vitro protein kinase assay was therefore used to determine the extent of reduction of EGF-stimulated protein kinase activity in variant cells. Membranes prepared from A431 and variant cells were solubilized with 1% Triton X-100/10% glycerol [20,34] and used as the enzyme source. These preparations contain EGF-stimulated protein kinase activity that catalyzes phosphorylation at tyrosine residues of a 170,000 M_r protein thought to be the EGF receptor. Because EGF receptors are reduced in variant cells, decreased EGF receptor phosphorylation could reflect either reduced enzyme activity or reduced substrate. Anti-$p60^{src}$ IgG, an exogenous substrate that is phosphorylated at tyrosine residues by EGF-dependent protein kinase [34,35], was therefore included as an exogenous phosphorylation substrate. The inclusion of this exogenous substrate did not inhibit phosphorylation of the EGF receptor. As shown in Table II, EGF-stimulated protein kinase activity was decreased in each of

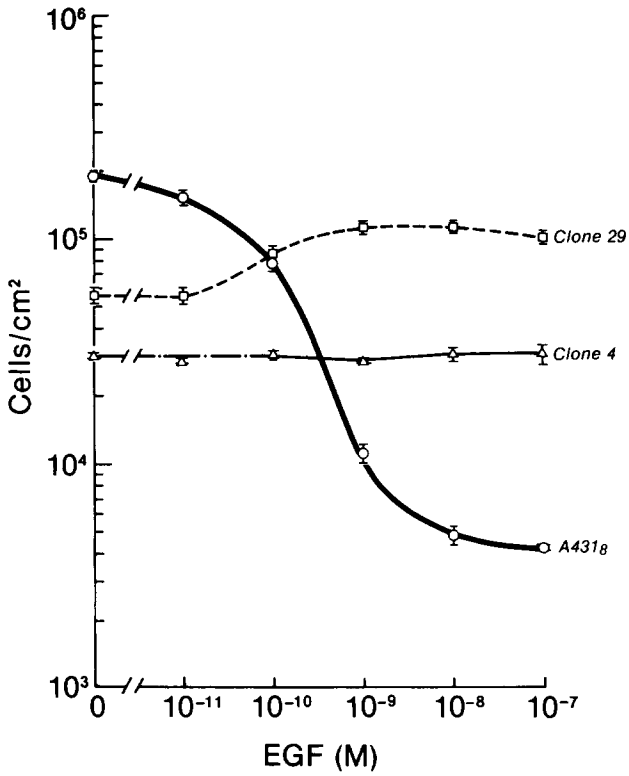

Fig. 1. Effect of varying concentrations of EGF on growth of variant A431 cells. A431-8, clone 29, and clone 4 were subcultured into 2 cm^2 Linbro wells at 10^3 cell/cm^2. After attachment, medium was replenished with DME–F-12 (1:1) containing 10% calf serum without or with varying concentrations on EGF. Medium with the indicated concentrations of EGF was replenished every 2 days and triplicate wells of cells counted on day 7. In the absence of EGF, A431-8 cells went through 7.1 population doublings, clone 29 cells went through 5.2 population doublings, and clone 4 cells went through 4.9 population doublings.

the variant cells compared to A431 cells. In clone 29, EGF-stimulated protein kinase activity was decreased to ~ 43% compared to the activity of A431 cells. In clone 4, kinase activity was further reduced to ~18% of the activity of A431 cells. The reductions in EGF-stimulated protein kinase activities were apparent in phosphorylation of both the EGF receptor and the heavy chain of anti-$p60^{src}$ IgG. Four additional clones resistant to the growth inhibitory effects of EGF were examined and there were significant reductions in EGF-stimulated protein kinase activity in all clones as measured by both in vivo and in vitro assays [32]. Clones that were resistant to the growth inhibitory effects of EGF thus uniformly exhibited diminished EGF-stimulated protein kinase activity.

