CELL REGULATION, Vol. 2, 599–612, August 1991

Epidermal growth factor and transforming growth factor-α: differential intracellular routing and processing of ligand-receptor complexes

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Two structurally related but different polypeptide growth factors, epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), exert their activities after interaction with a common cell-surface EGF/TGF-α-receptor. Comparative studies of the effects of both ligands have established that TGF-α is more potent than EGF in a variety of biological systems. This observation is not explained by differences in affinities of the ligands for the receptor, because the affinity-constants of both factors are very similar. We have compared the intracellular processing of ligand-receptor complexes using either EGF or TGF-α in two different cell systems. We found that TGF-α dissociates from the EGF/TGF-α-receptor at much higher pH than EGF, which may reflect the substantial difference in the calculated isoelectric points. After internalization, the intracellular TGF-α is more rapidly cleared than EGF, and a substantial portion of the released TGF-α represents undegraded TGF-α in contrast to the mostly degraded EGF. In addition, TGF-α did not induce a complete down-regulation of cell surface receptors, as observed with EGF, which is at least in part responsible for a much sooner recovery of the ligand-binding ability after down-regulation, in the case of TGF-α. These differences in processing of the ligand-receptor complexes may explain why TGF-α exerts quantitatively higher activities than EGF.

Introduction

Cell proliferation, function, and differentiation are regulated in response to numerous soluble growth factors. Epidermal growth factor (EGF), one of the earliest known polypeptide growth factors, acts as a mitogen for cells of ectodermal and mesodermal origin (Carpenter and Cohen, 1979) and may play a role in wound healing and early development (Cohen, 1962; Moore *et al.*, 1984; Brown *et al.*, 1986). On ligand binding, the EGF-receptor complexes are clustered and rapidly internalized through the endosome system, resulting in degradation of both receptor and ligand within the lysosomal compartment (Schlessinger, 1986). Binding of EGF to its cell-surface receptor induces a program of intracellular events that is initiated by the ligand-induced activation of the intrinsic kinase activity of the receptor and leads to a mitogenic stimulation of the cell (Ullrich and Schlessinger, 1990).

Transforming growth factor-α (TGF-α), originally isolated from retrovirus-transformed cells (DeLarco and Todaro, 1978), also binds to the EGF receptor. It is now generally accepted that the TGF-α-induced activities are mediated through the interaction of TGF-α with the common EGF/TGF-α receptor and that there may not be a unique TGF-α specific receptor (De Larco and Todaro, 1980; Massagué, 1983). It has more recently become evident that there are several other structurally related factors that can interact with the EGF/TGF-α receptor. These include the virus-encoded vaccinia virus growth factor (Stroobant *et al.*, 1985), amphiregulin (Shoyab *et al.*, 1989; Plowman *et al.*, 1990), and a heparin-binding factor from macrophages (Higashiyama *et al.*, 1991). Very little is as yet known about the biological activities and sites of synthesis of the latter two factors.

TGF-α is synthesized not only by retrovirus-transformed cell lines but by a large variety of tumor cells. Indeed, TGF-α synthesis is consistently observed in squamous and renal carcinomas, which have a high expression level for the EGF/TGF-α receptors, and is also frequently found in other ectodermally derived tumors (Bates *et al.*, 1988; Derynck *et al.* 1987; Nistér *et al.*, 1988). Overexpression of TGF-α in im-

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mortalized cell lines may be sufficient to induce transformation and tumorigenicity, especially in the presence of a high level of EGF/TGF-α receptors (Rosenthal *et al.,* 1986; Di Fiore *et al.,* 1987; Di Marco *et al.,* 1989). This finding and the spontaneous occurrence in vivo of tumors in TGF-α overexpressing transgenic mice (Jhappan *et al.,* 1990; Matsui *et al.,* 1990; Sandgren *et al.,* 1990) reinforces the belief that endogenous expression of TGF-α may contribute to the transformed phenotype of tumor cells. On the other hand, TGF-α expression is certainly not restricted to tumor cells but also occurs during fetal development (Han *et al.,* 1987; Wilcox and Derynck, 1988a) and in normal cells in the fully developed mammal, such as in macrophages (Madtes *et al.,* 1988; Rappolee *et al.,* 1988a); in the brain (Wilcox and Derynck, 1988b) and the pituitary (Kobrin *et al.,* 1988); and many types of epithelial cells, including keratinocytes (Coffey *et al.,* 1987; Beauchamp *et al.,* 1989; Valverius *et al.,* 1989). All of these observations taken together lead to the conclusion that TGF-α is a normal physiological ligand for the EGF/TGF-α receptor, which may be of major importance in the proliferation of normal epithelial cells and could play a role in tumor development. In contrast, EGF does not have such a widespread synthesis and is known to be released only by cells in the salivary gland. There is also synthesis of EGF in spleen and kidney, but EGF remains incorporated in the large ectodomain of the transmembrane EGF precursor and is not released by the cells (Rall *et al.,* 1985). Thus, whereas there may be additional sites of EGF synthesis, it appears that TGF-α expression is far more common than EGF expression.

It is striking how little is known about the interaction of TGF-α with the receptor, because almost all studies on ligand binding and intracellular trafficking have used EGF. On the other hand, various studies have examined the biological effects of TGF-α in comparison with EGF. TGF-α induces quantitatively similar results as EGF in some assays, like stimulation of DNA-synthesis in several cell lines (Schreiber *et al.,* 1986), induction of anchorage-independent growth (Anzano *et al.,* 1983; Salomon *et al.,* 1987), induction of eyelid opening in newborn mice (Smith *et al.,* 1985), or inhibition of parathyroid hormone-responsive adenylate cyclase in osteoblasts (Guiterrez *et al.,* 1987). In contrast, TGF-α is clearly more active than EGF in many more test systems. For example, TGF-α is more potent than EGF in causing transient ruffling of the cell membrane (Myrdal *et al.,* 1986), an increased rate of migration and monolayer formation in keratinocytes (Barrandon and Green, 1987), induction of neovascularization (Schreiber *et al.,* 1986), and increased arterial blood flow (Gan *et al.,* 1987). TGF-α also induces calcium-release from bones in culture and bone resorption (Stern *et al.,* 1985) and inhibits bone formation in vitro (Ibbotson *et al.,* 1986) at much lower concentrations than EGF. Furthermore, TGF-α is a stronger inhibitor of proliferation of endometrial carcinoma cells (Korc *et al.,* 1987) and a more powerful promoter of hepatocyte growth (Brenner *et al.,* 1989). Thus TGF-α frequently functions as a superagonist of EGF. Finally, TGF-α and EGF have been reported to induce differential activities on the proliferation of primary lung carcinoma cells (Siegfried, 1987), suggesting the possibility that some differences may be qualitative. The latter finding together with the nature of quantitative differences in several assays and a variety of control experiments indicate that differences in stability cannot be sufficient cause for all the differences in response to TGF-α and EGF.

