T3M4 human pancreatic carcinoma cells avidly bound and internalized $^{125}$I-labeled epidermal growth factor (EGF) but did not readily degrade the ligand. Pulse-chase experiments in which the cell-bound radioactivity was allowed to dissociate into the incubation medium in the presence of unlabeled EGF indicated that the majority of the released $^{125}$I-EGF consisted of intact EGF and a slightly processed species that readily bound to the cell. Omission of unlabeled EGF during the chase period markedly decreased the amount of radioactivity in the incubation medium, mainly as a result of the rebinding of EGF to the cells. In contrast, T3M4 cells readily degraded $^{125}$I-labeled transforming growth factor-$\alpha$ (TGF-$\alpha$), and the released radiolabeled products did not rebind to the cells. Both ligands were released from T3M4 cells under acidic conditions, complete dissociation occurring at a pH of 4.5 for EGF, and a pH of 6.5 for TGF-$\alpha$. A431 human epidermoid carcinoma cells and ASPC-1 human pancreatic carcinoma cells also failed to extensively degrade $^{125}$I-EGF, whereas Rat-1 fibroblasts markedly degraded the growth factor. As in the case of T3M4 cells, ASPC-1 cells extensively degraded $^{125}$I-TGF-$\alpha$. Degradation of either ligand was blocked by the lysosomotropic compound methylamine in all the tested cell lines. Immunoprecipitation of the EGF receptor with specific polyclonal antibodies and Western blot analysis revealed the anticipated 170-kDa protein in T3M4 cells. Both EGF and TGF-$\alpha$ enhanced EGF receptor degradation, but TGF-$\alpha$ was less effective than EGF. These findings indicate that in certain cell types EGF and TGF-$\alpha$ may be differentially processed.

Epidermal growth factor (EGF) is a mitogenic polypeptide that binds and activates the cell-surface EGF receptor, thereby regulating numerous cellular processes (1). The EGF receptor exhibits a strong sequence homology with the product of the avian erythroblastosis virus v-erbB oncogene (2), is autophosphorylated at tyrosine residues (3), and is internalized and degraded following activation by EGF (4). Transferring growth factor-$\alpha$ (TGF-$\alpha$) is a peptide growth factor that shares a 35% homology with EGF and that possesses 6 cysteine residues in the same relative positions as EGF (5, 6). Consequently, the receptor binding domains of EGF and TGF-$\alpha$ are believed to display highly similar three-dimensional configurations, enabling TGF-$\alpha$ to readily bind to the EGF receptor (7). It is generally accepted that the biological actions of TGF-$\alpha$ are mediated via the EGF receptor, inasmuch as TGF-$\alpha$ does not appear to bind to its own distinct receptor (6). Although EGF and TGF-$\alpha$ exert similar biological actions in many cell systems, there are several notable exceptions. TGF-$\alpha$ exerts a greater stimulatory effect than EGF with respect to calcium mobilization from fetal rat long bones (8), angiogenesis in the hamster cheek pouch model (9), skin wound healing (10), blood flow in the femoral artery of the dog (11), induction of cell ruffling (12), anchorage-independent growth of certain human pancreatic cancer cell lines (13), and formation of keratinocyte megacolonies in culture (14). In RL35-2 human endometrial cancer cells, TGF-$\alpha$ is a more potent inhibitor of cell proliferation than EGF when cells are plated at low seeding densities but is equipotent with EGF in stimulating proliferation when cells are plated at high seeding densities (15). In primary lung carcinoma cells, TGF-$\alpha$ enhances cell proliferation whereas EGF inhibits proliferation (16). Furthermore, the inhibitory effect of EGF on noradrenaline-induced contraction in arterial strips is diminished following repeated exposure of the strips to EGF but not to TGF-$\alpha$ (11). The mechanisms underlying these various quantitative and qualitative differences are not known.

In the present study we compared the degradation of EGF and TGF-$\alpha$ in the T3M4 human pancreatic carcinoma cell line. This cell line has 1.2 x 10$^5$ cell-surface EGF receptors (17) and releases significant amounts of TGF-$\alpha$ into the incubation medium (13). We now report that TGF-$\alpha$ is more rapidly and extensively degraded than EGF and that EGF induces a more significant degradation of the EGF receptor than TGF-$\alpha$ in these cells. These data may explain, in part, the ability of certain cells to concomitantly overexpress the EGF receptor and produce TGF-$\alpha$ without excessively down-regulating the receptor in an autocrine manner.

EXPERIMENTAL PROCEDURES

Materials—Sephadex G-25 and bovine serum albumin (BSA) were purchased from Sigma; carrier-free $^{131}$I and $^{32}$P and $^{35}$S)methionine (1100 Ci/nmol) were from DuPont-New England Nuclear; Pansorbin was from Calbiochem; IODO-BEADS were from Pierce Chemical Co.; sodium orthovanadate was from Alfa Products, Danvers, MA; horseradish peroxidase-conjugated IgG and color developing reagent were from Bio-Rad; Sykes-Moore culture chamber was from Bellco Glass, Inc., Vineland, NJ; and recombinant human insulin-like growth factor I (rhIGF-1) was from IMCERA, Inc., Terre Haute, IN. Recombinant human EGF (rhEGF) was a gift from Dr. C. George-Nascimento at Chiron Co. (Emeryville, CA). Recombinant human TGF-$\alpha$ (rhTGF-$\alpha$) was a gift from Dr. R. Derynck of Genentech, Inc. (South San Francisco, CA). Recombinant human insulin-like growth factor II (rhIGF-II) was a gift from Dr. R. Derynck of Genentech, Inc. (South San Francisco, CA). The abbreviations used are: EGF, epidermal growth factor; TGF-$\alpha$, transforming growth factor-$\alpha$; BSA, bovine serum albumin; IGF, insulin-like growth factor; rh, recombinant human; DME, Dulbecco’s modified Eagle’s medium; IEF, isoelectric focusing; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FCS, fetal calf serum; SDS, sodium dodecyl sulfate.

* This work was supported in part by United States Public Health Service Grant CA-40162 from the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of Medical Student Research Award T33HL07479 from the United States Public Health Service.

