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Articles

Structure and Properties of the Cellular Receptor for Transforming Growth Factor Type β[†]

Bradford O. Fanger,* Lalage M. Wakefield, and Michael B. Sporn
Laboratory of Chemoprevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892
Received October 31, 1985; Revised Manuscript Received December 31, 1985

ABSTRACT: Swiss 3T3 cells respond to picomolar concentrations of type β transforming growth factor (TGF-β) with a dose-dependent increase in the formation of colonies in soft agar, a decrease in the growth of cells in monolayer culture, and changes in morphology. This indicates that these cells have functional TGF-β receptors able to mediate a biological response. Binding analysis revealed a single class of TGF-β binding sites (80 000 per cell) with a $K_d \sim 50$ pM. Receptors were affinity-labeled by covalent attachment to ^{125}I-TGF-β with bis(sulfosuccinimidyl) suberate (BS^3). The complexes formed were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of 100 mM dithiothreitol and migrated as $M_r \sim 180\,000$ complexes in 3–10% linear gradient gels. The apparent size of these complexes was larger in gels with a higher percentage of acrylamide. The labeling of the ^{125}I-TGF-β–receptor complexes was inhibited by the presence of excess unlabeled TGF-β but was unaffected by other growth factors. These complexes could be formed by cross-linking whole cells, intact membranes, or solubilized membranes, demonstrating that the TGF-β receptor is located on the plasma membrane and can be solubilized without destruction of its ability to bind TGF-β. A larger $M_r \sim 360\,000$ complex was present in 3–10% linear gradient gels without reduction or after extensive cross-linking, suggesting that the receptor consists of two subunits of similar size attached by disulfide bonds. Since BS^3 is membrane-impermeable, at least a portion of both subunits is located on the outer surface of the plasma membrane. Unlike epidermal growth factor (EGF) receptors, which aggregate and form large complexes at temperatures >4 °C, no temperature-dependent increase in the size of TGF-β–receptor complexes was observed in either Swiss 3T3 or rat 1 cells. Furthermore, no TGF-β-induced phosphorylation was detected under conditions where the EGF receptor kinase was active. These observations indicate that the receptors for TGF-β may differ mechanistically from those of other growth factors, such as EGF.

Transforming growth factors (TGFs)[1] are defined operationally by their ability to cause anchorage-independent growth of NRK cells [for reviews, see Sporn & Todaro (1980), Todaro et al. (1981), and Roberts & Sporn (1986)]. This transforming activity was originally shown by De Larco and Todaro (1978) to be produced by cells in culture after viral transformation and is now known to be due to the cooperative action

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* Author to whom correspondence should be addressed.

[1] Abbreviations: BS^3, bis(sulfosuccinimidyl) suberate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EGF, epidermal growth factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NRK, normal rat kidney; PBS, Dulbecco's phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TGF, transforming growth factor; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

of two types of transforming growth factors: TGF-α and TGF-β (Anzano et al., 1983). TGF-α is homologous to, but immunologically distinct from, EGF (De Larco & Todaro, 1978; Marquardt et al., 1983; Derynck et al., 1984). Both EGF and TGF-α bind to the same receptor (De Larco & Todaro, 1978), stimulate its kinase activity (Reynolds et al., 1981), and have indistinguishable biological activities in vitro and in vivo (Massagué, 1983; Smith et al., 1985).

Investigations in this laboratory have identified a type β transforming growth factor (TGF-β) with biological and chemical properties distinctly different from TGF-α (Roberts et al., 1981; Assoian et al., 1983). It is composed of two apparently identical peptide chains (M_r ~12 500) whose amino acid composition and DNA sequence are unlike those of any other known growth factor (Assoian et al., 1983; Derynck et al., 1985). TGF-β has been shown to be a bifunctional regulator of cell growth, which can either stimulate or inhibit the proliferation of cells, depending on the culture conditions and growth factors present (Tucker et al., 1984b; Roberts et al., 1985).

Polypeptide growth factors act through specific receptors that are located on the plasma membrane of cells and are stimulated by the ligand to initiate a cellular response (Baxter & Funder, 1979; James & Bradshaw, 1984). The cellular signal appears to emanate from the receptor rather than the ligand because antibodies against the receptors for insulin (Kahn et al., 1977; Jacobs et al., 1978) and EGF (Schreiber et al., 1981) are able to stimulate a cellular response in the absence of the hormone. Recently, TGF-β has been radiolabeled and found to bind to NRK cells (Frolik et al., 1984) and AKR-2B cells (Tucker et al., 1984a) in a saturable and specific manner characteristic of hormone receptors (Baxter & Funder, 1979). Scatchard analysis indicated the presence of high-affinity binding sites with a K_d ~25–30 pM. In NRK cells, the number of receptors are "down-regulated" by a maximum of 50% after exposure to high concentrations of TGF-β (Frolik et al., 1984). These data suggest that the actions of TGF-β are mediated by a unique receptor system. Recently, the TGF-β receptor was affinity labeled and reported to be a disulfide-linked glycosylated M_r ~565 000 complex with a M_r ~280 000 binding subunit (Massagué & Like, 1985; Massagué, 1985). This paper confirms and extends those structural characteristics and, in addition, explores possible mechanisms of signal transduction for TGF-β.