Various studies have compared the receptor binding of both ligands and have revealed that the affinity constants of TGF-α and EGF are very similar or identical (Massagué, 1983; Winkler *et al.,* 1986; Lax *et al.,* 1988). Thus the observed quantitative differences between both factors cannot be explained by different binding affinities. We have therefore further examined the interaction of EGF and TGF-α with the EGF/TGF-α-receptor, focusing on events subsequent to initial binding. Starting from the observation that both ligands have a major difference in pH-dependent dissociation of the ligand-receptor complex, possibly related to differences in isoelectric point values, we have found marked differences in intracellular routing and down-regulation of the receptor-ligand complexes and in length of the refractory period. These observations may provide a basis to explain the quantitative differences in biological potency between EGF and TGF-α.

Results

Receptor binding

We decided to evaluate the ligand-receptor interactions of both TGF-α and EGF in a "monospecific" system using only human ligands and the human EGF/TGF-α receptors. Such comparison, which has as yet not been described, has obviously more physiological relevance than any other studies using ligands and receptors from different species. Recombinant human EGF (53 amino acids) and TGF-α (50 amino ac-

ids) were used on murine B82 L cells expressing human EGF/TGF-α-receptors (Chen *et al.*, 1989). This transfected cell line was chosen because of its high number of a single, well-characterized EGF-receptor species in a background devoid of endogenous murine EGF receptors. Moreover, the cells do not synthesize EGF or TGF-α, unlike, for example, A431 cells, which also display a high receptor number but have endogenous TGF-α synthesis (Derynck *et al.*, 1987). At a later stage we used human keratinocytes with a less elevated endogenous expression level of EGF/TGF-α receptors.

Because of the quantitative differences in biological responses between TGF-α and EGF,

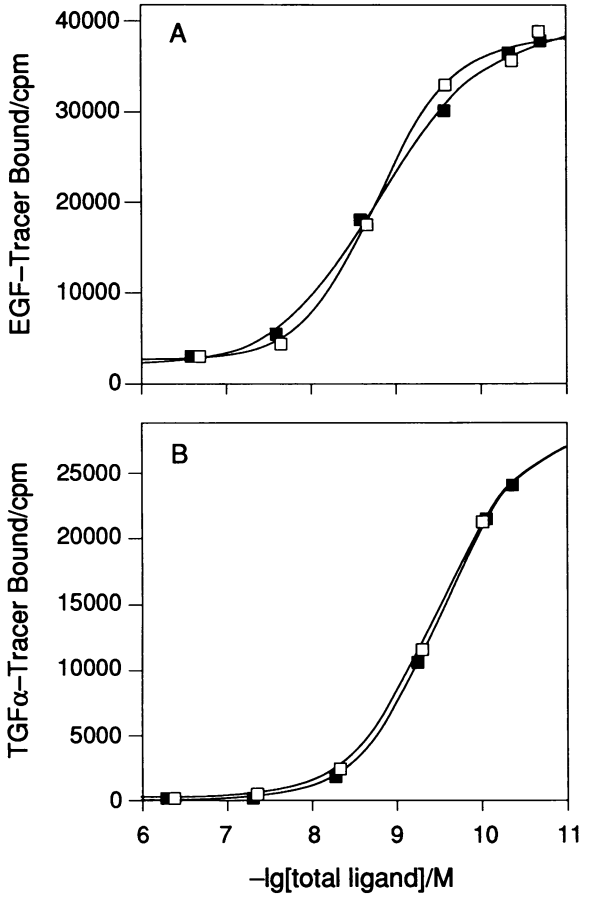

Figure 1. **Competitive inhibition of EGF and TGF-α binding.** Confluent 16-mm culture wells of B82 cells were washed and brought to equilibrium at 4°C with 21 pM ^{125}I-EGF (1251 Ci/mmol; A) or 45 pM ^{125}I-TGF-α (650 Ci/mmol; B) in the presence of increasing concentrations of cold EGF (□) or TGF-α (■). Total cell-bound cpm are averages of triplicate assays. Cold-ligand concentrations varied from 24 pM to 540 nM. Data for total cell-bound cpm are the average of triplicate determinations; divergence between triplicate values was 14% or less. Experiments were repeated twice with similar results.

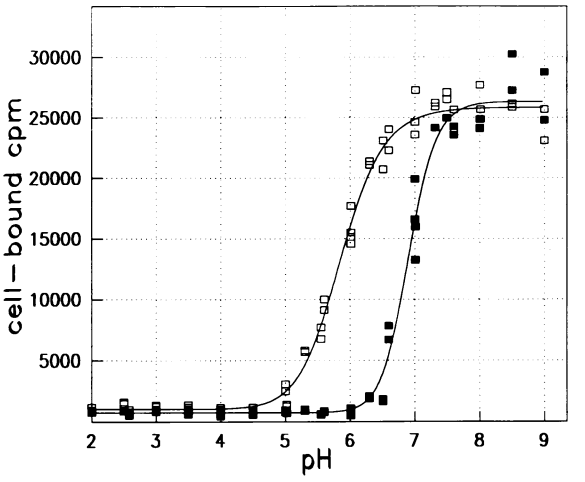

Figure 2. **Acid-dissociation of bound EGF and TGF-α.** Cell monolayers in 35-mm culture wells were brought to equilibrium with 214 pg/ml ^{125}I-EGF or 209 pg/ml ^{125}I-TGF-α (130 μCi/μg), washed three times with binding buffer, and then twice for 5 min with 0.5 ml/well of HEPES buffers at the various pH values indicated. Remaining cell-associated radioactivity was determined after lysis from the wells with NaOH/SDS. Each point represents the cpm from an individual well.

we first evaluated whether this could be due to differences in the affinity of either ligand for the receptor. Such studies had been done before, but, to our knowledge, not using ligands and receptors of the same species. We thus measured specific binding capacities of both ligands for the EGF/TGF-α receptors at the surface of the transfected cells. As it proved difficult to obtain radiolabeled EGF and TGF-α of identical specific activities, we performed competitive inhibition experiments in which we displaced constant amounts of ^{125}I-labeled ligand with identical molar concentrations of either unlabeled ligand. Figure 1 shows that competition of radioiodinated EGF and TGF-α by either unlabeled factor displays identical concentration curves. The determination of binding isotherms from measurements of the saturation of specific binding using varying concentrations of radiolabeled growth factors yielded dissociation constant (K_D) values of 2.6 nM for EGF and 2.2 nM for TGF-α. The receptor numbers were calculated to be 7–9 × 10^5/cell. All our estimates were based on a one-site receptor binding model. Discrete populations of high- and low-affinity sites for EGF have been reported (Massagué, 1983); yet, due to a lack of information on the number of receptor species present, we did not discriminate between multiple affinity classes. Thus, in confirmation of previous es-