‡ The abbreviations used are: EGF, epidermal growth factor; TGF-$\alpha$, transforming growth factor-$\alpha$; BSA, bovine serum albumin; IGF, insulin-like growth factor; rh, recombinant human; DME, Dulbecco’s modified Eagle’s medium; IEF, isoelectric focusing; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FCS, fetal calf serum; SDS, sodium dodecyl sulfate.
San Francisco, CA). Polyclonal antibodies against the EGF receptor and a specific blocking peptide (18) were a gift from Dr. L. Gentry at Medical College of Ohio (Toledo, OH).

Cell Culture—T2M, (19) and ASPC-1 (13) human pancreatic carcinoma cells were grown in RPMI 1640 medium supplemented with 5% newborn calf serum and 2% fetal bovine serum. A431 human vulvar carcinoma cells (20) were grown in DMEM with 10% fetal calf serum (v/v) supplemented with 1% HEPES, pH 7.4. Cells were maintained at 37 °C, T3H4 cells were incubated for 6 min at 4 °C in a buffer consisting of 135 mM NaCl, 5 mM KCl, and 5 mM acetic acid (27). Cells were maintained at 4 °C, and the buffer containing the surface-bound unlabeled ligands was removed. Cells were then rinsed twice with DME containing 10% FCS, prior to initiation of binding with BM containing either 125I-EGF or 125I-IGF-1. Binding was allowed to continue for 4 h at 4 °C prior to termination of incubation as in routine binding experiments.

Degradation of 125I-EGF and 125I-IGF-1 in incubation medium was monitored by determining the precipitability of the released radioactivity in the presence of 10% trichloroacetic acid and by analyzing supernatant radioactivity on Sephadex G-25 column chromatography.

To monitor 125I-EGF processing, dissociated radioactivity was also analyzed by IEF on horizontal 1% polyacrylamide gels (20). Gels containing 125I-labeled samples were prepared for fluorography by fixing in 10% trichloroacetic acid, 10% acetic acid, and 30% methanol. Gels were then washed twice with H2O, incubated for 30 min at 23 °C with 1 N sodium carbonate containing 0.5% glyceraldehyde, and 30% methanol. Gels were then washed twice with H2O, incubated for 30 min at 23 °C with 1 N sodium carbonate containing 0.5% glyceraldehyde, and 30% methanol. Gels containing 125I-labeled samples were treated as above except that the sodium carbonate step was omitted, and drying screens were utilized for the autoradiography. Immunoblotting was performed as described by Towbin et al. (31), but using 3% BSA as the blocking solution.

RESULTS

Characterization of 125I-EGF Binding and Processing—At 37 °C, T2M cells rapidly bound 125I-EGF. The amount of cell-bound radioactivity that was resistant to acid treatment increased continuously over the 180-min incubation period (Fig. 1A). The acid-sensitive compartment, taken to represent surface-bound radioactivity, increased during the initial 30 min of incubation, remained constant during the subsequent 30–60 min, and declined slightly thereafter (Fig. 1A). At 180 min, the vast majority of the radioactivity was localized inside the cell.

The fate of cell-bound EGF was determined by monitoring the degradation of 125I-EGF after a 30-min binding period at 37 °C, followed by a chase period. The chase was carried out in the presence of 34 nM unlabeled EGF to prevent the rebinding of dissociated radioactivity. Analysis of the 125I activity following incubation with 1 ng/ml 125I-EGF indicated that 70 ± 4% (mean ± S.E., n = 8) of the radioactivity released during a 180-min chase period co-migrated with authentic EGF on Sephadex G-25 column chromatography (Figs. 1B and 2A; Table I). The corresponding values at 30

Binding Studies—For most EGF binding experiments, biologically active EGF was prepared from mouse submaxillary glands by the method of Savage and Cohen (22) and iodinated by the use of IODO-BEADS to a specific activity of 200 μCi/μg. Briefly, 2 μCi of Na125I were incubated for 5 min at 25 °C with 5 μg EGF in the presence of two IODO-BEADS. The reaction was stopped by removal of the beads and addition of 0.03% BSA. The mixture was either applied to a Sephadex G-25 column, equilibrated with 50 mM phosphate buffer containing 3 mg/ml bovine serum albumin (BSA) for routine binding studies or subjected to isoelectric focusing (IEF) for processing studies (23). This iodination procedure routinely resulted in approximately 70% incorporation of 125I. The species of EGF (pl 4.55) representing authentic intact EGF by high performance liquid chromatography (25) was used in the processing studies in these comparative experiments were equipotent with respect to EGF receptor occupancy, competition-inhibition binding studies were carried out in T2M cells with 125I-labeled rhEGF and rhIGF-α. Both recombinant growth factors were iodinated under identical conditions by a modification of the chloramine-T method to an approximate specific activity of 120 μCi/μg for EGF and 150 μCi/μg for TGF-α, as described previously (25). One-half maximal inhibition of binding for 125I-rhEGF and 125I-rhIGF-α occurred at 4 ng/ml (0.7 nM) and 5.5 ng/ml (1.0 nM) unlabeled EGF, TGF-α, respectively. However, concentrations of TGF-α greater than 1 nM inhibited the binding of either labeled ligand to the same extent, with maximal inhibition of binding occurring at 17 nM unlabeled TGF-α. For experiments with 125I-IGF-1, rhIGF-1 was similarly iodinated to an approximate specific activity of 130 μCi/μg (25).

Binding studies were routinely performed in cells that were in monolayer culture (70–75% confluent). Cells were washed once in DME supplemented with 0.1% BSA and 20 mM HEPES, pH 7.4 (binding medium, BM), followed by incubation in BM in the presence of labeled ligand. In most binding experiments incubations were stopped by washing cells five times in Hank's balanced salt solution containing 0.1% BSA. Non-specific binding of either 125I-EGF or 125I-TGF-α was determined in the presence of a 1000-fold excess of unlabeled EGF, whereas non-specific binding of 125I-IGF-1 was determined in the presence of a 3000-fold excess of unlabeled IGF-1. Non-specific binding never exceeded 10% of total binding for any ligand. In pulse-chase experiments binding was stopped by subjecting cells twice in BM. Cells were then incubated with fresh BM supplemented with 34 nM unlabeled EGF in order to prevent rebinding of dissociated radioactivity. In some experiments this was accomplished by continuous replacement of the incubation medium with fresh medium. To this end, cells were seeded on a siliconized coverslip and allowed to adhere for 24 h. Cells were then placed in a Sykes Moore culture chamber that had an inflow and outflow port and incubated at 37 °C. The rate of flow of the fresh BM (prewarmed to 37°C) through the culture chamber was maintained at 1 ml/min.

