Transforming Growth Factor β Modulation of the Epidermal Growth Factor Ca\(^{2+}\) Signal and c-Fos Oncoprotein Levels in A431 Human Epidermoid Carcinoma Cells

Kathryn A. Elliget, Patricia C. Phelps, and Mary W. Smith

Department of Pathology, School of Medicine, University of Maryland, Baltimore, Maryland 21201

Abstract
Transforming growth factor β (TGFβ) was examined regarding its regulation of the mitogen EGF. A431 human epidermoid carcinoma cells were treated with TGFβ and epidermal growth factor (EGF) (10 ng/ml each) to determine if TGFβ modulates EGF-induced Ca\(^{2+}\) signaling and c-Fos oncoprotein levels. Changes in [Ca\(^{2+}\)]\(_i\), were determined by digital imaging analysis or photon counting. In HBSS + Ca\(^{2+}\) (1.37 mm), EGF treatment resulted in a transient increase in [Ca\(^{2+}\)]\(_i\), from 75 to 150 nm, which lasted approximately 3.5 min and re-equilibrated to 90 nm. In nominally Ca\(^{2+}\)-free (2–5 μm) HBSS, EGF caused a [Ca\(^{2+}\)]\(_i\) elevation that peaked at 140 nm and returned to baseline. TGFβ in HBSS + Ca\(^{2+}\) did not elicit a [Ca\(^{2+}\)]\(_i\) increase, although affinity labeling revealed types I, II, and III TGFβ receptors. TGFβ added simultaneously with EGF in HBSS + Ca\(^{2+}\) caused a gradual rise in [Ca\(^{2+}\)]\(_i\), from 50 to 100 nm over 16 min. Pretreatment with TGFβ (3 h; 10 ng/ml) abolished the EGF-induced [Ca\(^{2+}\)]\(_i\) elevation. EGF or TGFβ treatments increased c-Fos immunoreactivity by around 1 h. In summary, EGF elevated [Ca\(^{2+}\)]\(_i\), in the presence or absence of [Ca\(^{2+}\)]\(_e\), resulting in high [Ca\(^{2+}\)]\(_m\), associated with tyrosine and threonine phosphorylation, and increased c-Fos oncoprotein immunoreactivity. TGFβ did not increase [Ca\(^{2+}\)]\(_i\), but did increase c-Fos; TGFβ + EGF added simultaneously altered the EGF-induced [Ca\(^{2+}\)]\(_i\) elevation, and TGFβ pretreatment eliminated EGF-induced [Ca\(^{2+}\)]\(_i\) elevation. This suggests that TGFβ can regulate EGF in A431 cells and that increased c-Fos may not be mediated by Ca\(^{2+}\).

Introduction
The effects of growth factors are often dependent on interactions with other growth factors. TGFβ, a growth-regulating polypeptide, can exert a dominant role over mitogens such as EGF, the receptor of which is subject to regulation by TGFβ and other substances (1, 2). These types of growth factor interactions drive normal physiological processes and represent one mechanism by which TGFβ acts in pathophysiology, i.e., during wound healing. Thus, interactions between EGF and TGFβ are essential. This association was suggested when TGFβ was first identified and named based on cellular changes induced by a combined association with EGF (3, 4).

Two methods by which TGFβ can influence EGF are by enhancing the expression of the EGFR gene and by changing the number of high affinity EGFRs as well as the binding affinity of EGF (5). Goldkorn and Mendelschon (2) demonstrated that TGFβ increases phosphorylation of the EGFR and inhibits A431 cell growth, which is accompanied by prolongation of G0–G1, and cell shape changes. In epithelial cells other than A431, TGFβ has been shown to both prevent (6) and augment (7) EGF-induced mitogenesis. However, the specific mechanisms by which TGFβ regulate the EGFR have not been determined.