Experimental Procedures

Reagents and Factors. TGF-β was purified from fresh human platelets and radiolabeled as previously described (Assoian et al., 1983; Frolik et al., 1984). Laminin and fibronectin were provided by H. Kleinman (Laboratory of Developmental Biology and Anomalies, NIH, Bethesda, MD). Multiplication stimulating activity was obtained from M. M. Rechler (Laboratory of Biochemical Pharmacology, NIH, Bethesda, MD). EGF was prepared from male mouse submaxillary glands and iodinated as previously described (Roberts et al., 1980). Sodium insulin crystals (porcine) were obtained from Elanco Products Co. (Indianapolis, IN). High-range protein molecular weight standards and prestained protein standards were purchased from BRL (Gaithersburg, MD) and Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP (3 Ci/μmol) was obtained from New England Nuclear (Boston, MA). Aprotinin and leupeptin were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Pepstatin A and phenylmethanesulfonyl fluoride were obtained from Sigma Chemical Co. (St. Louis, MO). Iodoacetic acid, CHAPS, and BS^3 were obtained from Pierce Chemical Co. (Rockford, IL). All other chemicals used were reagent-grade or better.

Cell Cultures. Late-passage Swiss 3T3 cells (clone 42; Rose et al., 1975) were maintained at 37 °C under humidified 10% CO_2/90% air in Dulbecco's minimum essential medium with 0.5 mg/mL gentamicin sulfate and 5% heat-inactivated fetal calf serum (GIBCO, Grand Island, NY); rat 1 cells were provided by D. Stern (Center for Cancer Research, MIT, Cambridge, MA) and were grown similarly with 10% serum.

Preparation of Membranes. Membranes were prepared by a modification of the method of Atkinson (1973). Swiss 3T3 cells were detached at 37 °C with 0.2% EDTA in PBS and washed in cold PBS. All subsequent manipulations were performed at 0–4 °C in the presence of 3.3 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, and 50 μM phenylmethanesulfonyl fluoride (prepared fresh as a 50 mM stock solution in 2-propanol) to inhibit proteolysis. The purity and integrity of the membranes were monitored throughout the procedure by microscopic examination. Cells were swollen in 10 mM glycylglycine (pH 8.5) and lysed with a Dounce homogenizer (tight pestle) and the nuclei stabilized with 1/10 volume of 10× reticulocyte stabilizing buffer (100 mM NaCl, 30 mM $MgCl_2$, 100 mM glycylglycine, pH 8.5). Whole cells and nuclei were removed by acceleration at full speed to 1800 rpm in a TJ-6 centrifuge (Beckman Instruments, Palo, Alto, CA). The pellet was resuspended in reticulocyte stabilizing buffer, and both the pellet and the supernatant fractions were centrifuged again as above. Membranes were collected by centrifugation for 10 min at 10000g and resuspended in PBS, and protein was assayed by the Bio-Rad microassay (Bio-Rad Laboratories, Richmond, CA). Membranes were stored in small aliquots at −70 °C until needed.

Chemical Cross-Linking. For studies using intact cells, Swiss 3T3 cells were grown to confluency in six-well cluster plates. All subsequent steps were performed at 0–4 °C to minimize proteolysis and metabolism of the receptor. Cells were washed twice with PBS and exposed to ^{125}I-TGF-β (0.5 μCi), with or without a ≥100-fold molar excess of unlabeled growth factor, in 500 μL of PBS for 2–4 h. Unbound factors were removed by washing with PBS, and cells were cross-linked by a 2- or 15-min exposure to 1 mM BS^3 in PBS. For clustering experiments, cells were either washed with PBS at 37 °C or cross-linked with BS^3 at 37 °C to induce formation of high molecular weight complexes. The reaction was stopped by adding 1/5 volume of 500 mM glycine and incubating for 5 min.

Membranes were resuspended in PBS with 0.25 μCi ^{125}I-TGF-β and a 300-fold excess of unlabeled TGF-β or vehicle and then mixed in siliconized 1.5-mL polypropylene tubes for 2 h at 4 °C, with or without 0.5% CHAPS. Cross-linking was accomplished by the addition of 1/10 volume of 10 mM BS^3 in PBS and stopped by the addition of 1/5 volume of 500 mM glycine after 2 min at 4 °C.