Binding Studies, and Immunoprecipitation—To monitor 125I-EGF internalization, cultured cells were incubated with 125I-EGF and subsequently washed as in binding studies. Cells were then incubated for 4 min at 4 °C with 500 mM NaCl that was titrated to pH 2.5 with acetic acid (25). Radioactivity removed by acid treatment was approximately 50% of the acid-sensitive (internalized) radioactivity and was then determined separately. A milder surface stripping procedure was used in down-regulation experiments in order to remove surface-bound unlabeled ligand prior to incubations with labeled ligands (27). Following removal of the medium from cells previously incubated at 37 °C with either EGF or TGF-α, cells were incubated for 6 min at 4 °C in a buffer consisting of 135 mM NaCl, 5 mM KCl, and 5 mM acetic acid (27). Cells were maintained at 4 °C, and the buffer containing the surface-bound unlabeled ligands was removed. Cells were then rinsed twice with DME containing 10% FCS, prior to initiation of binding with BM containing either 125I-EGF or 125I-IGF-1. Binding was allowed to continue for 4 h at 4 °C prior to termination of incubation as in routine binding experiments.

Degradation of 125I-EGF and 125I-IGF-1 in incubation medium was monitored by determining the precipitability of the released radioactivity in the presence of 10% trichloroacetic acid and by analyzing supernatant radioactivity on Sephadex G-25 column chromatography.

To monitor 125I-EGF processing, dissociated radioactivity was also analyzed by IEF on horizontal 1% polyacrylamide gels (20). Gels containing 125I-labeled samples were prepared for fluorography by fixing in 10% trichloroacetic acid, 10% acetic acid, and 30% methanol. Gels were then washed twice with H2O, incubated for 30 min at 23 °C with 1 N sodium carbonate containing 0.5% glyceraldehyde, and 30% methanol. Gels were then washed twice with H2O, incubated for 30 min at 23 °C with 1 N sodium carbonate containing 0.5% glyceraldehyde, and 30% methanol. Gels containing 125I-labeled samples were treated as above except that the sodium carbonate step was omitted, and drying screens were utilized for the autoradiography. Immunoblotting was performed as described by Towbin et al. (31), but using 3% BSA as the blocking solution.

RESULTS

Characterization of 125I-EGF Binding and Processing—At 37 °C, T2M cells rapidly bound 125I-EGF. The amount of cell-bound radioactivity that was resistant to acid treatment increased continuously over the 180-min incubation period (Fig. 1A). The acid-sensitive compartment, taken to represent surface-bound radioactivity, increased during the initial 30 min of incubation, remained constant during the subsequent 30–60 min, and declined slightly thereafter (Fig. 1A). At 180 min, the vast majority of the radioactivity was localized inside the cell.

The fate of cell-bound EGF was determined by monitoring the degradation of 125I-EGF after a 30-min binding period at 37 °C, followed by a chase period. The chase was carried out in the presence of 34 nM unlabeled EGF to prevent the rebinding of dissociated radioactivity. Analysis of the 125I activity following incubation with 1 ng/ml 125I-EGF indicated that 70 ± 4% (mean ± S.E., n = 8) of the radioactivity released during a 180-min chase period co-migrated with authentic EGF on Sephadex G-25 column chromatography (Figs. 1B and 2A; Table I). The corresponding values at 30
Epidermal Growth Factor Binding by T3M4 Cells

Fig. 1. Binding, internalization, and release of 125I-EGF in T3M4 cells. A, T3M4 cells were incubated in 35-mm wells at 37 °C with 0.17 nM (100,000 cpm) 125I-EGF. Incubations were stopped at the indicated times. Surface-bound radioactivity was determined by elution into pH 2.5 buffer, and internalized radioactivity was determined by solubilization of cells in 0.5 N NaOH following removal of surface-bound radioactivity. B, T3M4 cells were incubated in 35-mm wells for 30 min at 37 °C with 0.17 nM (100,000 cpm) 125I-EGF, washed twice, and incubated in fresh medium at 37 °C in either the absence (closed circles) or presence (closed squares) of 34 nM unlabeled EGF. All of the radioactivity released into the medium during the subsequent 180 min (19,500 cpm) was analyzed by Sephadex G-25 chromatography. C, T3M4 cells were seeded on a 22.4-mm siliconized coverslip and incubated and washed as in B but with 0.34 nM (200,000 cpm) 125I-EGF. Cells were then incubated at 37 °C in either the absence (closed circles) or presence (closed squares) of 34 nM unlabeled EGF with fresh medium that was continuously removed at a rate of 1 ml/min using a Sykes-Moore flow-through apparatus. and 90 min after initiation of the chase were 86 and 75%, respectively (Table I).

To determine whether EGF released from T3M4 cells could rebinding to these cells, a pulse-chase experiment in which the chase was carried out in the absence of unlabeled EGF was performed next. When the radioactivity released into the incubation medium during a 180-min chase period was analyzed by Sephadex G-25 chromatography, the high molecular weight peak was greatly reduced (Fig. 1B). In contrast, the amount of low molecular weight radioactivity was relatively small and was similar to that observed when the chase was performed in the presence of unlabeled EGF (Fig. 1B).

To determine whether the presence of unlabeled EGF during the chase period resulted in enhanced dissociation of cell-bound radioactivity, the pulse-chase experiment was repeated under conditions that allowed for continuous replacement of the incubation medium with fresh medium (Fig. 1C). The

Table I

Degradation of 125I-labeled EGF and TGF-α in T3M4 cells

| Time | 125I-EGF | 125I-TGF-α |
|------|----------|------------|
| min  | 10 ng/ml | 100 ng/ml  | 1 ng/ml | 10 ng/ml | 100 ng/ml |
| 30   | 0.54 ± 0.02 | 0.67 ± 0.03 | 0.32 ± 0.02 | 0.44 ± 0.03 | 0.58 ± 0.04 |
| 90   | 0.64 ± 0.05 | 0.76 ± 0.06 | 0.45 ± 0.05 | 0.57 ± 0.06 | 0.72 ± 0.07 |
| 180  | 0.75 ± 0.06 | 0.87 ± 0.07 | 0.56 ± 0.06 | 0.70 ± 0.07 | 0.82 ± 0.08 |
disassociated radioactivity was thus prevented from rebinding to the cells irrespective of the presence or absence of unlabeled EGF in the medium. Under these incubation conditions, unlabeled EGF (34 nM) caused a slight increase in the amount of radioactivity in the medium during the initial 60 min of the chase period. However, during the remainder of the chase period, there was a greater release of radioactivity into the medium in the absence of unlabeled EGF. After 180 min, 50 and 41% of the cell-bound radioactivity was released into the medium in the presence or absence of unlabeled EGF, respectively.