The dynamics of EGF Ca\(^{2+}\) signaling in A431 cells have been investigated (8–10). In these and other studies, EGF caused Ca\(^{2+}\) release from intracellular stores, presumably from the ER, as well as Ca\(^{2+}\) entry across the plasma membrane. Other studies, however, report that EGF does not induce Ca\(^{2+}\) release (11, 12). Most reports suggest that TGFβ signal transduction is not coupled to an early [Ca\(^{2+}\)]\(_i\), transient, but Hahn and Cooper (13) showed that a small increase in [Ca\(^{2+}\)]\(_i\) occurred 30 s after adding TGFβ to hepatocytes. Much later responses have been reported for other cell types (14).

It is well known that the induction of early response genes, such as c-fos, is a fundamental consequence of growth factor receptor binding in A431 cells (15). It is also well known that TGFβ regulates gene expression, including the early response genes, the cytoskeleton, and some matrix proteins (16, 17). TGFβ may inhibit growth by decreasing the expression of growth-promoting genes that function in G0–G1 of the cell cycle (18–20).

In this study, we used A431 human epidermoid carcinoma cells to examine Ca\(^{2+}\) signaling and the c-Fos oncprotein in response to EGF and TGFβ. A431 cells were chosen because they have an extremely high number of EGFRs, and because EGF is mitogenic at low concentrations but is growth inhibitory at high concentrations (21). It was, therefore, of interest to investigate signaling events in tumor cells with a biphasic response to EGF. Our experiments were designed to determine if TGFβ alters these early EGF effects, with the broad objective of clarifying
some of the interactions of TGFβ and EGF in pathophysiological processes.

**Results**

**Receptor Affinity Labeling.** Using cross-linking technology and polyacrylamide gel electrophoresis, $^{125}$I-labeled TGFβ$^3$ and $^{125}$I-labeled EGF were covalently bound to subconfluent cultures of A431 cells to label TGFβ and EGF receptors (Fig. 1). TGFβ types I ($M$, 60,000–70,000), II ($M$, 85,000–95,000), and III ($M$, 280,000–330,000) receptors were identified (Fig. 1A, Lane 2). No radioactivity was detected when a 200-fold excess of unlabeled TGFβ was included to inhibit the specific binding of $^{125}$I-labeled TGFβ to its receptor (Fig. 1A, Lane 1). As a positive control, the TGFβ receptor profile of a TGFβ-responsive human bronchial epithelial cell line, BEAS-2B (22), is presented (Fig. 1A, Lane 3). The A431 M, 170,000 EGF is shown in Fig. 1A, Lane 4. Controls for this experiment included deletion of the chemical cross-linker BS$^3$, which inhibited $^{125}$I-labeled EGF binding (Fig. 1A, Lane 5), and the addition of 200-fold excess EGF (Fig. 1A, Lane 6).

**EGFR Clustering.** Immunocytochemical visualization of receptor clustering, using an antibody to the EGFR and immunoperoxidase staining, is shown (Fig. 1B). EGFR clusters appeared as discrete, dense spots at cell-cell junctions and at leading cell edges (Fig. 1B, arrows), and there was a predictable variability in receptor number among cells in a colony.

**[$Ca^{2+}$], Measurements.** Using DFM, we obtained gray-level computer images depicting [$Ca^{2+}$], in live fura 2-loaded A431 cells (Fig. 2). In untreated control cells, [$Ca^{2+}$], was fairly uniform throughout the cell, but with many nuclei having much lower [$Ca^{2+}$] (Fig. 2, Aa and Ba). EGF or TGFβ were added to cells, and images were captured at frequent intervals. Fig. 2A represents control cells (Fig. 2Aa) and cells after the addition of EGF (Fig. 2Aa, b–i). [$Ca^{2+}$], peaked at around 3 min (Fig. 2Ae) and declined to near baseline by 6 min (Fig. 2Ai). The rate of increase of both cytosolic and nuclear [$Ca^{2+}$], was variable among cells in the same population. TGFβ did not cause an elevation in [$Ca^{2+}$], when viewed up to 20 min (Fig. 2B, b–i).