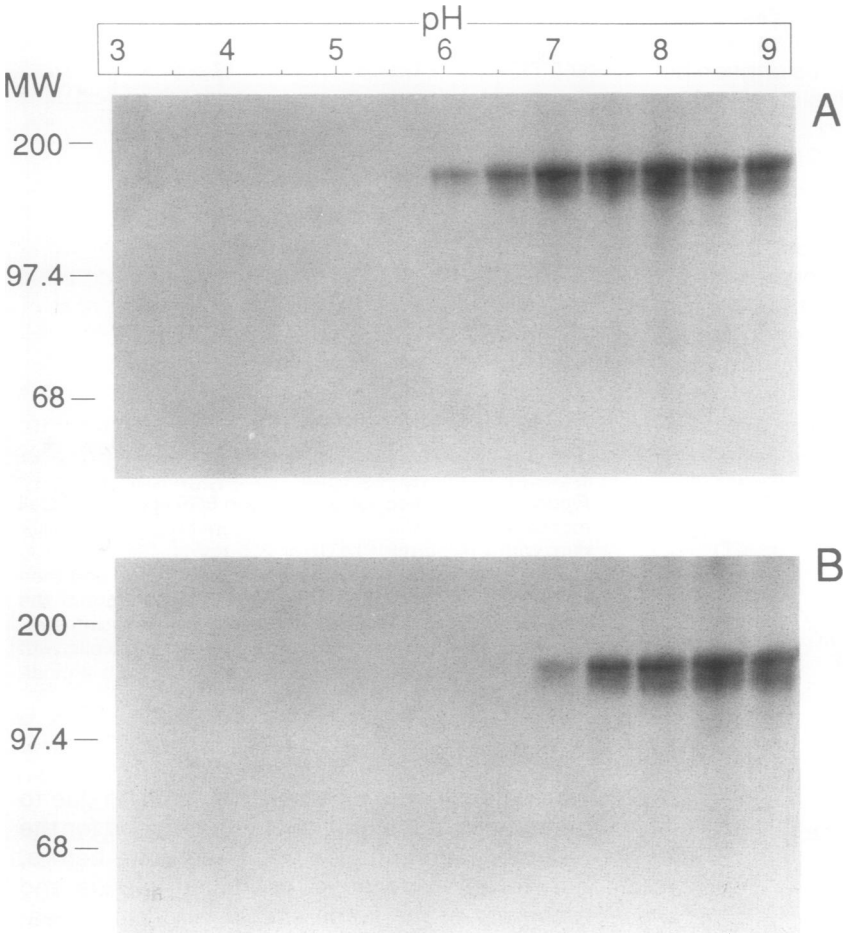

Figure 3. **Affinity-labeling of surface-receptors after ligand stripping.** ^{125}I-EGF or -TGF-α were bound to cells and the cells were washed with HEPES buffers of different pH values as described for Figure 2. After subsequent washes with neutral buffer, the cells were incubated with 0.15 mM disuccinimidylsuberate in PBS for 15 min, washed again, and scraped. Covalently cross-linked proteins were visualized by reducing SDS-PAGE of the solubilized cells and autoradiography. The position of the EGF/TGF-α-receptor (170 kDa) is marked by an arrow.

timates, there is no major difference in affinity constants of EGF and TGF-α for the receptor.

Stability of ligand-receptor complexes

Because the quantitative differences in response between EGF and TGF-α cannot be explained by differences in affinity constants, we evaluated several other aspects of the ligand-receptor interactions. Calculations of the isoelectric point (pI) values of both ligands revealed a major difference: EGF has a pI of 4.6, whereas the pI of TGF-α is 5.9. This suggested that TGF-α may dissociate from the receptor at a higher pH than EGF, in accordance with the differences in pI values. We thus saturated the EGF/TGF-α receptor overexpressing cells with radiolabeled ligand at 0°C and then exposed the cells to washes with buffers of gradually decreasing pH. We used buffers of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.5 M acetic acid, 0.5 M NaCl, adjusted to the desired pH using NaOH, instead of the more commonly used acetate-acetic acid buffers (Haigler *et al.*, 1980; Glenney *et al.*, 1988), because of the low-buffering capacity of the latter buffers at the higher pH values. Figure 2 shows that TGF-α dissociates from the receptor at much higher pH than EGF, with half-maximal dissociation values of pH 6.9 for TGF-α and pH 5.6 for EGF. In addition, the slope of the dissociation curve is slightly steeper for TGF-α than for EGF. TGF-α binding was also more sensitive to alkaline conditions than EGF binding (data not shown), which may be of little physiological relevance.

Because the measurements in Figure 2 were made in the cold, virtually all radiolabel associated with the cells should reflect receptor binding sites at the cell surface. This was confirmed by affinity-cross-linking of radiolabeled ligands to cells using the cross-linker disuccinimidylsuberate (Figure 3). Gel electrophoresis under reducing conditions revealed that, as in the previous experiment, there is no detectable receptor-TGF-α interaction below pH 7,

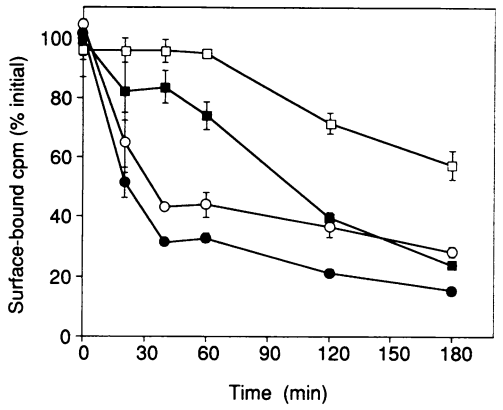

***Figure 4.* Off-rates in the presence of phenylarsinoxide.** B82-cells were brought to equilibrium at 4°C with 0.4 μg/ml of ^{125}I-EGF(○, □) or TGF-α (●, ■), washed, and incubated at 37°C without ligand in the medium. Specific ligand off-rates were determined over a 3-h period by measuring the radioactivity from the acid-strippable ("surface-bound") fraction of the washed cells in the absence (circles) or presence (squares) of 0.15 mM phenylarsinoxide.

whereas receptor-EGF complexes remain detectable down to pH 5.5.

We also evaluated the stability of the receptor-ligand interactions under physiological conditions. To prevent internalization and subsequent intracellular processing of receptor-ligand complexes, which occurs readily under these conditions, we incubated the cells in the presence of phenylarsinoxide, a potent inhibitor of trafficking of membrane proteins (Kaplan *et al.*, 1985). We thus saturated the cells with radiolabeled ligand in the presence and absence of phenylarsinoxide and monitored the loss of ligands from the cells in neutral medium at 37°C. TGF-α dissociated from the surface of the phenylarsinoxide-treated cells more rapidly than EGF. After 3 h, the remaining cell-associated TGF-α was only 20% of the originally bound ligand, compared with 60% for EGF (Figure 4). In the absence of phenylarsinoxide, the ligand disappears from the cell surface as a result of internalization and degradation (discussed below).

Ligand traffic

After initial binding of EGF, occupied receptors are clustered and receptor-ligand complexes are internalized by endocytosis. We thus compared the time course of internalization of both EGF and TGF-α at 37°C as well as at room temperature, in the presence and absence of monensin. Incubation at room temperature causes intracellular enrichment of ligand due to an inhibition of resurfacing (Felder *et al.*, 1990). Monensin has been shown to inhibit recycling of several receptors (Basu *et al.*, 1981), including the EGF/TGF-α receptor (Gladhaug and Christofferson, 1988), although it can have various other activities as well and is known to inhibit lysosomal targeting. Both ligands followed similar internalization kinetics at 37°C and room temperature (Figure 5). However, repeated experiments consistently showed that TGF-α is faster released from the cells than EGF after the initial internalization. The ligands remained internalized in the presence of monensin.