EGF Receptors in Variant A431 Cells

Each of the six variant cell lines examined had a reduced number of EGF receptors as measured both in intact cells and in isolated membranes [32]. The affinity of EGF binding to EGF receptors was similar in both parental and variant cell lines [32]. The decrease in EGF receptors as measured by ligand binding was confirmed

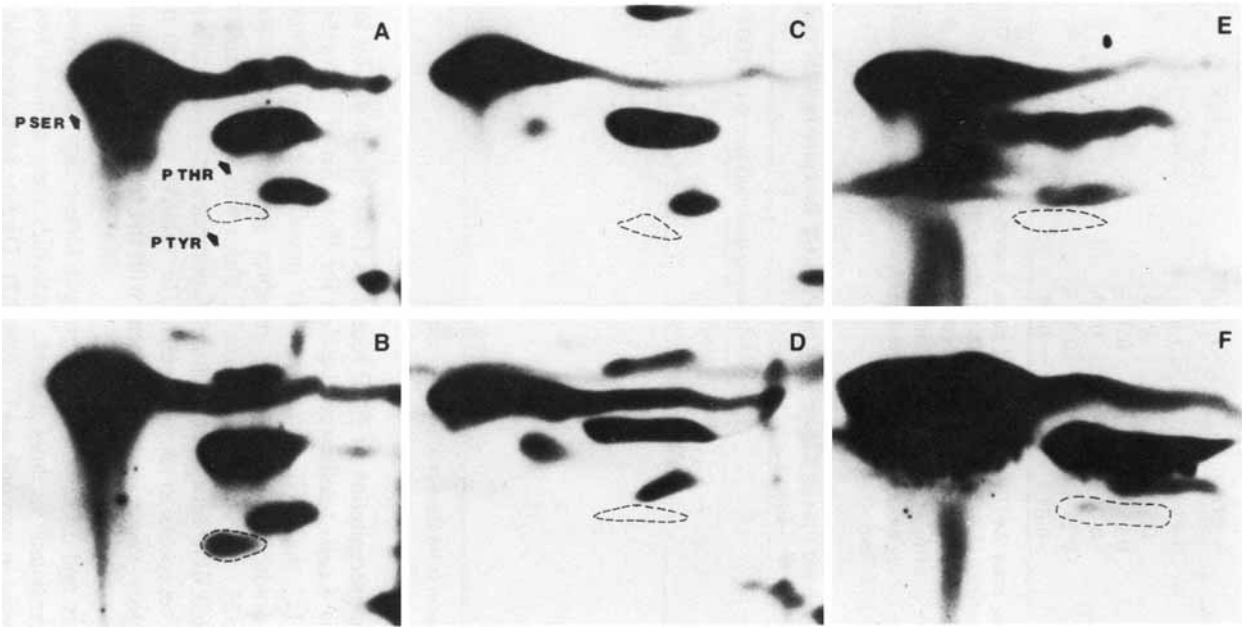

Fig. 2. Effect of EGF on phosphotyrosine content of parental A431 cells (A and B) and resistant clones numbers 4 (C and D) and 29 (E and F). Exponentially growing cells were labeled with 1 mCi/ml ^{32}P-orthophosphate for 18 hr prior to treatment without (A, C, E) or with 0.1 μM EGF for 2 hr (B, D, F). Extraction and electrophoretic separation of phosphorylated amino acids was carried out as described [23,32]. Internal authentic phosphorylated amino acids were included for precise localization. Approximately 2 $\times$ 10^6 cpm were applied to each plate and autoradiographic exposure was for 17 hr. Reprinted with permission from Buss et al [32].

TABLE II. EGF-Stimulated Protein Kinase Activities in Solubilized Membranes Prepared From A431 and Variant Cells

	pmol ^{32}P incorporated/min/mg membrane protein[a]			
	EGF receptor phosphorylation		Anti-p60^{src} IgG phosphorylation	
Cell	− EGF	+ EGF	− EGF	+ EGF
Parental A431	0.43 ± 0.12	1.80 ± 0.30	0.12 ± 0.01	0.96 ± 0.14
Clone 29	0.25 ± 0.01	0.77 ± 0.03	0.08 ± 0.02	0.42 ± 0.04
Clone 4	0.15 ± 0.05	0.32 ± 0.05	0.05 ± 0.03	0.17 ± 0.06

[a]Solubilized membranes from A431, clone 29, and clone 4 were assayed for EGF-stimulated protein kinase activities as described [32]. Reactions were terminated with RIPA buffer containing 0.1% SDS and formalin fixed protein A-containing Staphylococcus aureus was added for 1 hr at 4°C to adsorb IgG. The mixtures were centrifuged to separate the immune complex from the EGF receptor-containing supernatant. Data represent the mean ± SD of three to five separate assays using two different membrane preparations. Modified from Buss et al [32].