The cross-linked moieties were solubilized at room temperature in sample buffer (8 M urea, 2% sodium dodecyl sulfate, 62.5 mM Tris, pH 6.8). Viscous samples were homogenized for 10 s in a sonifer cell disruptor on a setting of 5 (Heat Systems-Ultrasonics, Inc., Planview, NY). Reduction of disulfide bonds was accomplished by boiling the samples for 1 min in the presence of 100 mM dithiothreitol. Electrophoresis was performed by the method of Laemmli (1970) with 3–10% linear gradient gels. If needed, 0.4% Coomassie Brilliant Blue R-250 was added to the upper buffer to stain proteins (Borejdo & Flynn, 1984). Gels were fixed/destained for 2 h in 40% methanol/10% acetic acid followed by 10%

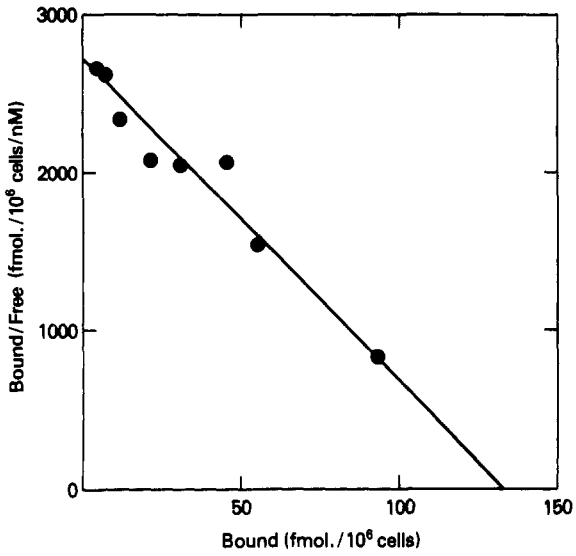

FIGURE 1: Analysis of TGF-β binding to Swiss 3T3 cells. Varying concentrations of ^{125}I-TGF-β were incubated for 2 h at 25 °C and analyzed as previously described (Frolik et al., 1984). Nonsaturable binding was determined by the addition of 10 nM unlabeled TGF-β. This is one of four experiments performed on Swiss 3T3 cells giving essentially identical results.

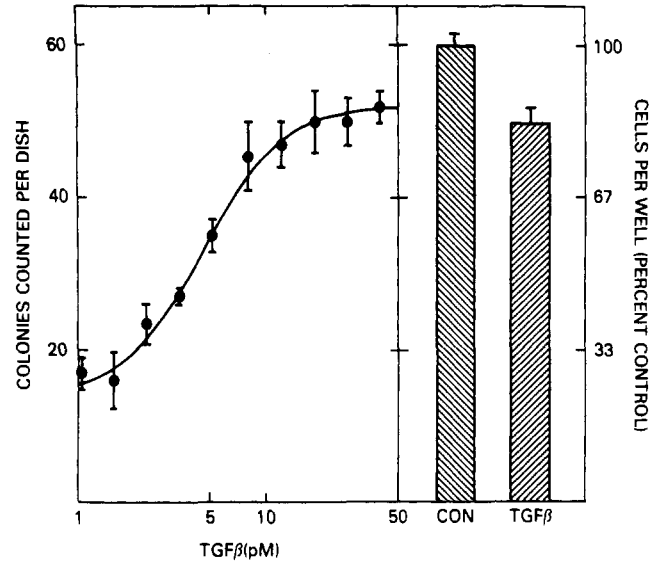

FIGURE 2: Growth response of Swiss 3T3 cells to TGF-β. (Left panel) Anchorage-independent growth. Colonies were quantitated after growth in medium containing 0.3% agar, 10% calf serum, and 1–50 pM TGF-β. (Right panel) Growth in monolayer culture. Cells were seeded at 4 × 10^4 cells per well and grown for 4 days in 24-well cluster plates, with or without 50 pM TGF-β, and then were detached with trypsin and counted. The data in both panels are the mean ± SE of three to four determinations and are representative of more than four similar experiments.

acetic acid overnight. They were then dried by heat and vacuum for 3 h and autoradiographed at −70 °C.