We also performed chase experiments in which we monitored cell surface and internalized ligand, as well as resurfaced radioactivity. The latter was separated by trichloroacetic acid (TCA) precipitation into undegraded resurfaced factor and TCA-soluble, degraded peptides (Figure 6). As in previous experiments, the internalization of the ligands was followed by a clearance from the cell, whereby TGF-α was removed faster than EGF. The level of degraded peptides released from cells was similar, although a slightly higher initial rate of degradation of TGF-α seen was apparent in several ex-

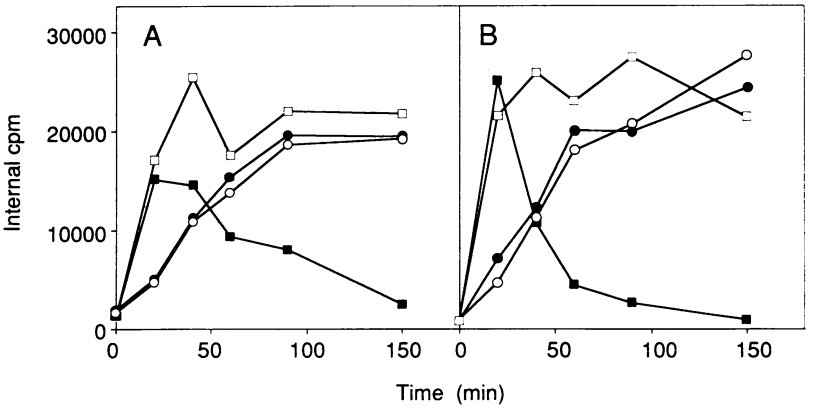

***Figure 5.* Effects of temperature and monensin on ligand internalization.** B82 cells were preincubated with ^{125}I-EGF (A) or -TGF-α (B) for 6 h at 4°C. After removal of unbound radiolabeled ligand, the temperature was shifted to 20°C (circles) or 37°C (squares), in the presence (○, □) or absence (●, ■) of 100 μM monensin, and incubation proceeded for the times indicated. Internalized cpm were determined after acid-stripping the cells at pH 2.5.

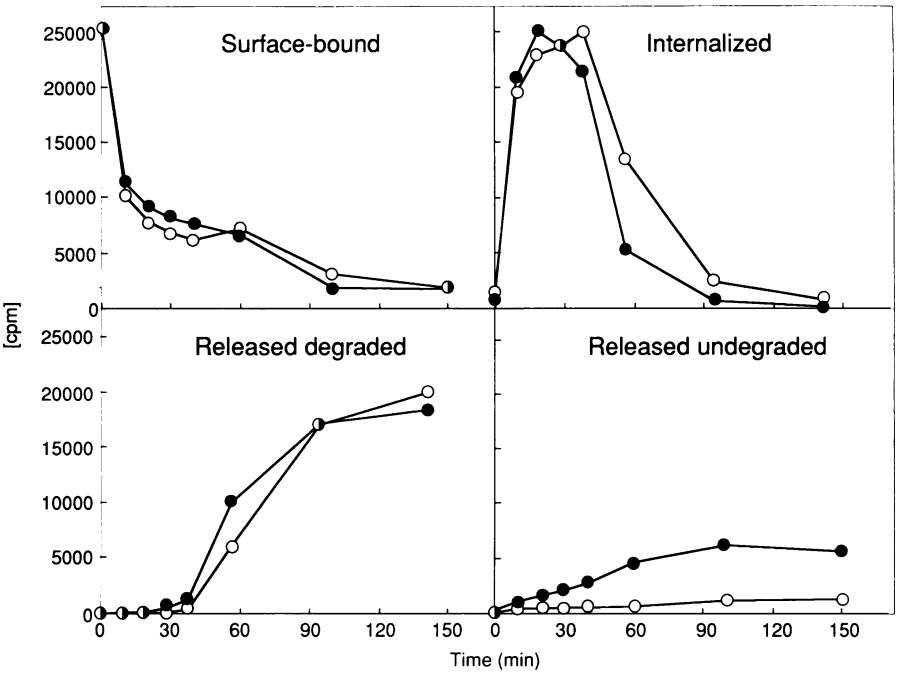

***Figure 6.* Chase of surface-bound ligands.** Confluent cells in 35-mm wells were brought to equilibrium at 4°C with 300 ng/ml of ^{125}I-EGF (○) or -TGF-α (●), washed, and then incubated without additional ligand at 37°C for different times. At these time points, the TCA-precipitable ("released undegraded") and TCA-soluble ("released degraded") cpm in the medium, as well as the cpm stripped from the cells at pH 2.5 ("surface-bound") and the remaining cpm in the cells solubilized with 0.5 M NaOH/1%SDS ("internalized"), were measured. Data shown are averages of triplicate determinations. The divergence between triplicate values was 15% or less.

periments. It is striking that, whereas hardly any undegraded EGF is released into the medium, a significant portion of undegraded TGF-α is returned to the medium (Figure 6). This finding does not agree with previously published data (Korc and Finman, 1989) from pancreas carcinoma cells.

In previous experiments, the continuous internalization of the ligand from the cell surface may have drastically influenced the levels of radioactivity in the fractions monitored. To minimize this interference, we carried out a chase experiment in which the fate of only the internalized factors was monitored. Cells were surface-saturated with radiolabeled ligand, but then incubated at room temperature to allow ligand internalization (Felder *et al.*, 1990) before incubation at 37°C and analysis as in Figure 6. It is again obvious that TGF-α is more rapidly cleared from the cell interior than EGF and that a considerable fraction of undegraded TGF-α is released in contrast to only a minimal level of undegraded EGF (Figure 7).

Down-regulation and refractory period

To further analyze the movement of ligands and receptors after initial ligand-receptor binding, we monitored the down-regulation of available cell-surface receptors. After treatment of cells with an excess of unlabeled ligand, we monitored receptor down-regulation by measuring the time-dependent disappearance of binding sites from the cell surface. The bound unlabeled ligands were removed at pH 4 rather than the often used pH 2.5 to fully preserve the ability of the cell to rebind ligand. This procedure was sufficient to remove 85–95% of bound tracer (data not shown). Figure 8 shows that EGF induces a virtually complete down-regulation of the receptors, but that the TGF-α induced down-regulation is markedly less. Monensin does not change EGF-induced down-regulation, indicating that there is only a minimal level of recycling of the receptor after internalization of the receptor-ligand complex. In contrast, monensin enhances the TGF-α induced internalization to the level of the EGF-induced internalization, indicating that receptor recycling is at least in part responsible for the lower level of down-regulation caused by TGF-α. There was no difference whether ^{125}I-EGF or -TGF-α was used to determine residual binding sites.