TABLE III. EGF-Stimulated Protein Kinase Activity Per EGF Receptor in A431 and Variant Cells

	(pmol ^{32}P incorporated/min)/(nmol [^{125}I]EGF bound)	
Cell	EGF receptor phosphorylation	Anti-p60^{src}IgG phosphorylation
Parental A431	91.3	48.7
Clone 16	75.9	39.8
29	108.4	59.2
24	71.7	35.8
33	84.2	36.8
4	82.1	43.6
18	77.8	36.1

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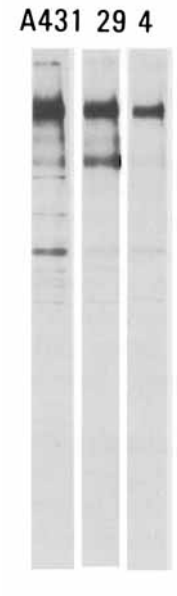
by specific immunoprecipitation of EGF receptors from metabolically labeled A431, clone 29, and clone 4 cells. Cells were labeled for 16 hr with [^{35}S]methionine, lysed with RIPA buffer [1], and EGF receptors were quantitated by immunoprecipitation with monoclonal antibodies to the EGF receptor prepared and characterized by Waterfield et al [36]. As shown in Figure 3, there was a significant reduction in metabolically labeled EGF receptors in clone 29 compared to A431 cells and a further reduction of EGF receptors in clone 4 cells. The relative decrease in metabolically labeled EGF receptors was in good agreement with the reduction measured by ligand binding.

EGF receptor and EGF-stimulated protein kinase activities were reduced in parallel in the six resistant variants studied. Activities were reduced from ~50% to ~10% of those found in parental A431 cells [32]. Despite up to ten-fold reduction in EGF-stimulated protein kinase activity per mg membrane protein, variant cells retained the same relative activity per EGF binding site as the parental A431 cells (Table III). This was true when either EGF receptor or anti-p60^{src} IgG phosphorylation was examined.



Cell	Total Incorporation (CPM in TCA Insoluble Material)	EGF Receptor (CPM in Immune Precipitate)	EGF Receptor (% Total Cell Protein)
A431	9.84×10^6	4052	0.041
CL 29	7.59×10^6	2008	0.026
CL 4	7.69×10^6	828	0.011

Fig. 3. A431, Cl 29, and Cl 4 cells were subcultured at a density of 2×10^5 cells per 35 mm dish. Twenty-four hr later, medium was replaced with DME containing 5% of the standard level of methionine (0.75 mg/L) including 100 μCi [^{35}S]methionine and 4% calf serum. After 16 hr, medium was removed, cells were washed with PBS and lysed with 200 μl of RIPA buffer containing 1 mM EDTA. Total [^{35}S]methionine incorporation into protein was determined by TCA precipitation of duplicate aliquots (20 μl) that had been treated with NaOH to hydrolize [^{35}S]met·tRNA. [^{35}S]met-labeled EGF receptors were quantitated in duplicate in equal size aliquots (20 μl) by adding monoclonal anti-EGF IgG prepared and characterized by Dr. Bradford Ozanne [36] and incubating for 15 min at 25°C. Goat antiserum to mouse IgG was then added for 30 min at 0°C followed by addition of Pansorbin (Calbiochem) and incubation for an additional 60 min at 0°C. Immunoprecipitates were collected by centrifugation, washed, redissolved in a 20 μl sample of buffer, and proteins were analyzed by polyacrylamide gel electrophoresis in slab gels containing 15% acrylamide and 0.087% bisacrylamide. After autoradiography the [^{35}S]methionine-labeled EGF receptor protein bands were excised, dissolved in 1 ml NCS (Amersham Searle) plus 20 ml toluene-based scintillant, and radioactivity was determined. Precipitations using control antisera yielded no detectable immunoprecipitated radioactivity [36]. These monoclonal anti-EGF antibodies also precipitate in vitro ^{32}P-labeled EGF receptors.