The 125I activity in the postincubation culture medium from A431 cells exhibited a similar profile as that observed in T3M4 cells when analyzed by chromatography on Sephadex G-25 (Fig. 2C). In three experiments, 65 ± 4% (mean ± S.E.) of the 125I activity that was released into the incubation medium during a 180-min chase period co-migrated in the void volume with authentic EGF. In contrast, the majority of the postincubation radioactivity from Rat-1 fibroblasts appeared as low molecular weight degradation products (Fig. 2B), and only 25 ± 3% (mean ± S.E., n = 3) of the released radioactivity co-migrated in the void volume with authentic EGF.

Following binding to target cells EGF is processed by the sequential removal of several carboxyl-terminal amino acids prior to eventual degradation (32). The first species thus formed represents EGF that is lacking either one or two amino acids (pI 4.20). Further EGF processing results in the removal of an additional three amino acids (pI 4.35) and another high molecular weight species lacking the last seven carboxyl-terminal amino acids (pI of 4.0). When the high molecular weight peak obtained following chromatography of radioactivity released by T3M4 cells was subjected to IEF, it consisted mainly of either the pI 4.55 or 4.20 form, whereas the pI 4.35 and 4.0 moieties were only faintly visible (Fig. 2A, inset 1). IEF analysis of the high molecular weight peaks from both A431 cells (Fig. 2C, inset 1) and Rat-1 fibroblasts (Fig. 2B, inset 1) again revealed the preponderant presence of the pI 4.55 and 4.20 moieties. However, the 4.35 moiety was more distinctly visible than in the case of T3M4 cells. All three cell lines bound and internalized both authentic EGF (pI 4.55 species) and the pI 4.2 species (Table II) but did not readily bind the pI 4.35 species and failed to bind the pI 4.0 species (data not shown).

Methylamine, at a concentration of 10 mM, markedly attenuated the appearance of low molecular weight metabolites in the case of all three cell lines and increased the relative proportion of the pI 4.55 species of EGF in the high molecular weight peak (Fig. 2, inset 2). The effects of methylamine (10 mM) on the kinetics of dissociation of 125I radioactivity from T3M4 cells (Fig. 3) and Rat-1 cells (Fig. 4) were compared next. During a 30-min pulsing period, approximately equal amounts of 125I activity were bound to control and methylamine-treated cells. However, the rate of release of bound 125I activity from

### Table II

| Cell type | 125I-EGF bound | 125I-EGF bound |
|-----------|----------------|----------------|
|           | cpm/10⁶ cells | cpm/10⁶ cells |
| T3M4      | 4.5           | 4,225 ± 139   |
|           | 4.2           | 4,369 ± 223   |
| Rat-1     | 4.5           | 361 ± 4       |
|           | 4.2           | 406 ± 36      |
| A431      | 4.5           | 9,381 ± 20    |
|           | 4.2           | 9,376 ± 588   |
methyamine-treated cells during the subsequent postincubation period was approximately 50% of that of control activity in both cell types (Figs. 3A and 4A). Furthermore, the methyamine-induced delay in the loss of cell-bound ^125I activity was observed in both the cell-surface (Figs. 3B and 4B) and intracellular (Figs. 3C and 4C) compartments.

Characterization of ^125I-TGF-α Degradation—Following binding with 1 ng/ml ^125I-TGF-α, only a small amount of radioactivity was released into the incubation medium during the initial 10 min of the chase period (Fig. 5). Analysis of the postincubation medium on Sephadex G-25 column chromatography indicated that after 30 min the majority (64%) of the released radioactivity consisted of low molecular weight degradation products (Table I). In six experiments in which the chase period was extended to 180 min, 23 ± 5% (mean ± S.E.) of the ^125I activity co-migrated with authentic TGF-α (Fig. 5A). Methyamine completely blocked the appearance of TGF-α degradation products (Fig. 5A).

To determine whether TGF-α released from T3M4 cells could rebind to these cells, a pulse-chase experiment was carried out in which the chase was performed in both the presence and absence of 34 nM unlabeled EGF. In contrast to the findings with ^125I-EGF, omission of unlabeled EGF from the medium resulted in only a slight decrease in the amount of ^125I activity that was present in the void volume peak, and this was associated with a slight increase in the amount of low molecular weight radioactivity (Fig. 5C). Furthermore, the percentage of radioactivity in the low molecular weight degradation peak did not decrease when binding with ^125I-TGF-α was performed in the presence of 1 ng/ml unlabeled EGF (data not shown).

It is generally accepted that cellular degradation of EGF is initiated after it dissociates from its receptor within the acidic lysosomal milieu (32, 33). Therefore, the relative resistance of EGF to degradation in T3M4 cells by comparison with TGF-α suggested that the two ligands may dissociate from the EGF receptor at different pH optima. To determine the pH dependence of ligand dissociation, T3M4 cells were incubated for 5 h at 4 °C with either ^125I-EGF or ^125I-TGF-α, washed at 4 °C, and incubated at 4 °C in fresh medium that had been titrated to varying pH levels. Chromatographic analysis of the radioactivity released into the medium during the subsequent 2 h indicated that neither ligand was degraded at 4 °C (data not shown). Decreasing the pH of the medium from 7.4 to 5.0 resulted in a progressive increase in the amount of ^125I-EGF that was released into the medium (Fig. 6), complete dissociation of surface-bound ^125I-EGF occurring at pH 4.5. In contrast, even at neutral pH (7.4), the vast majority of ^125I-TGF-α readily dissociated from the surface of T3M4 cells, with virtually complete dissociation occurring at a pH of 6.5 (Fig. 6).