Phosphorylation. Membranes (50 μg of protein) were resuspended in 55 μL of phosphorylation buffer (150 mM NaCl, 10 mM $MnCl_2$, 25 mM MOPS, pH 7.4) and incubated for 20 min at 4 °C in the presence of 145 nM TGF-β, 145 nM EGF, or vehicle. The phosphorylation reaction was initiated by the addition of 10 μL of [γ-^{32}P]ATP (1 μCi/tube; final concentration 9 μM). The reaction was stopped after 60 s by addition of 75 μL of 2× sample buffer containing 200 mM dithiothreitol and boiling for 10 min. Phosphoproteins were separated on 3–10% linear gradient gels and fixed as above. To enrich for phosphotyrosine, gels were washed with distilled water and 1 N KOH and then incubated at 55 °C for 2 h in 1 N KOH (Cooper et al., 1983). The KOH solution was changed several times to prevent neutralization by residual acetic acid from the fixing procedure. Gels were dried and autoradiographed as above.

Binding Analyses and Soft Agar Assays. These were performed as previously described (Frolik et al., 1984; Roberts et al., 1981) accept that soft agar assays were incubated under 10% CO_2 with 0.5 mg/mL gentamicin sulfate as the antibiotic.

RESULTS

Source of TGF-β Receptors. Previous investigations in this laboratory have identified receptors for TGF-β on NRK cells (Frolik et al., 1984). However, there are only ~17 000 receptors per cell; therefore, these studies were initiated by searching for a cell type that expressed a higher level of TGF-β receptors. Binding analyses were performed on more than 50 cell lines, including normal and transformed cells from a variety or organisms and tissues [for a partial listing, see Assoian et al. (1985)]. Of these, murine 3T3 cells had the highest number of receptors, and Swiss 3T3 cells were selected for use in these studies. The binding analysis for these cells, plotted in the manner of Scatchard (1949), is shown in Figure 1 and indicates the presence of a single class of sites with a K_d ~50 pM. There are 80 000 receptors per Swiss 3T3 cell, which represents a 5-fold increase over NRK cells.

Response of Swiss 3T3 Cells to TGF-β. The effects of TGF-β on these Swiss 3T3 cells were studied in order to determine if they possessed receptors able to mediate a biological response. Since induction of anchorage-independent growth is the definitive assay for TGF-β, its ability to promote the growth of Swiss 3T3 colonies in soft agar was investigated. Figure 2 (left panel) shows that TGF-β induces a dose-dependent increase in the number of colonies of these Swiss 3T3 cells; there was also a small increase in the average size of the colonies. The response plateaued by 50 pM, and the half-maximal response occurred at 5 pM.

TGF-β has also been shown to inhibit the growth of a number of cell types in monolayer cultures (Tucker et al., 1984b; Roberts et al., 1985). Cultures of Swiss 3T3 cells grown for 4 days in the presence of 50 pM TGF-β had an 18% decrease in the number of cells per well (Figure 2, right panel); concentrations of TGF-β as low as 1 pM are able to elicit an effect. TGF-β also produced a change in the morphology of these cells: they became elongated and formed a pattern not seen in control cells (Figure 3). At confluency, a secondary layer of cells began to grow over the original monolayer with a pattern perpendicular to those below. The enhanced colony formation, reduced cell number, and morphological changes induced by TGF-β imply that Swiss 3T3 cells contain functional TGF-β receptors that are able to mediate a biological response. Furthermore, these morphological changes (Figure 3) are exhibited by the entire population of Swiss 3T3 cells, demonstrating that all these cells are able to respond to TGF-β.

Affinity Labeling of TGF-β Receptors. The binding of ^{125}I-TGF-β to only one class of sites (Figure 1) suggested that it could be used for specific affinity labeling of its receptor. Since there are 16 lysine residues per molecule of TGF-β (Derynck et al., 1985), bis(sulfosuccinimidyl) suberate (BS^3), a water-soluble bifunctional cross-linking reagent that reacts with amino groups (Staros, 1982), was chosen as the cross-linking reagent. Whole cells were used for better preservation of the normal membrane environment, and metabolic and degradative changes were minimized by the use of low temperatures. The presence of 15 mM iodoacetic acid, 50 μM phenylmethanesulfonyl fluoride, 3.3 μg/mL aprotinin, and/or

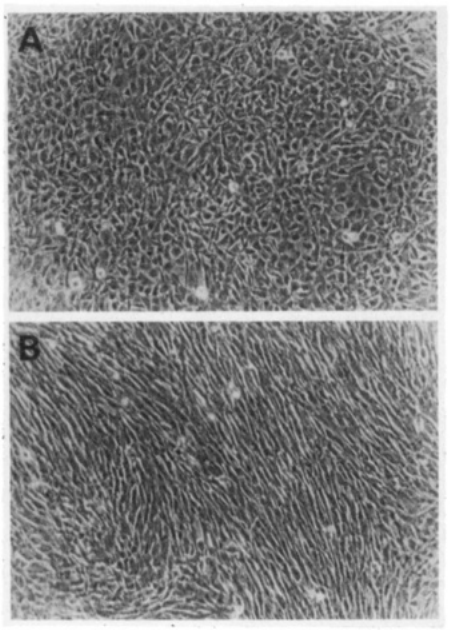

FIGURE 3: Morphological response of Swiss 3T3 cells to TGF-β. Cells were seeded at 5×10^4 cells per well and grown for 3 days in 24-well cluster plates with (B) or without (A) 50 pM TGF-β and photographed. The pictures are representative of more than four similar experiments.