EGF-mediated down-regulation of the receptors is known to result in a temporary unresponsiveness to additional ligand due to an unavailability of cell surface receptors. After this refractory period, the cells return to their initial state of responsiveness, by recycling of undegraded receptors and/or new synthesis. We compared the length of the refractory period after down-regulation of the receptor by either ligand (Figure 9). Unlike the experiment in Figure 8, we did not remove the unlabeled ligands used

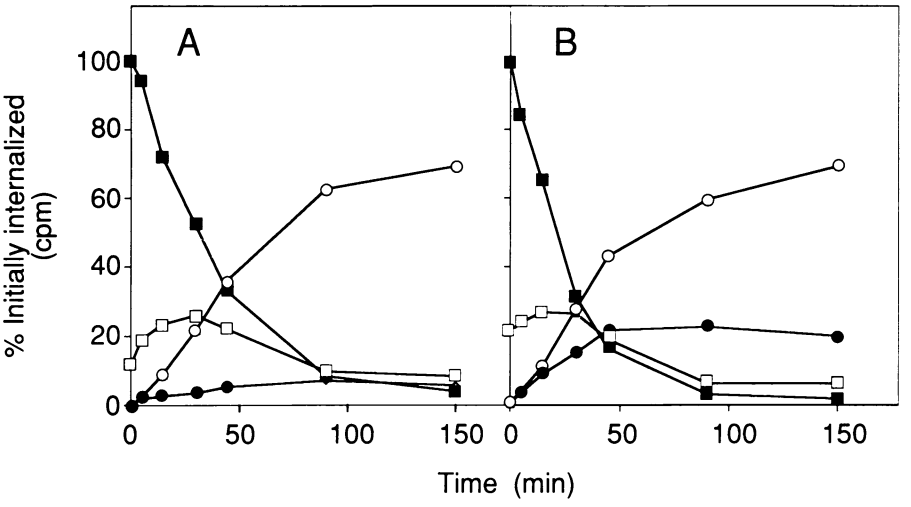

Figure 7. **Chase of internalized ligands.** Cells were preincubated with ^{125}I-EGF (A) or -TGF-α (B) for 2 h at 4°C and then for 2 h at room temperature. After removal of unbound ligand and acid-stripping of surface-associated tracer and subsequent washing, the incubation proceeded at 37°C for the times indicated. Assignment of radiolabel to the four experimental compartments—surface (□), internal (■), released degraded (○), and released undegraded (●)—was performed as in Figure 6.

for pretreatment by acid-stripping, but allowed for their degradation by incubation at 37°C in full growth media (Carpenter and Cohen, 1976). The refractory period, defined as the time span necessary to regain the binding capacity of the untreated control at time zero, was 2.5 h in the case of TGF-α compared with 5 h for EGF. We do not know whether this major difference is a mere consequence of the differential down-regulation or whether there are additional mechanistic differences involved in the recovery, especially because the rates of recovery differ only slightly. However, the recovery after EGF treatment was somewhat more sensitive to cycloheximide, an inhibitor of protein synthesis, suggesting that more new protein synthesis is required in the case of EGF.

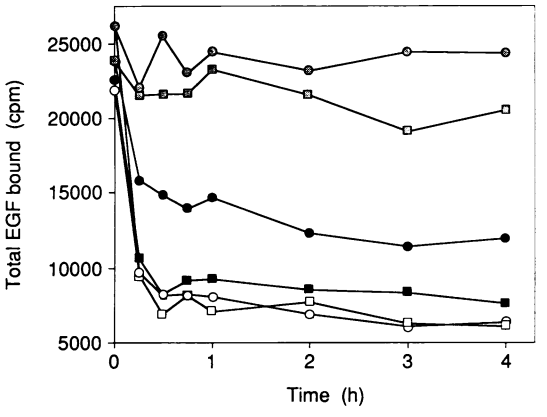

Figure 8. **Down-regulation of EGF-receptors.** B82 cells were incubated in binding medium for 4 h at 37°C. At the indicated times before the end of this incubation, 0.2 μM unlabeled EGF (○, □) or TGF-α (●, ■) were added in the absence (circles) or presence (squares) of 0.3 mM monensin. Gray symbols represent values for the controls not pretreated with either factor. After acid-stripping (pH 4) and washing monolayers in the cold, binding capacities for ^{125}I-TGF-α (not shown) or ^{125}I-EGF (1160 Ci/mmol) were determined. Counts per minute shown are means of triplicate measurements.

Ligand traffic in keratinocytes

Recombinant cells overexpressing a transfected receptor gene are often used to evaluate binding

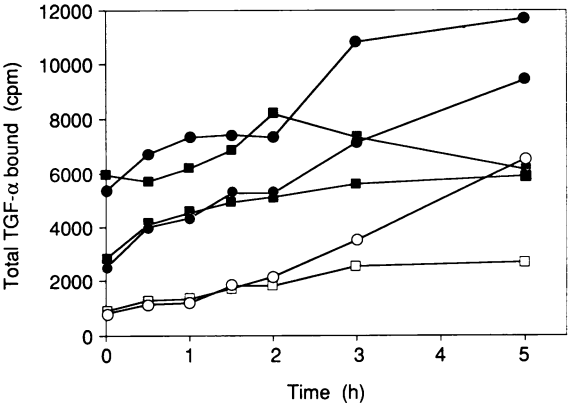

Figure 9. **Recovery of binding capacity after down-regulation.** An excess of unlabeled EGF (○, □; 1.5 μg/ml) or TGF-α (●, ■; 1.5 μg/ml) or no growth factor (gray symbols) was added to triplicate confluent culture wells in binding medium (no serum) at 37°C in a 5% CO_2 atmosphere. After 3 h, the cells were washed to remove unbound ligand and reincubated for another 3 h in serum-free binding buffer to allow degradation of the bound factors. The buffer was then replaced at the 0 h time point with serum-containing growth medium with (squares) or without (circles) cycloheximide (30 μg/ml). At the times indicated, triplicate wells from each treatment were washed and assayed for their capacity to bind ^{125}I-EGF (not shown) or ^{125}I-TGF-α (812 Ci/mmol). The cycloheximide-sensitive increase in binding capacity of the controls (no growth factor added) is presumably a consequence of enhanced protein synthesis after refeeding the washed cells in serum-containing growth medium.

characteristics of ligands and receptors. On the other hand, their high receptor number may result in anomalies in processing of receptor-ligand complexes and in cellular responsiveness (Wiley *et al.*, 1989). We therefore examined the processing of both EGF and TGF-α in cells with a normal level of EGF/TGF-α receptors, i.e., in normal human HFK1321 keratinocytes. Determination of binding isotherms by Scatchard-analysis revealed K_D values of 3.62 for EGF and 3.67 for TGF-α with a receptor number of 0.6–1.0 $\times$ 10^5 sites/cell, roughly an order of magnitude less than the B82 cells used above.