[$Ca^{2+}$], measurements of fura 2-loaded A431 cells were also obtained with photon counting techniques. Results from untreated cells in HBSS are presented in Fig. 3a (normal HBSS + Ca$^{2+}$; 1.37 mM) is represented by +Ca$^{2+}$ on the graph and nominally Ca$^{2+}$-free HBSS (2–5 μM) by −Ca$^{2+}$]. The [$Ca^{2+}$], of cells incubated in HBSS + Ca$^{2+}$ stayed near basal levels (approximately 75 nm) for at least 20 min. When cells were incubated in nominally Ca$^{2+}$-free HBSS, the [$Ca^{2+}$], decreased slightly or remained constant at basal levels for 20 min. However, these cells appear to be sensitive to Ca$^{2+}$-free conditions, as indicated by morphological signs

---

3 The abbreviations used are: TGFβ, or TGF-β, transforming growth factor β; or α; EGF, epidermal growth factor; EGFR, EGF receptor; [Ca$^{2+}$], concentration of intracellular ionized Ca$^{2+}$, presumable cytosolic [Ca$^{2+}$], and nuclear [Ca$^{2+}$],; [Ca$^{2+}$], extracellular Ca$^{2+}$; DFM, digital imaging fluorescence microscopy; BS$^3$, bis(sulfosuccinimidyl) suberate; ER, endoplasmic reticulum; FBS, fetal bovine serum.

Growth Factor Effects on c-Fos Oncoprotein. To understand the early events whereby TGFβ regulates EGF, c-Fos oncoprotein was studied by indirect immunofluorescence microscopy. Cell cultures were serum- and growth factor-starved for 4 h prior to adding EGF or TGFβ. Control cultures in serum- and growth factor-free medium showed low basal levels of c-Fos (Fig. 4a). Low levels of fluorescence were also noted in the serum- and growth factor-free medium control cells at 1 h (Fig. 4b) and in the TGFβ vehicle-treated cultures (4 mM HCl containing 1 mg/ml BSA) at 1 h (Fig. 4c). For Fos-positive controls, cells were treated with the Ca^{2+} ionophore ionomycin (5 μM; 1 h; Fig. 4d) or 10% FBS (Fig. 4e), which resulted in extremely bright nuclear fluorescence in all cells and cytoplasmic fluorescence in many cells. Treatment with 10 ng/ml EGF resulted in both nuclear and cytoplasmic Fos immunoreactivity. The level of Fos increased between 15–30 min, peaked at around 1 h (Fig. 4f), and decreased to basal levels by 4 h (Fig. 4g). Treatment with TGFβ alone (Fig. 4g) resulted in the same pattern of nuclear and cytoplasmic Fos immunoreactivity as seen with EGF, including the return to baseline by 4 h (Fig. 4k). The simultaneous addition of TGFβ and EGF (Fig. 4h) increased Fos levels to the same extent and cellular distribution as with either growth factor added alone (Fig. 4, f and g). Pretreatment with TGFβ for 2 h, followed by TGFβ + EGF, yielded similar results (Fig. 4i). Pretreatment for 2 h with TGFβ was, therefore, inconclusive with respect to the abrogation of EGF-induced Fos levels since TGFβ independently caused Fos to increase.

EGF-induced Protein Phosphorylation. To correlate the EGF-induced [Ca^{2+}]i, elevation with protein phosphorylation, A431 cells were treated with EGF and examined by immunofluorescence microscopy for phosphorylation of tyrosine and threonine residues (Fig. 5). Baseline phosphorylations were low on both tyrosine and threonine residues; Fig. 5a represents baseline phosphotyrosine. After a 1-min treatment with EGF, there was phosphorylation of both tyrosine (Fig. 5b) and threonine (Fig. 5, c and d) residues. Phosphorylated tyrosine had a cytoplasmic or perinuclear location (Fig. 5b), and phosphorylated threonine was localized in the nucleus (Fig. 5, c and d). By 5 min, phosphorylation on tyrosine and threonine residues returned to near baseline (Fig. 5e); Fig. 5e represents recovered phosphothreonine.