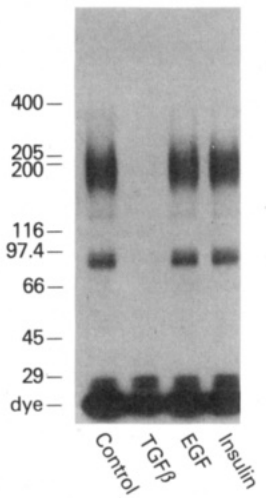

FIGURE 4: Specific formation of TGF-β–receptor complexes. Swiss 3T3 cells were incubated for 2 h with 40 pM ^{125}I-TGF-β with or without 40 nM unlabeled growth factors as indicated. Proteins were cross-linked and separated by SDS–PAGE through 3–10% linear gradient gels in the presence of 100 mM dithiothreitol. This is representative of the five experiments performed.

1.0 μg/mL leupeptin did not alter the apparent size of the TGF-β–receptor complexes formed, suggesting that no significant proteolysis occurred during the cross-linking procedure.

Figure 4 shows the radioactive complexes separated on 3–10% linear gradient gels in the presence of 100 mM dithiothreitol. The affinity labeling of an M_r ~180 000 band is blocked by the presence of 40 nM (1000-fold excess) unlabeled TGF-β (saturable) and is unaffected by the presence of 40 nM EGF or insulin (specific); multiplication stimulating activity was also without effect (data not shown). This saturable and specific displacement of ^{125}I-TGF-β parallels observations in whole cell binding experiments (Frolik et al., 1984; Tucker et al., 1984a) and suggests that the M_r ~180 000 band is the TGF-β receptor. The radioactivity at the bottom of the gel runs with the dye front, as does reduced TGF-β, and thus represents ligand that was bound but was not cross-linked. The slightly larger M_r ~25 000 band represents TGF-β with its two subunits attached by BS^3. The apparent partial saturability of the TGF-β bands is due to the removal of unbound TGF-β before cross-linking.

A faint M_r ~125 000 band and a more prominent M_r ~ 90 000 band, which are saturable and specific, are also present. The intensity of these bands differed from one experiment to another, and their size and number varied widely between cell types (M. O'Connor-McCourt, unpublished observations). These could result from partial proteolysis of the receptor despite the presence of protease inhibitors or proteolysis during the solubilization of receptor complexes for electrophoresis, when no protease inhibitors were present. Rapid proteolysis of the EGF receptor is known to occur after disruption of cells (Cohen et al., 1982; Cassel & Glaser, 1982). The M_r ~90 000 band was not always completely saturated by unlabeled TGF-β (see Figure 5), suggesting that it may represent a low-affinity binding site. This is supported by the observations of Massagué and Like (1985) that affinity labeling of the M_r ~90 000 band was unaffected by low concentrations (1.5 nM) of TGF-β, which could reduce the affinity labeling of the receptor. A similar M_r ~90 000 band was detected by cross-linking bovine serum albumin with ^{125}I-TGF-β in the absence of cells (data not shown). Thus, bovine serum albumin, present in the ^{125}I-TGF-β storage buffer, could account for this band, as could the attachment of TGF-β to a serum protein that was not removed by washing the cells. It is very unlikely, therefore, that these smaller bands represent intact TGF-β receptors.

The relative mobility of the ^{125}I-TGF-β–receptor complex was dependent on the gel system used; estimations of size ranged from M_r ~180 000–200 000 on 3–10% linear gradient gels to M_r ~250 000–300 000 on 7.5% gels, with intermediary sizes observed with gels of 4.5, 5, 6, and 7% (data not shown). This did not appear to result from altered migration of the protein standards, as the migration of prestained and unstained protein molecular weight standards from two different sources was linearly proportional to the log of their reported sizes on all gel systems used. In addition, laminin (M_r ~200 000 and 400 000) and fibronectin (M_r ~220 000 and 240 000) were used to improve estimations of size in the high molecular weight range. The same preparations of affinity-labeled TGF-β receptors migrated differently on gels with varying concentrations of acrylamide, while different preparations migrated similarly on the same gel. A similar but less pronounced effect was observed with both the M_r ~125 000 band and the EGF receptor; however, the M_r ~90 000 complex migrated similarly in all electrophoresis systems used (data not shown). The TGF-β receptor, like the EGF receptor, is a glycoprotein (Massagué, 1985; Fanger & Sporn, 1986), and anomalous migration of glycoproteins in SDS–PAGE has been reported due to altered binding of detergent, although this results in a decrease in the electrophoretic mobility as the concentration of acrylamide increases (Segrest et al., 1971). The complexes will be referred to as M_r ~180 000 proteins for consistency with the data presented in Figures 4–7, which use 3–10% linear gradient gels.