We repeated a number of experiments shown above, but now using the keratinocytes. In a chase experiment of surface-bound ligand (Figure 10), the difference between both ligands in loss of factor from the cell interior is even more obvious than with the B82 cells (Figure 6). Not even one-half of the internalized EGF is released after 2 h, but almost 90% of the internal TGF-α is cleared. And again, significantly more TGF-α is released in an undegraded state than EGF. With respect to the ligand-mediated down-regulation, we observed an overall lower level of residual sites than with the B82 cells, presumably due to the lower receptor number (Figure 11A). However, TGF-α also caused in these cells a much weaker down-regulation, and the cells recovered sooner from the refractory period (Figure 11B). Thus the keratinocytes behaved similarly to the B82 cells and showed in some cases even more outspoken differences than the B82 cells.

Discussion

In this study we have compared EGF and TGF-α for their interactions with the common EGF/TGF-α receptor using a "monospecific" system consisting of human ligands and the human receptor. Our current knowledge on the sites of synthesis of both ligands indicates that TGF-α is a widely expressed ligand in solid tumors and in many normal cells, especially epithelial cells, and should thus be considered as a normal physiological ligand for the receptor. In contrast, EGF is expressed at only few selected sites. It is thus striking in retrospect that EGF, and not TGF-α, has been widely used as ligand to study the ligand-receptor interactions and the subsequent signal transduction and modulation of gene expression (Ullrich and Schlessinger, 1990). This is obviously a consequence of the much earlier discovery and the wide availability of EGF.

A variety of studies have compared biological activities of TGF-α and EGF in a diversity of assay systems. As outlined in the introduction, TGF-α and EGF share most if not all biological actions, but TGF-α is often a more potent agonist than EGF. In some cases, both ligands induce quantitatively similar cellular responses, but TGF-α is more potent than EGF, whereas in other cases the TGF-α-induced response is stronger. Interestingly, there are also differences in the time course of action. TGF-α has been reported to cause effects of greater duration (Myrdal *et al.*, 1986) and, in another case,

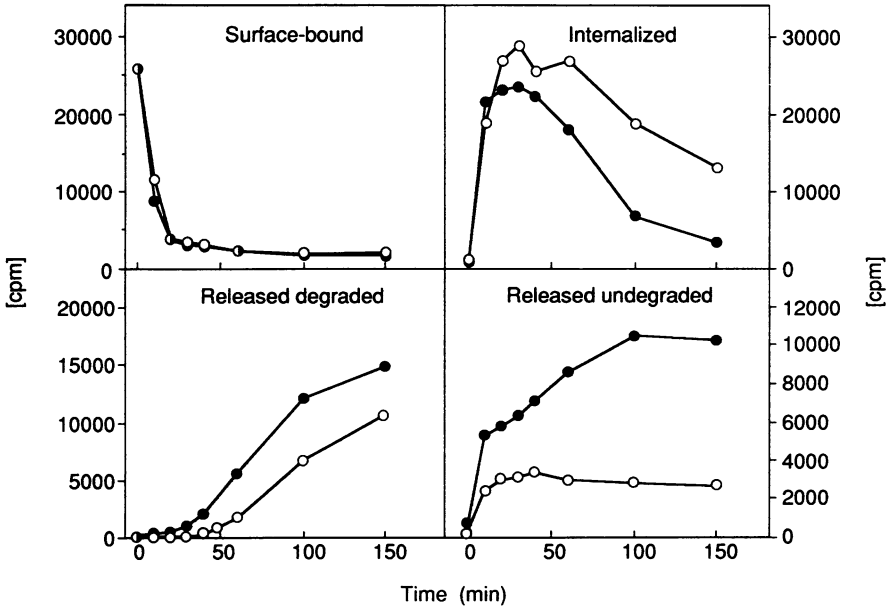

***Figure 10.* Chase of surface-bound ligands in keratinocytes.** HFK1321 keratinocytes (10^6/well) in triplicate were used. Binding and monitoring of radioactivity in the four fractions and the symbols are as described in Figure 6.

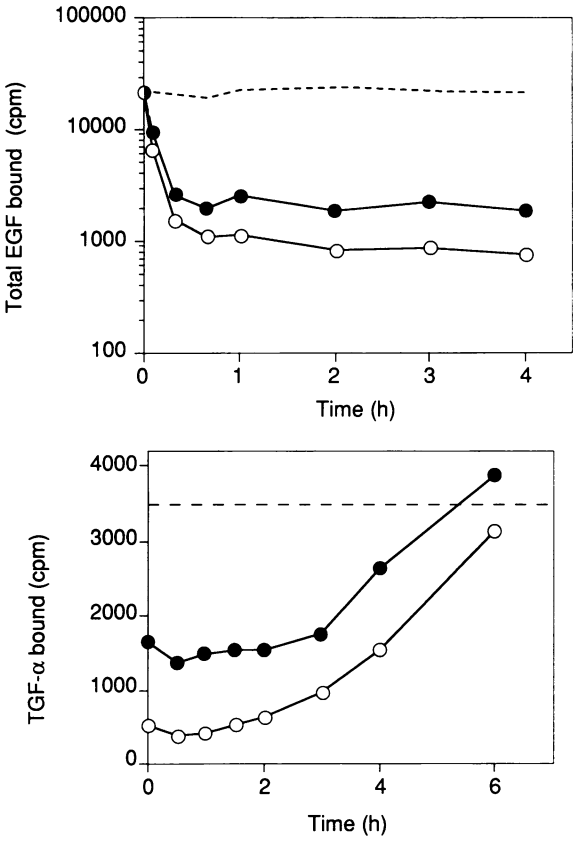

Figure 11. **Down-regulation of and recovery of surface-receptors in keratinocytes.** (A) Confluent monolayers of HFK1321 were treated with excess unlabeled EGF (○) or TGF-α (●) or were incubated without growth factor (---) for the times indicated. The cell surface binding capacities were assayed after acid-stripping and washing as described for Figure 7. Data shown are averages of triplicate determinations. (B) Down-regulation of the receptors with 30 μg/ml cold EGF (○) or TGF-α (●) was followed by removal of unbound factor and degradation of the bound factor. The recovery and assay for binding capacity were done as described for Figure 8. The value of binding without pretreatment with cold ligand is indicated by the dashed line.

does not induce a refractory period as EGF does (Gan *et al.*, 1987). In contrast to a previous report (Massagué *et al.*, 1982), it is now considered unlikely that these differences are due to interactions of TGF-α with a specific receptor, different from the common EGF/TGF-α receptor. However, the existence of a specific TGF-α receptor has not been ruled out.

Different potencies of analogous ligands are traditionally ascribed to different affinities for the receptor. However, several other mechanistic aspects could also provide a basis for differences in potencies between ligands that interact with a common receptor. These include 1) differential stabilities of the growth factor in the assay system, 2) differences in the modulation of the internalization rate, 3) differential receptor-ligand traffic after internalization (down-regulation, degradation vs. reuse of the ligand), 4) differential regulation of receptor synthesis or degradation, 5) differences in adaptation of the target cell, and 6) differential auto-induction of ligand synthesis. It is clear that differences in affinity cannot explain the quantitative differences in biological activities, because TGF-α and EGF bind to the receptor with a similar K_D. This was observed not only in our system (Figure 1), but also in published work (Lax *et al.*, 1988). In addition, both ligands induce receptor autophosphorylation at similar concentrations (Lax *et al.*, 1988).