Discussion

A better understanding of the mechanisms that control epithelial cell growth and differentiation can be obtained by studying growth factor interactions. A431 cells are excellent as a model for growth factor study because they have a high number of EGFRs, EGF binding results in a Ca^{2+} signal, and the presence of TGFβ receptors has been confirmed by chemical cross-linking (Fig. 1A). The high number of EGFRs makes receptor clustering into coated pits readily visualized and, therefore, can be considered a characteristic of these cells. The use of a carcinoma cell line has important implications in tumor cell growth and regulation, since the cellular responses to growth factors can be either amplified or diminished. Concentrations of EGF and TGFβ, which are growth inhibitory to A431 cells (2, 23), were selected specif-
Fig. 3. [Ca\(^{2+}\)] measurements in A431 cells using photon counting. a, the untreated controls in HBSS + Ca\(^{2+}\) (1.37 mM; + Ca\(^{2+}\)) and nominally Ca\(^{2+}\)-free HBSS (0-5 mM, −Ca\(^{2+}\)) stayed at or near basal levels for 20 min. b, treatment with EGF (10 ng/ml) in HBSS + Ca\(^{2+}\) resulted in an immediate [Ca\(^{2+}\)] increase (from 75 to 150 nM), which peaked at 2.5 min before declining to 90 nM, where it was maintained until thapsigargin (10 nM) was added. Thapsigargin mobilized more [Ca\(^{2+}\)] (to 220 nM) than seen with EGF. c, after EGF treatment in nominally Ca\(^{2+}\)-free HBSS, there was a lag period of 1 min before [Ca\(^{2+}\)] started to rise. [Ca\(^{2+}\)] was elevated for 3 min, peaking at 2 min to 142 nM, and then sharply declining to baseline. The timing of the EGF-induced [Ca\(^{2+}\)], elevation varied among cells within this small colony, resulting in multiple Ca\(^{2+}\) peaks on the photon counter output. d, treatment with TGFβ (10 ng/ml) in HBSS + Ca\(^{2+}\) did not result in increased [Ca\(^{2+}\)]. e, simultaneous addition of TGFβ and EGF resulted in a slow [Ca\(^{2+}\)] elevation from 50 to 100 nM, over a 15-min period. f, preincubation of TGFβ (10 ng/ml; 3 h), followed by EGF + TGFβ (10 ng/ml each), negated the EGF-induced [Ca\(^{2+}\)] elevation.

Physically to establish whether there is an association or disso-
ciation between ligand binding, Ca\(^{2+}\) as a second mes-
enger, and early response gene expression. At this time, there
is still much to be learned about cell signal pathways and the
role of Ca\(^{2+}\) in specific gene expression.

[Ca\(^{2+}\)] Measurements. Ca\(^{2+}\) signaling in non-excitable
cells involves release from intracellular stores, which acti-
vates plasma membrane Ca\(^{2+}\) entry (24). Photom counting is
more sensitive for [Ca\(^{2+}\)] measurement than digital imaging
methods and is useful for treatments that result either in
rapid responses or slight changes in fluorescent signals.
DIFM, however, gives information about spatial changes in
[Ca\(^{2+}\)] in individual cells.

Conflicting data exist in the literature concerning the re-
lease of Ca\(^{2+}\) from intracellular stores in A431 cells or
whether EGF causes [Ca\(^{2+}\)] to increase in the absence
of [Ca\(^{2+}\)]. Our results show that treatment with EGF causes an
increase in [Ca\(^{2+}\)], both in the presence and absence of
[Ca\(^{2+}\)], which concur with results from others (9, 25).
Gonzales et al. (11) and Moolenaar et al. (12), however,
reported that EGF does not always trigger a [Ca\(^{2+}\)] elevation
in the absence of [Ca\(^{2+}\)], suggesting there is no release from
intracellular stores. There are several variables that can in-
fluence intracellular Ca\(^{2+}\) stores and expression of growth
factor receptors including: (a) differences in A431 cell strains;
(b) cell density differences (which would affect receptor num-
bers and affinities and, thus, could greatly affect the results
of growth factor studies); and (c) the presence of other sub-
stances that can regulate the EGFR. In addition, not every-
one recognizes that Ca\(^{2+}\)-free medium contains Ca\(^{2+}\)
present in other ingredients, such as NaCl, and is correctly
termed nominally Ca\(^{2+}\)-free. As mentioned in “Results,”
when extracellular Ca\(^{2+}\) was chelated with EGTA, the A431
cells contracted and rounded within 10 min. This disruption
of cell shape and of the cell monolayer is typical of epithelial
cells exposed to EGTA. Cell vacuolization is also commonly
seen. This toxic response introduces artifacts in Ca\(^{2+}\) mea-
surements and thus invalidates results.