Since BS^3 randomly attaches amino groups within 1.1 nm of each other, large protein aggregates containing multiple proteins can form, which lead to an artificially high estimation of their actual size. The detergent CHAPS was used to determine if the M_r ~180 000 complexes contained more than TGF-β and its receptor, since this detergent has previously been shown both to dissociate protein subunits that remain associated in other detergents and to solubilize hormone receptors in an active state when other detergents lead to in-

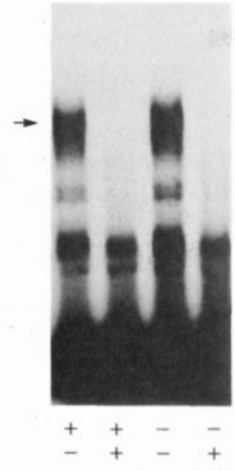

FIGURE 5: TGF-β–receptor complexes from intact and solubilized Swiss 3T3 membranes. Membranes were incubated with ^{125}I-TGF-β in PBS for 2 h at 4 °C, in the absence or presence of 0.5% CHAPS and excess unlabeled TGF-β as indicated. Proteolysis was inhibited with 10 μg/mL aprotinin, 3 μg/mL leupeptin, and 3 μg/mL pepstatin A. Complexes were formed by the addition of 1/10 volume 10 mM BS^3 and separated by SDS–PAGE through 3–10% linear gradient gels in the presence of 100 mM dithiothreitol. The arrow indicates the position of the reduced ^{125}I-TGF-β–receptor complex. This is representative of more than 10 similar experiments.

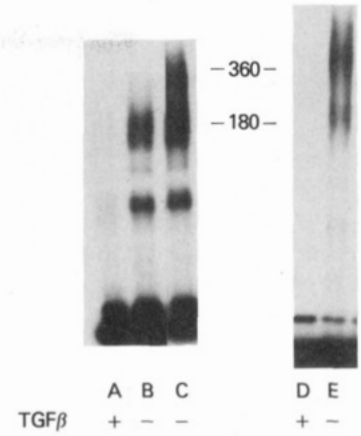

FIGURE 6: Subunit structure of the TGF-β receptor. ^{125}I-TGF-β, with or without excess unlabeled TGF-β as indicated, was cross-linked to Swiss 3T3 cells and separated by SDS–PAGE through 3–10% linear gradient gels. (A and B) Complexes formed by exposure to 1 mM BS^3 for 2 min and reduced by boiling in the presence of 100 mM dithiothreitol before electrophoresis; (C) complexes formed by exposure to 1 mM BS^3 for 15 min and reduced by boiling in the presence of 100 mM dithiothreitol before electrophoresis; (D and E) unreduced complexes formed by exposure to 1 mM BS^3 for 2 min. This is representative of more than five similar experiments.

activation (Hjelmeland, 1980; Simonds et al., 1980; Bitonti et al., 1982; Liscia et al., 1982). Membranes were prepared with a modification of the differential centrifugation technique described by Atkinson (1973). All manipulations were performed at 0–4 °C in the presence of protease inhibitors to minimize degradation of the receptor. As in whole cells, these membranes bound TGF-β in a saturable manner (data not shown) and formed saturable M_r ~180 000 complexes with ^{125}I-TGF-β (Figure 5); thus, the TGF-β receptor is a membrane protein.

^{125}I-TGF-β–receptor complexes are formed in solubilized membranes (Figure 5), demonstrating that CHAPS did indeed preserve the ability of receptors to bind TGF-β. Note that the M_r ~90 000 band (but not the M_r ~125 000 band) remains labeled in the presence of excess unlabeled TGF-β, indicating that it has a relatively low affinity for TGF-β. These complexes migrated similarly to those obtained from intact membranes and whole cells with 5% (data not shown) and 3–10 (Figures 4 and 5) polyacrylamide gels. The failure to observe new saturable bands in the presence of a detergent known to dissociate subunits suggest that the M_r ~180 000 complex represents only TGF-β and its receptor, although very tight association of another protein with the receptor cannot be ruled out. Further support for this conclusion was obtained by decreasing the extent of cross-linking used to form the complexes, a condition that should reduce cross-linking of large aggregates and shift radioactivity to smaller bands; however, no such smaller bands were detected (data not shown).