The emphasis of our comparison between EGF and TGF-α was on events occurring after initial ligand-receptor binding at the cell surface. We have observed that EGF has a pI of 4.6, whereas the pI of TGF-α is 5.9, and that there is a major difference in pH-dependent dissociation of the ligand-receptor complex. A difference in pH sensitivity was suggested before (Massagué, 1983; Korc and Finman, 1989). This finding implies that, during endocytosis and concomitant acidification of the endosomes, TGF-α is released from the receptor much sooner than EGF. The former ligand will thus dissociate from the receptor very shortly after internalization, whereas EGF dissociates from the receptor only in lysosomes or in a prelysosomal compartment. We also observed other differences between both ligands. At the neutral physiological pH, TGF-α dissociates more rapidly and to a much larger extent than EGF (Figure 4). Also, TGF-α, although internalized at about the same rate as EGF, is released from the cell more rapidly than EGF. In addition, a significant release from cells of undegraded TGF-α is in contrast to undetectable levels of undegraded EGF. Perhaps the most important difference is that TGF-α does not induce a virtually complete down-regulation of cell surface receptors, as observed with EGF, due to the monensin-sensitive recycling of the receptor to the cell surface (Figures 8 and 11). This difference in down-regulation is at least in part responsible for the major difference between EGF and TGF-α in the time needed for full recovery of the original ligand-binding ability. This period is considerably shorter for TGF-α than for EGF (Figures 9 and 11). Thus the cell retains the ability to interact with TGF-α at any time, whereas this is not the case for EGF, due to a complete down-regulation of the receptors and a long refractory period.

Based on our current knowledge on the interactions of EGF with the receptor and the data presented here, we propose that there are several differences in the processing of the receptor-ligand complex. These are schematically represented in Figure 12. After interaction of EGF with the receptors, the clustered receptor-ligand complexes are internalized and remain associated in the gradually acidifying endosomes. The large majority of these complexes are then targeted toward the prelysosomal and lysosomal compartments in which receptor and ligand are degraded. As a consequence, the receptor down-regulation results in a virtual absence of cell surface receptors, thus leading to a greatly diminished responsiveness or refractory period. After removal of the ligand, the level of cell surface receptors is increased as a result of new synthesis of the receptors and the recycling to the cell surface of the fraction of undegraded receptors, eventually returning the cell to a fully responsive state. The scenario of the TGF-α interaction with the receptor is somewhat different. After internalization of the clustered receptor-ligand complexes, the ligands and receptors dissociate in the acidifying vesicles. Only a fraction of the vesicles are targeted to the lysosomes, resulting in degradation of ligands and receptors. The other fraction recycles to the cell surface, resulting in immediate resurfacing of newly available receptors and in a release of undegraded ligand into the medium. It is not known whether the dissociation of the ligand receptor complex is the signal for the recycling to the cell surface. The lower frequency of targeting of receptors to lysosomes should result in a lower level of receptor degradation compared with EGF, as observed in a pancreatic carcinoma cell line (Korc and Finman, 1989). Because of this difference in routing, the time required to clear the ligand from cells is shorter than for EGF, and there is no complete down-regulation of receptors as seen with EGF. The incomplete down-regulation results in the continuous availability of a fraction of cell surface receptors and in only a short refractory period. It should be pointed out that treatment of the cell with ligand may result in an increase in synthesis of the EGF/TGF-α receptor.

It is possible to explain many of the observed quantitative differences in biological responses between TGF-α and EGF as a consequence of this differential processing of ligand-receptor complexes. The complete receptor down-regulation by EGF and the incomplete down-regulation by TGF-α are consistent with and may explain the observation that TGF-α does not induce a refractory period of responsiveness, whereas EGF does, as described for the effects of both growth factors on arterial blood flow (Gan *et al.,* 1987). The induction of a refractory period by EGF and the lack of a refractory period and a resulting continuous responsiveness in the presence of TGF-α may also be the basis of observations that lower levels of TGF-α than of EGF are needed to obtain quantitatively similar biological effects. The need for a continuous stimulation by the growth factor to induce various responses, together with the above mentioned differences in continuous cellular responsiveness, may also explain how TGF-α can induce a higher maximum response than EGF. This difference is perhaps most apparent in the ability of TGF-α to induce larger anchorage-dependent keratinocyte colonies than EGF (Barrandon and Green, 1987). Thus the pronounced

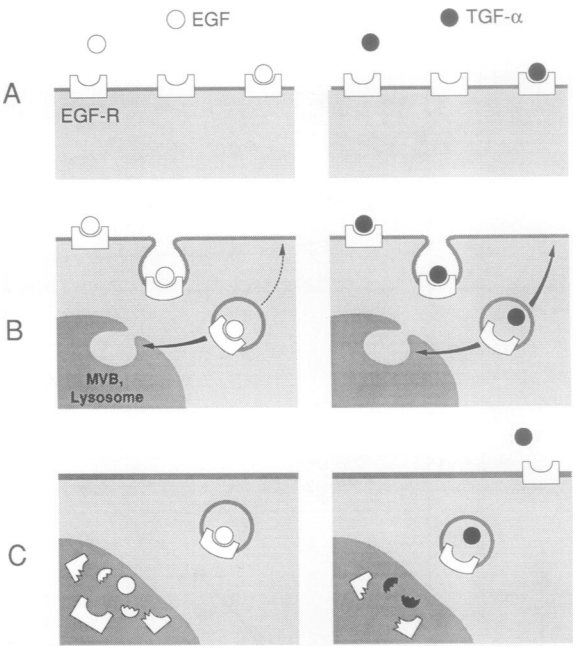

Figure 12. **Schematic diagram of the differential intracellular routing pathways followed by receptor-complexes of EGF or TGF-α.** External EGF (left) and TGF-α (right) bind equally well to EGF-receptors (EGF-R) on the surface of responsive target cells (A). It is proposed that, after internalization, ligand-receptor complexes enter one of two basic pathways (B): 1) Rapid resurfacing of intact receptor together with undegraded ligand, possibly triggered by dissociation of the latter from the membrane receptor in the gradually acidified endocytotic vesicle and 2) sequestering into the luminal space of the multivesicular body (MVB) and degradation after fusion with or maturation to a lysosomal vacuole. Although most of the bound EGF ends up degraded, a more significant portion of TGF-α-containing vesicles is recycled to the cell surface along with their receptors, thereby maintaining responsiveness to further signaling (C).

down-regulation of receptors by EGF may be the basis for an attenuated response to EGF in comparison with the TGF-α-induced response. This is reminiscent of the experiments of Chen *et al.* (1989) and Wells *et al.*, (1990), who showed that exposure at the cell surface of an internalization-defective receptor mutant to EGF induces a stronger biological response than the internalized and down-regulated normal receptor. Thus receptor down-regulation provides a mechanism to obtain an attenuated response.