It is noteworthy that the high [Ca\(^{2+}\)] observed in EGF-
treated cells (Fig. 2A) has been reported recently in smooth
muscle cells and was discussed in terms of the role of protein
phosphorylation regulation of Ca\(^{2+}\) homeostasis (26). Our
results showed that by 1 min, phosphorylation of both ty-
rrosine and threonine residues occurred. This phosphoryla-
tion is temporally related to the EGF-induced rise in [Ca\(^{2+}\)].
These findings agree with those of Himpes et al. (26) in that
we also observed high [Ca\(^{2+}\)], and phosphorylated protein
residues in the nuclear or perinuclear areas, supporting cou-
pling between Ca\(^{2+}\) homeostasis and protein phosphoryla-
tion. Our results are also in agreement with Tidball and
Spencer (27), who found that after 10 min stimulation with
platelet-derived growth factor in skeletal muscle cells,
phosphotyrosine distribution changed from focal contacts to being located in "granules concentrated in perinuclear regions." Interestingly, in this study the granules also immunostained for platelet-derived growth factor receptor. These two events may be linked through Ca\(^{2+}\) binding to calmodulin, which would activate many proteins, including protein kinases (28). These data show that EGF induces phosphorylation of cytoplasmic and nuclear proteins, which are presumably directly related to the transcription of genes in response to EGF binding. It is noteworthy that the [Ca\(^{2+}\)]\(_{\text{exo}}\) increased more rapidly than [Ca\(^{2+}\)]\(_{\text{cyt}}\). The associated perinuclear and nuclear localization of [Ca\(^{2+}\)]\(_{\text{cyt}}\), is probably derived from unloading of the nuclear/perinuclear ER, perhaps related to Ca\(^{2+}\) influx factor, which may be regulated by protein phosphatase and kinase activity (29, 30).

Most reports suggest that TGF\(\beta\) receptor binding does not result in Ca\(^{2+}\) fluxes or phosphoinositide metabolism. Our results are in agreement with others who found that TGF\(\beta\) treatment of A431 cells for <1 h had no effect on [Ca\(^{2+}\)]\(_{\text{cyt}}\), either by influx or redistribution of Ca\(^{2+}\) stores (14, 31), indicating that phosphoinositide metabolism is not involved. However, early (13) and late (14) Ca\(^{2+}\) responses have been reported. A delayed TGF\(\beta\)-induced Ca\(^{2+}\) response was shown in the work of Muldoon et al. (31) in which [Ca\(^{2+}\)]\(_{\text{cyt}}\) increased after 2–4 h in conjunction with elevated inositol-1,4,5-trisphosphate levels. Experiments by Muldoon et al. (31), however, were performed using fibroblasts in which TGF\(\beta\) acts as a stimulatory growth factor. In the study by Muldoon et al. (31), TGF\(\beta\) had no effect on the EGF-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation in fibroblasts, which is contrary to what we have demonstrated for epithelial cells. These findings emphasize the strong multifunctional nature of TGF\(\beta\). The absence of an early TGF\(\beta\) Ca\(^{2+}\) signal, however, does not preclude an essential role for Ca\(^{2+}\) in epithelial cell signaling, since basal levels of Ca\(^{2+}\) may be sufficient for recruitment.
by TGFβ. Ca²⁺ has recently been shown to enhance the expression of the TGFβ gene in transformed bronchial epithelial cells (32), suggesting that Ca²⁺ may be integral to TGFβ action.