Subunit Structure of TGF-β Receptor. Most receptors for growth factors, including EGF, nerve growth factor, insulin-like growth factor II, and platelet-derived growth factor, consist of a single subunit; however, receptors for insulin and somatomedin C have multiple subunits (James & Bradshaw, 1984). To determine if the receptor for TGF-β consists of one or more subunits, ^{125}I-TGF-β–receptor complexes were solubilized under reducing (boiling in 100 mM dithiothreitol) and non-reducing conditions and separated by SDS–PAGE. The majority of the unreduced ^{125}I-TGF-β–receptor complexes migrated as a M_r ~360 000 band (Figure 6, lanes D and E), in contrast to the M_r ~180 000 complexes present in reduced samples (Figure 6, lanes A and B). As with the M_r ~180 000 band, the apparent size of the unreduced complexes was dependent on the concentration of acrylamide in the gels but was always approximately twice the size of the reduced complex. This larger band could also be detected under reducing conditions after extensive cross-linking with BS^3 in intact cells (Figure 6, lane C). These data confirm that the M_r ~180 000 subunit is associated with another protein and, since BS^3 cannot cross the plasma membrane (Staros, 1982), show that at least a portion of both proteins is present on the outer surface of the plasma membrane. The presence of more than two distinct proteins in the larger complex should result in the formation by extensive cross-linking of complexes with sizes in between the M_r ~180 000 and 360 000 complexes. No such complexes were detected after varying degrees of cross-linking (data not shown), although it is possible that their presence is masked by the diffuse nature of the band. These data suggest that the TGF-β receptor consists of similarly sized subunits covalently linked by disulfide bonds.

Clustering and Kinase Activity of TGF-β Receptor. Growth factor receptors undergo ligand-dependent clustering and internalization that may play a role in the generation and regulation of the cellular response (Schreiber et al., 1983). Clustering of EGF receptors can be detected by the formation of high molecular weight complexes after warming of occupied receptors (O'Connor-McCourt & Hollenberg, 1983; Fanger et al., submitted for publication). A similar approach was taken to determine if TGF-β induced the clustering of its receptors. Cells to which TGF-β was bound were warmed for 2 or 10 min at 37 °C to allow clustering to occur. Figure 7 (lanes A–D) shows that warming produced no difference in the complexes formed in Swiss 3T3 cells, as compared to complexes from cells that had not been warmed. Extensive cross-linking, which should enhance the formation of these larger complexes, resulted in the covalent attachment of the two subunits of the TGF-β receptor but did not reveal any temperature-dependent high molecular weight complexes (data not shown).

The low numbers of EGF receptors and the low efficiency of cross-linking due to the lack of lysine residues in murine EGF (Taylor et al., 1972) precluded the detection of EGF

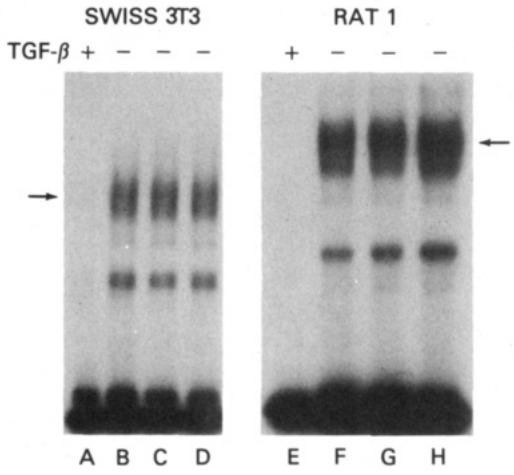

FIGURE 7: Clustering of TGF-β receptors in Swiss 3T3 and rat 1 cells. ^{125}I-TGF-β, with or without excess unlabeled TGF-β as indicated, was bound to cells for 2 h at 4 °C. Cells either remained at 4 °C or were warmed to 37 °C and then cooled on ice and then cross-linked with 1 mM BS^3 at either 0 or 37 °C as indicated below. Complexes were reduced by boiling in 100 mM dithiothreitol and separated by SDS–PAGE through 3–10% linear gradient gels. The arrow indicates the position of the M_r ~180 000 ^{125}I-TGF-β–receptor complex. (A and B) Not warmed; (C) warmed for 2 min; (D) warmed for 10 min; (E and F) not warmed; (G) warmed for 2 min; (H) not warmed but cross-linked at 37 °C. These data are representative of the nine experiments performed.