DISCUSSION

To identify biochemical steps involved in EGF-mediated inhibition of A431 cell growth, variants were selected that were resistant to this effect of EGF. All six resistant variants examined had reduced EGF-stimulated tyrosine-specific protein kinase activity. This correlation suggests that increased EGF-stimulated protein kinase activity mediates the growth inhibitory effect of EGF on A431 cells. If this hypothesis is correct, phosphorylation of tyrosine residues in proteins may result in either inhibition or in stimulation of cell proliferation.

A431 cells possess an unusually large number of EGF receptors per cell [37,38]. Two lines of evidence suggest that EGF-stimulated protein kinase is closely coupled if not identical to these EGF receptors. First, Cohen el al [20,21] reported that EGF-binding and EGF-stimulated protein kinase activity remained associated through

purification on EGF affinity columns, electrophoresis in nondenaturing polyacrylamide gels, and immunoprecipitation with anti-EGF receptor serum. Second, EGF binding and EGF-stimulated protein kinase activities varied in parallel over a tenfold range in variant A431 cells [32].

Without EGF treatment variant cells with decreased EGF receptors and EGF-stimulated protein kinase do not grow more rapidly than A431 cells. Inhibition of proliferation therefore does not result merely from the presence of large amounts of these proteins but from their activation. From the variants examined, a reduction in EGF-stimulated protein kinase activity appears essential for escape from growth inhibition. It is possible that an inhibitory pathway is activated in the parental cells because receptors and tyrosine kinase activity are excessively elevated. When EGF-stimulated protein kinase is reduced, activation of the inhibitory pathway may no longer predominate.

Precise analysis of the quantitative relationship between EGF-stimulated protein kinase activity and cell growth control is difficult. A reduction in EGF receptors and EGF-stimulated protein kinase activity in clone 29 of 40–50% compared to parental A431 appears to be sufficient not only for escape from growth inhibitory effects but also for expression of growth stimulatory effects of EGF. In clone 4 where activity was reduced 80–90% compared to parental A431 cells, growth in serum was not affected by EGF, but a mitogenic response to EGF became evident in serum-free medium. No qualitative differences in EGF receptors and EGF-stimulated protein kinase have been detected in the variants. The ED_{50} for biological response, the K_D for EGF binding, and the molecular weight of both phosphorylated and metabolically labeled EGF receptors were identical among variants with widely differing quantities of enzyme.

Although EGF receptor and EGF-stimulated protein kinase activities are reduced in all resistant variants examined, this reduction may not be sufficient to explain the shift in biological response from strong inhibition to strong stimulation of proliferation. Additional differences may exist between parental A431 cells and variants whose growth is stimulated by EGF. In clone 29, where enzymes activities were reduced ~50%, and in unselected A431-8 cells the appropriate growth stimulatory or inhibitory responses were observed at all EGF concentrations between 0.01 nM and 100 nM. In particular, the growth of A431-8 cells was always inhibited by EGF, even at subsaturating concentrations. EGF also inhibited growth of the parental A431 cells when EGF receptors varied up to ten-fold in response to changes in cell density [23]. Therefore, in the parental A431 cells the activation of fewer receptors does not convert growth inhibition to growth stimulation. Protein substrates and metabolic control points may thus also differ between A431 cell variants with opposing growth responses to EGF.

The development of A431 variants with differing biological responses to EGF provides a new avenue for investigation of the role of EGF-stimulated protein kinase and tyrosine phosphorylation in control of cell proliferation. Available data support a close correlation between enzyme concentration and the biological effect of EGF. Comparison of these stable variants may reveal additional critical variables and pathways leading to growth stimulation or inhibition.

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