Two types of experiments were carried out to determine whether the extensive degradation of ^125I-TGF-α in T3M4 cells was a consequence of the use of a tracer amount of ligand in the face of marked overexpression of the EGF receptor in this cell line. First, the degradation of TGF-α and EGF following binding with high ligand concentrations was examined in T3M4 cells. Increasing the concentration of ^125I-TGF-α to 30 and 100 ng/ml did not alter the degradation profile of the

Fig. 5. Binding and release of ^125I-TGF-α in T3M4 cells. A and B, T3M4 cells were incubated for 60 min at 37 °C in 35-mm wells in the absence (A) or presence (B) of 10 mM methylamine. ^125I-TGF-α (0.17 nM, 110,000 cpm) was added 30 min prior to termination of incubation. Cells were then washed twice and placed in fresh medium supplemented with 34 nM unlabeled EGF for a second incubation at 37 °C in the absence (A) or presence (B) of 10 mM methylamine. Medium was collected 10 min (open circles) and 180 min (closed circles) later and lymphoplasmic, and the released radioactivity was analyzed by Sephadex G-25 chromatography. C, T3M4 cells were incubated in 35-mm wells for 30 min at 37 °C with 0.34 nM (^220,000 cpm) ^125I-TGF-α, washed twice, and incubated in fresh medium in either the absence (closed circles) or presence (closed squares) of 34 nM unlabeled EGF. Radioactivity released into the incubation medium was then analyzed by Sephadex G-25 chromatography.

Fig. 6. Effect of pH on ligand dissociation in T3M4 cells. T3M4 cells were incubated for 5 h at 4 °C with 0.17 nM of either ^125I-EGF or ^125I-TGF-α and washed twice at 4 °C. Cells were then incubated at 4 °C for 2 h in the presence of fresh medium that had been titrated to the indicated pH levels. The amount of ^125I-EGF (circles) and ^125I-TGF-α (triangles) that dissociated from the cells during the 2-h incubation was expressed as a percentage of the respective radioactivity that bound to the cells during the 2-h incubation. Data are the means ± S.E. of three separate experiments. S.E. values smaller than the symbols are not shown.
released radioactivity at either 30, 90, or 180 min following initiation of the chase period (Table 1). In contrast, increasing the concentration of \(^{125}\text{I}-\text{EGF}\) resulted in a progressive increase in the amount of low molecular weight degradation products (Table 1). However, at all ligand concentrations, the degradation of EGF was always less extensive than that of TGF-\(\alpha\). Second, pulse-chase experiments were performed with ASPC-1 cells, a human pancreatic cancer cell line that has \(2.2 \times 10^5\) surface EGF receptors per cell (13). Comparison of the postincubation profiles following 30 min binding with either \(^{125}\text{I}-\text{EGF}\) (Fig. 7) or \(^{125}\text{I}-\text{TGF-\(\alpha\)}\) (Fig. 8) indicated that in the case of \(^{125}\text{I}-\text{EGF}\) the majority of the radioactivity co-

![Diagram](https://example.com/diagram1.png)

**FIG. 7.** Chromatographic analysis of dissociated \(^{125}\text{I}\) activity following binding with \(^{125}\text{I}-\text{EGF}\) in ASPC-1 cells. Cells were incubated for 60 min at 37 °C in 35-mm wells in the absence (A) or presence (B) of 10 mM methylamine. \(^{125}\text{I}-\text{EGF}\) (0.17 nM, 100,000 cpm) was added 30 min prior to the termination of incubation. Cells were then washed twice and placed in medium supplemented with 34 nM unlabeled EGF for a second incubation at 37 °C in the presence or absence of 10 mM methylamine. Medium was collected 10 min (closed circles) and 180 min (open circles) later and lyophilized. One-half of each sample was analyzed by Sephadex G-25 chromatography. At the 180-min collection, 3,900 cpm (A) and 2,100 cpm (B) were applied to the columns.

![Diagram](https://example.com/diagram2.png)

**FIG. 8.** Chromatographic analysis of dissociated \(^{125}\text{I}\) activity following binding with \(^{125}\text{I}-\text{TGF-\(\alpha\)}\) in ASPC-1 cells. Cells were incubated as in Fig. 7 but in the presence of 0.17 nM (110,000 cpm) \(^{125}\text{I}-\text{TGF-\(\alpha\)}\). Cells were then treated as in Fig. 7. Medium was collected 10 min (closed circles) and 180 min (open circles) later, and one-half of each sample was analyzed as in Fig. 6. At the 180-min collection, 7,600 cpm (A) and 2,500 cpm (B) were applied to the column.

migrated with authentic EGF, whereas in the case of \(^{125}\text{I}-\text{TGF-\(\alpha\)}\) the majority of the radioactivity appeared as low molecular weight degradation products. In the case of both ligands, methylamine (10 mM) completely inhibited the appearance of degradation products released by ASPC-1 cells (Figs. 7B and 8B). Furthermore, Rat-1 cells, which only have 100,000 surface EGF receptors per cell, also extensively degraded \(^{125}\text{I}-\text{TGF-\(\alpha\)}\) (data not shown).

**Effects of EGF and TGF-\(\alpha\) on EGF Receptor Degradation—** Immunoprecipitation of the EGF receptor revealed the anticipated 170-kDa protein when \(T_3M_4\) cells were radiolabeled with \(^{35}\text{S}\)methionine (Fig. 9, lane 1) and \(^{32}\text{P}\) (Fig. 9, lane 5), as well as when the cell homogenate was subjected to gel electrophoresis and transferred to a nitrocellulose filter prior to incubation with the antibody (Fig. 9, lane 3). In these
Fig. 9. SDS-polyacrylamide gel analysis of the EGF receptor. T3M4 cells were washed twice in methionine-free (lanes 1-2) or phosphate-free (lanes 5-7) DME containing 0.5% FCS, prior to labeling with [35S]methionine (150 µCi/ml) for 6 h (lanes 1-2) or 32P (500 µCi/ml) for 17 h (lanes 5-7). A431 cells (lanes 8-10) were labeled similarly with 32P. In lanes 6 and 9 EGF (17 nM) was present during the last 60 min of incubation with 32P. T3M4 cells were not labeled for electrophoretic transfer of the receptor (lanes 3-4). Cell lysates were prepared as described under “Experimental Procedures.” Immunoprecipitations and immunological detection of the immobilized receptor were carried out with 5 µl of a polyclonal anti-EGF receptor antibody in the absence (lanes 1, 3, 5-6, 8-9) or presence (lanes 2, 4, 7, 10) of 1 µg of blocking peptide. Gel exposure times were 16 h for lanes 1-2, 24 h for lanes 5-7, and 4 h for lanes 8-10. The molecular weight markers and the band representing the EGF receptor (EGFR) are shown to the left of the figure.