A major focus of our investigation was to determine if TGFβ would abolish the EGF Ca²⁺ signal. The simultaneous addition of EGF and TGFβ resulted in a gradual [Ca²⁺], increase. The reason for this gradual increase is unclear. In many cases, TGFβ exposure must be over a long time period in order to see certain effects. For example, the elimination of EGF-induced [Ca²⁺], elevation was observed only after a preincubation with TGFβ. Humes et al. (6) and others (5) also noted this. We have indications that on other cell types, short TGFβ incubations are not adequate to see these effects.

c-Fos Oncoprotein. To better understand the role of proto-oncogenes in the growth regulation of A431 cells, we investigated the c-Fos oncprotein in relation to EGF and TGFβ stimulation. We showed with indirect immunofluorescence microscopy that EGF induced the appearance of Fos oncprotein in a time-dependent manner, which reached a peak at around 1 h. In another study, growth-inhibitory concentrations of EGF increased Fos in A431 cells independent of the growth state of cells (15). The significance of using growth-inhibitory concentrations is that EGF-induced increases in the Fos oncprotein are independent of proliferation, which also supports the role of c-fos expression in differentiation.

Some of the earliest effects of TGFβ receptor binding are the induction of proto-oncogenes and induction of genes coding for cytoskeletal and extracellular matrix proteins (16, 17). We and others (15) have shown that TGFβ induces c-fos in A431 cells with kinetics similar to EGF, indicating a signal pathway coupled to the TGFβ receptors and one which overlaps with EGFR binding. Importantly, TGFβ receptor binding does not result in a Ca²⁺ signal, suggesting a dissociation between c-fos induction and Ca²⁺ regulation. It is not clear from these experiments whether Ca²⁺ is integral to c-fos induction in general or for these growth factor effects. As suggested by some, they may be independent pathways, or it has been proposed that Ca²⁺ is critical to c-fos induction (33). We propose that c-fos induction by TGFβ is independent of Ca²⁺ elevation and agree with others (34), who have implicated a TGFβ signaling pathway exclusive of classical second messengers such as Ca²⁺.

A431 is a tumor cell line that is not resistant to the potent growth inhibitor TGFβ and is growth inhibited by the potent mitogen EGF. Since TGFβ inhibits the growth of A431 cells, induces the cell differentiation-related c-fos gene but does not elevate [Ca²⁺], an integral role for Ca²⁺ in cell proliferation pathways is substantiated. The mechanisms by which TGFβ can alter the cellular response to a mitogen are not clear, although TGFβ phosphorylation of the EGFR is known to occur. However, this study and others which show that pretreatment with TGFβ is necessary for TGFβ dominance over EGF suggest that TGFβ may act on targets downstream of EGFR binding or receptor autophosphorylation. Our study points to TGFβ modulation of Ca²⁺ regulation in mitogenic pathways and the differentiating properties of high concentrations of growth factors through the induction of c-fos.
Materials and Methods

Materials. Mouse EGF was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY); human TGFβ was from R & D Systems, Inc. (Minneapolis, MN); 125I-labeled EGF and 125I-labeled TGFβ were from NEN Dupont (Wilmington, DE); BS3 was from Pierce Chemical Co. (Rockford, IL); fura 2/AM was from Calbiochem (La Jolla, CA); and DMEM with 4.5 g/liter glucose and FBS were from Paragon Biotech, Inc. (Baltimore, MD).

Antibodies included FoS Ab-2, a rabbit, affinity-purified, polyclonal antibody raised against the NH₂-terminal domain and specific for the human p62 TM. Like MoAb, and biotinylated antimouse IgG; they were purchased from Oncogene Science, Inc. (Uniondale, NY). The VECTASTAIN ABC System kit was purchased from Vector Laboratories, Inc. (Burlingame, CA). The monoclonal antihuman EGFR and polyclonal antiphosphotyrosine antibodies were obtained from Upstate Biotechnology, Inc. Monoclonal antiphosphotyrosine was obtained from Sigma Chemical Co. (St. Louis, MO). Fluorescein-conjugated goat, affinity-purified antibody to rabbit IgG (whole molecule) and rabbit, affinity-purified antibody to mouse IgG (whole molecule) were obtained from Cappel (Organon Teknika Corp., West Chester, PA).