This continuous responsiveness of a cell to TGF-α, and not EGF, may also have relevance for the physiological role of TGF-α. It is conceivable that tumor cells or other cells that have a constitutive synthesis of TGF-α and receptor are continuously subject to autocrine mitogenic stimulation by TGF-α, which would not be achieved to the same extent if EGF were the produced ligand. The differences between EGF and TGF-α, described in this report, may even be the basis for the observation that EGF can have growth inhibitory effects on selected cell types. EGF has the ability to inhibit some types of epithelial cells (Vonderhaar, 1987; Coleman and Daniel, 1990), which are now known to constitutively secrete TGF-α (Derynck, 1988). Addition of EGF would then result in a complete down-regulation of the receptors and prevent the cells from being constitutively stimulated in an autocrine fashion by the TGF-α. This could also explain in part the inhibition by EGF of the proliferation of squamous and renal carcinoma cells with high EGF/TGF-α receptor levels (Kawamoto *et al.*, 1983). These cell types synthesize TGF-α, making it probable that there is a continuous autocrine stimulation of these cells by TGF-α. This stimulation could then be attenuated by exogenous EGF administration. Further work is obviously needed to evaluate and understand the physiological consequences of the differences in receptor-ligand processing between EGF and TGF-α. These studies may provide insight into the differential physiological roles of both ligands.

Materials and methods

Cell lines and growth conditions

B82K murine L cells expressing human wild-type EGF-receptors were obtained from G. Gill (University of California-San Diego) and were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) with 10% dialyzed calf serum supplemented with antibiotics and 2 mM methotrexate to select for maintenance of the transfected phenotype. Immortalized human HFK1321 keratinocytes (Münger *et al.*, 1989) were obtained from P. Howley (National Cancer Institute, National Institutes of Health, Bethesda, MD) and grown in keratinocyte growth medium (Clonetics, San Diego, CA). After trypsinization, proteases were inhibited by centrifuging the cells through a layer of 10% serum-containing Eagle's minimum essential medium. Cells were routinely propagated in monolayer culture at 37°C in a 5% CO_2-95% humidified air atmosphere.

Chemicals

Recombinant human EGF was from Boehringer Mannheim (Indianapolis, IN) and recombinant human TGF-α from Genentech, Inc. (San Francisco, CA). ^{125}I-human TGF-α and ^{125}I-murine or human EGF were radioiodinated using a slightly modified chloramine T-method (Frolik *et al.*, 1984) or purchased from Amersham (Arlington Heights, IL). Monensin, methylamine, and cycloheximide (actidione) were from Serva Biochemicals (Westbury, NY). Phenylarsinoxide was from Sigma (St. Louis, MO). Disuccinimidylsuberate was purchased from Pierce Chemical (Rockford, IL).

Binding assays

Monolayers grown to confluency in plastic wells were in most cases changed to serum-free medium about 12 h before experiments. Cells were then washed in binding medium (high glucose DMEM, 20 mM HEPES pH 7.2, and 0.2% bovine serum albumin [BSA]) and incubated in the same medium with the indicated amounts of inhibitors and unlabeled or radiolabeled ligands. During incubations, the wells were slowly shaken on a rocker platform. To reach equilibrium conditions, incubation proceeded for at least 4 h at 4°C. After removal of unbound ligand in the medium, washing the cells several times in binding medium, lysing the monolayers in 0.5 M NaOH/1% sodium dodecyl sulfate (SDS) (>1 h/37°C) and transfer into 5-ml plexiglass tubes, the total cell-bound radioactivity was determined in a γ-counter.

Acid stripping of surface-bound tracers was done by two subsequent 5-min incubations on ice with 5 mM acetic acid, 135 mM NaCl, 2.5 mM KCl, pH 2.5 (Glenney *et al.*, 1988), which in all cases removed over 95% of all radiolabeled ligand bound in the cold. However, the washes were performed at pH 4 if newly added tracers had to bind after surface stripping (down-regulation experiments).

Chase experiments

Cells were allowed to bind radiolabeled ligands at 4°C and incubated further at the temperatures indicated. At the end of incubations, the cells were washed three times with binding medium. Surface-bound ligand was measured after the collection of two pH 2.5 washes. The quantity of internal ligand was determined by NaOH/SDS-lysis as described above.

To measure the extent of tracer degradation, incubation media were removed from the wells and diluted with ice-cold TCA (to 10% final). Carrier BSA (1 mg/ml) was added and precipitation of the mixed suspensions proceeded for 1 h at 4°C. After spinning in a microcentrifuge for 15 min, pellets were washed once with binding medium-10% TCA. Precipitates were referred to as undegraded, supernatants as degraded, ^{125}I-ligands. Eighty-five to 95% of EGF or TGF-α tracer was recovered in the precipitate in a control assay.

Affinity labeling protocol

Cells, bound to equilibrium, washed and acid-treated as indicated, were washed for 10 min at room temperature with phosphate-buffered saline and then incubated with 0.15 mM disuccinimidyl suberate and added to saline from a 15 mM stock solution in dimethylsulfoxide. Without quenching un-

reacted cross-linker, cells were washed again and scraped from the plastic well. Cell suspensions were transferred to microfuge tubes and spun down for 30 s. Precipitated cells were resuspended in reducing electrophoresis sample buffer (Laemmli, 1970) and homogenized by repeatedly passing through a syringe needle to decrease viscosity. After electrophoresis on a 7.5%/0.75 mm polyacrylamide gel using a discontinous pH system, gels were fixed, dried without staining, and subjected to autoradiography using X-Omat Kodak R films (Rochester, NY) and DuPont LightningPlus intensifying screens (Wilmington, DE). Molecular size protein standards were ^{14}C-labeled myosin (H-chain, 200 kDa), phosphorylase b (97.4 kDa), BSA (68 kDa), and ovalbumin (43 kDa).

Statistics

All determinations of binding capacity were done in duplicate to quadruplicate parallel wells. Average values of these determinations are presented with the corresponding deviations mentioned. Isoelectric points were estimated with calculation programs by D. Burdick and S. Shire (Genentech, Inc.). Curve fits for competitive inhibition and acid-dissociation experiments were by least-square analysis using nonlinear, four-parameter functions of the kind $y = [A - D/(1 + x/C)^B] + D$. Binding isotherms using varying radiolabeled tracer concentrations were determined with computerized nonlinear regression methods after plotting data according to the method of Scatchard (1949), and all affinity estimates were calculated with the LIGAND-program of P. Munson rewritten in Fortran by R. Vandlen (Genentech, Inc.).

Acknowledgments

We are grateful to Dr. Richard Vandlen for helpful discussions, advice, and assistance with analysis of our data, and to Dr. Gordon Gill (U.C. San Diego) for advice. We also thank Patricia B. Lindquist for expert technical assistance in the early stages of the project, Drs. Gordon Gill and Peter Howley for providing cell lines, and Carol Morita for computer graphics. R.E. is a research fellow of the Deutsche Forschungsgemeinschaft (DFG).

Received: May 4, 1991.
Revised and accepted: June 14, 1991.

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