Fig. 10. Dose-dependent effect of EGF on EGF receptor phosphorylation in T3M4 cells. Cells were incubated with 32P (500 µCi/ml) for 17 h as in Fig. 9, except that EGF was present during the last 10 min of incubation with 32P. Immunoprecipitation of the EGF receptor (EGFR) was carried out as in Fig. 9. Equal amounts of radioactivity (15,000 cpm/lane) were subjected to SDS-polyacrylamide gel electrophoresis using a 7% running gel. Gel exposure time was 70 h. C, control; lane 1, 1.7 nM EGF; lane 2, 3.4 nM EGF; lane 3, 17 nM EGF; lane 4, 34 nM EGF.

Experiments, the band representing the EGF receptor was not present when the antibody was first preincubated with a specific blocking peptide (Fig. 9, lanes 2, 4, 7, and 10). Furthermore, EGF receptor phosphorylation was enhanced when either T3M4 (Fig. 9, lane 6) or A431 (Fig. 9, lane 9) cells were incubated for 60 min with 17 nM EGF. The stimulatory effect of EGF receptor phosphorylation was dose-dependent (Fig. 10), maximal stimulation of phosphorylation occurring at 17 to 34 nM EGF. In three experiments, 17 nM EGF (a concentration that maximally inhibited the binding of 125I-EGF in T3M4 cells) enhanced EGF receptor phosphorylation by 40%.

When T3M4 cells were incubated with [35S]methionine for 6 h and then incubated for 12 h in fresh medium devoid of radioactivity and supplemented with unlabeled methionine, there was a gradual decrease in the amount of radioactivity in the band representing the EGF receptor (Fig. 11, lanes a). When the second incubation was performed in the presence of 34 nM EGF, there was a marked acceleration in the rate of decrease of radioactivity in this band (Fig. 11, lanes b). In contrast, the decrease in radioactivity was not as pronounced in the presence of 34 nM TGF-α as with EGF (Fig. 11, lanes c). Although these concentrations of EGF and TGF-α were in excess of the concentration needed to maximally inhibit

125I-EGF binding, there was a difference between the growth factors in their ability to induce the degradation of the EGF receptor. Thus, after a 12-h chase period with EGF, the band representing the EGF receptor was barely visible by comparison with control or TGF-α treated cells, irrespective of whether equal amounts of radioactivity (Fig. 11, lanes 3) or protein (Fig. 11, lanes 4) were analyzed by gel electrophoresis. Densitometric analysis of the bands representing the EGF receptor at the beginning of the chase and at 2, 4, 6, and 12 h into the chase from three separate experiments indicated that the half-life of the EGF receptor was 16.0 h (Fig. 12). EGF and TGF-α significantly decreased the half-life of the receptor to 4.4 ± 0.15 and 7.2 ± 0.4 h (mean ± S.E.), respectively, and these values were significantly different from each other (p <
Effects of EGF and TGF-α on the binding of ¹²⁵I-EGF and ¹²⁵I-IGF-1 in T₃M₄ cells

Cells were plated in 35-mm wells (75% confluent) and incubated for 60 min at 37°C with the indicated additions. After removal of medium, cells were incubated for 6 min at 4°C in a buffer consisting of 135 mM NaCl, 5 mM KCl, and 5 mM acetic acid. Cells were maintained at 4°C and rinsed twice with DME and once with DME containing 10% FCS. Binding with either ¹²⁵I-EGF (100,000 cpm) or ¹²⁵I-IGF-1 (100,000 cpm) was then carried out for 4 h at 4°C. Values are mean ± S.D. of triplicate determinations from a representative experiment.

| Addition | ¹²⁵I-EGF | ¹²⁵I-IGF-1 |
|----------|----------|-----------|
| None     | 5,778 ± 449 | 10,796 ± 744 |
| EGF      | 1,763 ± 127 | 10,533 ± 635 |
| 1        | 1,271 ± 154 | 9,752 ± 674 |
| 5        | 1,094 ± 36 | 11,300 ± 450 |
| 10       | 992 ± 84 | 10,569 ± 634 |
| TGF-α    | 2,357 ± 24 | 10,195 ± 661 |
| 2        | 1,880 ± 142 | 11,305 ± 329 |
| 5        | 1,580 ± 26 | 10,931 ± 714 |
| 10       | 1,679 ± 134 | 10,623 ± 837 |

0.001) as determined by analysis of variance and Student’s t-test (Fig. 12).

To study EGF- and TGF-α-induced down-regulation of the EGF receptor in T₃M₄ cells, incubations were carried out at 37°C for 60 min with equimolar concentrations of each growth factor (1–10 nM). These concentrations of EGF and TGF-α were also equivalent at inhibiting the binding of either of the ¹²⁵I-labeled growth factors. Following removal of the surface-bound unlabeled ligands, the cells were incubated for 4 h at 4°C with either ¹²⁵I-EGF or ¹²⁵I-IGF-1. Both EGF and TGF-α caused a dose-dependent decrease in the subsequent binding of ¹²⁵I-EGF. However, the effects of EGF were always greater than the corresponding effects of TGF-α (Table III). In contrast, under the same incubation conditions, neither growth factor altered the binding of ¹²⁵I-IGF-1.