Cell Culture. A431 human epidermoid carcinoma cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM with 10% FBS (termed complete growth medium). Exponentially growing cells (20–30% confluent) were washed five times in serum- and growth factor-free DMEM and were incubated in the same medium for 4 h prior to adding EGF and TGFβ. The growth factors were diluted from concentrated stocks (for 125I-EGF, 4 μCi/ml; for 125I-TGFβ, 1 μCi/ml) in DMEM (5% FBS) with serum-free medium for all experiments except for [Ca²⁺]ᵢ measurements, for which HBSS was used as the diluent (see below).

Affinity Labeling. A431 cell monolayers were affinity labeled with 150 pm 125I-labeled TGFβ or 125I-labeled EGF according to the method of Massague and Lague (35). To detect plasma membrane receptors, cells were chemically cross-linked with BS3 to cell-bound 125I-labeled TGFβ or 125I-labeled EGF. In competitive binding experiments, 200-fold excess unlabeled TGFβ or EGF were added. Labeled receptors were resolved by polyacrylamide gel electrophoresis (36) under reducing conditions, followed by autoradiography.

Immunoperoxdase Staining for EGFR Clustering. Cells on 4-well glass chamber slides were fixed in 1.5% formaldehyde in PBS and lightly permeabilized in 0.3% Triton X-100, 10 mM Tris and 1 mM EDTA (pH 7.0) for 2 min. The cells were reacted with the primary antibody to the EGFR, secondary biotinylated antimouse IgG antibody, and ABC reagents using standard immunohistochemical staining techniques. The immunoperoxidase reaction was terminated by the addition of water, and the cells were lightly counterstained in hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted with a coverslip using Permount.

[Ca²⁺]ᵢ Measurements. A431 cells were cultured on 25-mm round, glass coverslips in complete medium for 2–3 days. Cells were washed five times in serum- and growth factor-free DMEM, followed by incubation in the same medium for 4 h at 37°C. The cells were loaded with fura 2/AM (5 μM; 1–1.5 h at room temperature) in DMEM minus NaHCO₃ and phenol red. For [Ca²⁺]ᵢ measurement, cells were rinsed and placed in HBSS (pH 7.2), with or without the addition of 1.37 mM CaCl₂. HBSS with CaCl₂ (1.37 mM) was termed “normal” or “HBSS + CaCl₂”; and without added CaCl₂ (<5 μM) was termed “nominally Ca²⁺-free HBSS.”

[Ca²⁺]ᵢ measurement by DIFn was performed as described previously (37, 38) using a Tracor Northern Fluoroplex III and a TN-8002 image analysis system (Tracor Northern, Middleton, WI). At 5-min intervals, image pairs were collected at 340 and 380 nm excitation with a 520-nm wide, band pass (40 nm) emission filter.

To obtain continuous measurement of [Ca²⁺]ᵢ, the Tracor Northern photon counting system (TN6600) was used in conjunction with the Fluoroplex III system. A beam splitter was inserted to divert one-half of the light from the DIFn system camera to the photomultiplier tube. Camera were recorded with an IBM compatible computer and software. During experiment, the fluorescent signals (340 and 380 nm excitation) were collected continuously at the rate of 1 image pair/2 s. Colonies of cells large enough to fill the emission field were selected for measurement, and the resulting fluorescent signals represented the average fluorescence of the 4–10 cells in the field. Background was determined using coverslips of unloaded cells. Ratios were calculated, and graphics and statistical analyses were performed using Axum graphics software (TriMetrix, Seattle, WA).

Immunofluorescence Microscopy of c-Fos Oncoprotein, Phosphotyrosine, and Phosphothreonine. For indirect immunofluorescence microscopy, low density A431 cells on glass were treated with EGF or TGFβ for 30 to 4 h. Cells were fixed in 1.5% formaldehyde in PBS, lightly permeabilized with 0.3% Triton X-100 (as described in “Materials and Methods”) and stained using standard immunofluorescent techniques. Primary antibodies included polyclonal antibodies against human p62 TM and phosphotyrosine and a monoclonal antibody against phosphothreonine. The secondary antibodies were fluorescein-conjugated antibodies to rabbit or mouse IgGs. The final cell preparations were covered with a coverslip using non-quenching medium and viewed with an Olympus microscope.

References