DISCUSSION

The first step in the action of EGF is represented by its binding to a specific cell-surface glycoprotein receptor (34). The binding process is associated with EGF receptor phosphorylation and with the generation of other signals that participate in mediating the biological effects of EGF (1–4). Following binding, the EGF-receptor complex translocates through the membrane (35) and internalizes into the cell by a receptor-mediated endocytosis (36). Because the mechanisms whereby cells process bound EGF may be an important aspect of the modulation of EGF receptor activation, the fate of cell-bound EGF has been extensively studied in many cells. In most cell types, the majority of internalized EGF is rapidly transported to lysosomes or lysosome-like organelles and, subsequently, readily degraded (1, 26). In spite of considerable ligand endocytosis, the degradation of EGF is attenuated in rat pancreatic acini (37), PANC-1 human pancreatic carcinoma cells (38), rat neonatal intestinal cells (39), and Madin-Darby canine kidney cells (40). Furthermore, approximately 25% of internalized EGF is transported through a nonlysosomal pathway in the liver and is consequently protected from degradation (41).

In the present study we have determined that EGF is not rapidly degraded in T₃M₄ or ASPC-1 human pancreatic carcinoma cells or in A431 vulvar carcinoma cells. It is unlikely that this attenuated degradation is due merely to a low ratio of EGF to cell-surface EGF receptors, inasmuch as ASPC-1 cells do not markedly overexpress the receptor. This conclusion is supported by the observation that increasing the EGF concentration 100-fold in binding studies with T₃M₄ cells did not lead to the type of extensive degradation that was observed following binding with tracer amounts of TGF-α.

EGF was processed in T₃M₄, A431, and Rat-1 cells to a number of high molecular weight moieties. However, in the case of T₃M₄ and A431 cells, the majority of the radioactivity that was present in the incubation medium after a 180-min chase period consisted of high molecular weight products that were mainly represented by the pl 4.20 and 4.55 species. In contrast, in the case of Rat-1 fibroblasts, the majority of the radioactivity consisted of low molecular weight degradation products, and the preponderant high molecular weight species was the pl 4.2 moiety. Inasmuch as the pl 4.20 species, like authentic EGF (pl 4.55 species), readily bound and internalized in all three cell types, these observations suggest that the release of cell-bound EGF as either intact EGF or its pl 4.20 species is a more widespread phenomenon than previously suspected.

Of the four cell lines that were examined in the present study, T₃M₄ cells were the least efficient in degrading EGF. Although there was an avid and sustained internalization of ¹²⁵I-EGF in this cell line, several lines of evidence indicate that EGF was released between the intracellular compartments and the incubation medium. Internalized ¹²⁵I-EGF was readily released from the cells into the incubation medium, and the majority of this radioactivity consisted of intact or slightly processed EGF. The preponderant species of processed EGF that was released (pl 4.20) bound and internalized in T₃M₄ cells as extensively as authentic EGF. In the presence of competing unlabeled EGF during pulse-chase experiments, there was a marked increase in the amount of high molecular weight products in the incubation medium. The experiments with the Sykes-Moore culture chamber (Fig. 1C) indicate that this increase was primarily due to the inhibition of rebinding of dissociated EGF and its pl 4.2 derivative, rather than to a marked increase in the release of bound ¹²⁵I-EGF as a result of the presence of unlabeled EGF.

Following endocytosis, polypeptide ligands dissociate from their receptors in acidic intracellular organelles prior to being subsequently degraded (33). Methylamine, at a concentration of 10 mM, blocks lysosomal enzyme activity and thereby retards the degradation of many peptide ligands (42). This concentration of methylamine blocked the degradation of EGF in T₃M₄ and ASPC-1 cells as effectively as in A431 and Rat-1 cells and increased the relative proportion of authentic EGF in the incubation medium of all three cell lines in which EGF processing was examined. Furthermore, the kinetics of dissociation of bound EGF were altered by methylamine to a similar extent in T₃M₄ and Rat-1 cells. Taken together, these observations suggest that the degradation of EGF in T₃M₄ and ASPC-1 cells most likely occurred within cellular organelles that have an acidic internal milieu.

TGF-α was extensively degraded in T₃M₄ and ASPC-1 cells. Furthermore, in the case of T₃M₄ cells, radioactivity released into the incubation medium during pulse-chase experiments did not rebind to the cells. Therefore, it is unlikely that the attenuated degradation of EGF was due to some nonspecific inability of T₃M₄ and ASPC-1 cells to degrade peptide growth factors. It is also unlikely that the marked degradation of TGF-α was due to the inability of tracer amounts of the ligand to saturate cellular degradative pathways, inasmuch as increasing the ligand concentration 100-fold was not associated with a decrease in TGF-α degradation. However, it is con-
ceivable that the enhanced degradation of TGF-α was due to its ability to dissociate readily from the EGF receptor under relatively mild acidic conditions (Fig. 6).

Methyamine readily blocked the degradation of TGF-α in both T3M4 and ASPC-1 cells, indicating that the processing of this ligand was occurring within lysosomal or lysosome-like organelles. In contrast, methyamine fails to inhibit TGF-α degradation in RL95-2 cells (15). Therefore, in some cell lines TGF-α may also be processed in extra-lysosomal compartments. Taken together, these observations indicate that there is a marked divergence in the fate of EGF and TGF-α in a variety of cell lines and point to a difference in either the lysosomal regulation or the intracellular routing of the complexes formed between the EGF receptor and each of the two homologous growth factors.

The structural or molecular aspects of the EGF receptor protein that dictate the extent of ligand endocytosis and degradation following binding are not known. However, substitutions at lysine 721 within the ATP binding domain of their EGF receptors. TGF-α, at a concentration of TGF-α exhibited an attenuated ability by comparison to EGF receptor recycling. Furthermore, lower concentrations of TGF-α in T3M4 cells, was not as effective as EGF in decreasing EGF receptor half-life. Furthermore, lower concentrations of TGF-α exhibited an attenuated ability by comparison with EGF to induce the down-regulation of the surface EGF receptor. The failure of TGF-α to completely mimic these actions of EGF may be due to two reasons. First, the propensity of TGF-α to readily dissociate from the EGF receptor may allow the unoccupied receptor to recycle to the cell surface. Second, the recycling of EGF by T3M4 cells may allow a single EGF molecule to induce the internalization and degradation of more than one EGF receptor.

Irrespective of the underlying mechanisms, the effects of EGF and TGF-α on EGF receptor down-regulation were relatively specific, inasmuch as neither growth factor caused the down-regulation of the iGF-1 receptor. The present findings raise the possibility that the attenuated degradation of the EGF receptor by TGF-α represents a mechanism that spares cells from excessive autocrine degradation of this receptor. Conversely, the marked degradation of TGF-α may partly explain the discrepancies between TGF-α mRNA and protein levels in a variety of cells (13, 46). Additional reasons for these differences include the failure to release TGF-α (47, 48) and the production of variant TGF-α molecules that are not detected by available assays (6).

Acknowledgments—We thank Dr. Barry Komm for many helpful discussions during the course of this work and Jenny Barff for excellent technical assistance.

REFERENCES

1. Carpenter, G., and Cohen, S. (1979) Annu. Rev. Biochem. 48, 193-216
2. Downward, J., Yarden, Y., Mayes, E., Scrase, G., Totty, N., Stockwell, P., Ulrich, A., Schlessinger, J., and Waterfield, M. D. (1984) Nature 307, 521-527
3. Hunter, T. (1984) Nature 311, 414-416
4. Stoscheck, C. M., and Carpenter, G. (1984) J. Cell Biol. 98, 1048-1053
5. Marquardt, H., Hankapiller, M. W., Hood, L. E., and Todaro, G. J. (1984) Science 225, 1079-1082
6. Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y., and Goeddel, D. V. (1984) Cell 38, 287-297
7. Massagué, J. (1983) J. Biol. Chem. 258, 13614-13620
8. Ibbotson, K. J., Harrod, J., Gowen, M., D’Souza, S., Smith, D. D., Winkler, M. E., Derynck, R., and Mundy, G. R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 2229-2232
9. Schreiber, A. B., Winkler, M. E., and Derynck, R. (1986) Science 232, 1250-1253
10. Schultz, G. S., White, M., Mitchell, R., Brown, G., Lynch, J., Twardzik, D. R., and Todaro, G. J. (1987) Science 235, 350-352
11. Gan, B. S., Hollenberg, M. D., MacCannell, K. C., Lederis, K., Winkler, M. E., and Derynck, R. (1987) J. Pharm. Exp. Ther. 242, 331-337
12. Myrdal, S. E., Twardzik, D. R., and Auerberg, N. (1985) J. Cell Biol. 102, 1230-1234
13. Smith, J. J., Derynck, R., and Korc, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7567-7570
14. Barrandon, Y., and Green, H. (1987) Cell 50, 1131-1137
15. Korc, M., Hausler, C. A., and Troxman, N. S. (1987) Cancer Res. 47, 4909-4914
16. Siegfried, J. M. (1987) Cancer Res. 47, 2905-2910
17. Korc, M., Meltzer, P., and Trent, J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5141-5144
18. Gentry, L. E., and Lawton, A. (1986) Virology 152, 421-431
19. Okabe, T., Yamaguchi, N., and Ohsawa, N. (1983) Cancer 51, 662-668
20. Fabricant, R. N., Delarco, J. E., and Todaro, G. J. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 565-569
21. Frasad, I., Zouzias, D., and Basilico, C. (1976) J. Virol. 18, 436-444
22. Savage, C. R., Jr., and Cohen, S. (1972) J. Biol. Chem. 247, 5900-5911
23. Matrisian, L. M., Planck, S. R., and Magun, B. E. (1984) J. Biol. Chem. 259, 3047-3052
24. Winkler, M. E., Brngman, T., and Marks, B. J. (1986) J. Biol. Chem. 261, 13888-13843
25. Korc, M., and Magun, B. E. (1985) Cell Physiol. 124, 344-348
26. Haigler, H. T., Maxfield, F. R., Williams, M. C., and Pastan, I. (1980) J. Biol. Chem. 255, 1239-1241
27. Glenney, J. R., Jr., Chen, W. S., Lazar, C. S., Walton, G. M., Zoka, L. M., Rosenfeld, M. G., and Gill, G. N. (1988) Cell 52, 675-684
28. Rohrschreiber, L. R. (1979) Cell 16, 11-24
29. Kessler, S. W. (1975) J. Immunol. 115, 1617-1624
30. Laemmli, U. K. (1970) Nature 227, 680-685
31. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350-4354
32. Planck, S. R., Finch, J. S., and Magun, B. E. (1984) J. Biol. Chem. 259, 3053-3057
33. Hanover, J. A., Williams, M. C., and Pastan, I. (1984) Cell 39, 293-293
34. Soderquist, A. M., and Carpenter, G. (1984) J. Biol. Chem. 259, 12586-12594
35. Zidovetzki, R., Yarden, Y., Schlessinger, J., and Jovin, T. M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6831-6835
36. Pastan, I. H., and Williams, M. C. (1981) Science 214, 504-509
37. Korc, M., Matrisian, L. M., and Magun, B. E. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 461-465
Epidermal Growth Factor Binding by T3M4 Cells

38. Korc, M., and Magun, B. E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6172–6175
39. Gonnella, P. A., Siminoski, K., Murphy, R. A., and Neutra, M. R. (1987) J. Clin. Invest. 80, 22–32
40. Maratos-Flier, E., Kao, C-Y. Y., Verdin, E. M., and King, G. L. (1987) J. Cell Biol. 105, 1595–1601
41. Burwen, S. J., Barker, M. E., Goldman, I. S., Hradek, G. T., Raper, S. E., and Jones, A. L. (1984) J. Cell Biol. 99, 1259–1265
42. Maxfield, F. R. (1982) J. Cell Biol. 95, 676–681
43. Honegger, A. M., Dull, T. J., Felder, S., Obberghen, E. V., Bellot, F., Szapary, D., Schmidt, A., Ullrich, A., and Schlessinger, J. (1987) Cell 51, 199–209
44. Stoscheck, C. M., and Carpenter, G. (1984) J. Cell Physiol. 120, 296–402
45. Cooper, J. A., Scolnick, E. M., Ozanne, B., and Hunter, T. (1983) J. Virol. 48, 752–764
46. Derynck, R., Goeddel, D. V., Ullrich, A., Gutterman, J. U., Williams, R. D., Bringman, T. S., and Berger, W. H. (1987) Cancer Res. 47, 707–712
47. Wong, S. T., Winchell, L. F., McCune, B. K., Earp, H. S., Teixido, J., Massague, J., Herman, B., and Lee, D. C. (1989) Cell 56, 496–506
48. Brachmann, R., Lindquist, P. B., Nagashima, M., Kohr, W., Lipari, T., Napier, M., and Derynck, R. (1989) Cell 56, 691–700