receptor clustering in these cells (data not shown); therefore, the possibility that all receptors in Swiss 3T3 cells cluster abnormally could not be ruled out. Thus, clustering was also examined in NRK and rat 1 cells, which are known to respond to both TGF-β and EGF (Anzano et al., 1983; Racker et al., 1985) and which should therefore possess functional receptors for both growth factors. We found that large EGF–receptor complexes can be formed in these cells by cross-linking with BS^3 at 37 °C (data not shown). However, no larger ^{125}I-TGF-β–receptor complexes were observed when NRK cells (data not shown) or rat 1 cells (Figure 7, lanes E–H) were cross-linked at 37 °C or warmed to 37 °C for 2 min just prior to cross-linking. These data suggest that TGF-β receptors do not undergo ligand-dependent clustering (defined here as the movement of the amino groups on the TGF-β receptor to within 1.1 nm of an amino group on another protein).

A number of growth factor receptors have been shown to undergo ligand-dependent tyrosine-specific autophosphorylation (James & Bradshaw, 1984). Therefore, the ability of TGF-β to induce similar phosphorylation was investigated. Swiss 3T3 membranes were prepared as before and incubated for 20 min with TGF-β or vehicle; as a positive control, an equivalent molar amount of EGF was used in parallel to stimulate the autophosphorylation of its receptor. These membranes maintained the ability to bind TGF-β in a saturable manner, as determined by a soluble TGF-β binding assay developed in this laboratory (Fanger & Sporn, 1986). Proteins were labeled by exposure for 60 s at 0 °C to [γ-^{32}P]ATP; the reaction was stopped by the addition of 2× electrophoresis buffer (8 M urea, 4% sodium dodecyl sulfate, 125 mM Tris, pH 6.8) with 200 mM dithiothreitol and boiling. Phosphoproteins were separated by SDS–PAGE through 3–10% linear gradient gels and digested for 2 h at 55 °C with 1 N KOH to enrich in phosphotyrosine (Cooper et al., 1983). Figure 8 shows that no TGF-β-induced phosphoproteins were detected under conditions where the EGF-induced autophosphorylation of its receptor was observed. Furthermore, TGF-β did not influence the activity of other kinases, including the EGF receptor.

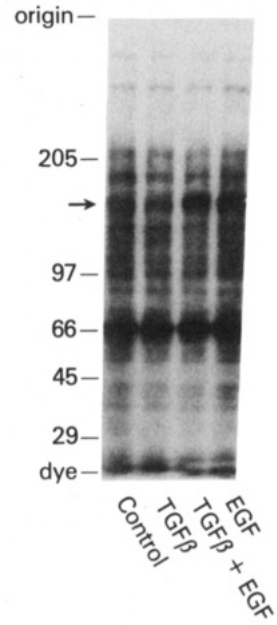

FIGURE 8: TGF-β- and EGF-induced kinase activity in Swiss 3T3 membranes. Membranes were incubated at 0 °C for 20 min with 145 nM TGF-β, 145 nM EGF, or vehicle as indicated and exposed to labeled ATP for 60 s at 0 °C. Phosphoproteins were reduced by boiling in 100 mM dithiothreitol, separated by SDS–PAGE through 3–10% linear gradient gels, and digested for 2 h at 55 °C with 1 N KOH. The arrow indicates the position of the EGF receptor. This is one of more than 10 experiments using numerous different conditions (see text).

An exhaustive effort was made to determine if a TGF-β-induced kinase activity could be detected under different conditions. Two different methods for preparing membranes (Atkinson, 1973; Thom et al., 1977) were used with numerous protease inhibitors (iodoacetic acid, leupeptin, aprotinin, and/or phenylmethanesulfonyl fluoride) to protect receptors from inactivation. The kinase reaction itself was repeated with the following changes: different ATP and [γ-^{32}P]ATP concentrations, different temperatures, several different detergents to solubilize membranes, different buffer systems (Cohen et al., 1980; Rubin et al., 1982; Pike et al., 1983; PBS; 10 mM MOPS, with or without 150 mM NaCl, pH 7.4), several concentrations of Mn^{2+}, Mg^{2+}, and/or Ca^{2+}, different incubation times to balance receptor occupancy with any loss of kinase activity, and *p*–nitrophenyl phosphate to inhibit dephosphorylation. Electrophoresis was performed under reducing and nonreducing conditions, with and without KOH digestion of phosphoproteins. In addition, numerous cell types known to possess TGF-β receptors were examined, including NRK, NIH 3T3, and BALB/c 3T3 cells. TGF-β produced no reproducible alteration in protein phosphorylation under any of these conditions (data not shown). These data suggest that TGF-β receptors lack a ligand-induced kinase activity, although it is possible that none of these conditions were sufficient for its detection.

Discussion

These investigations were designed to study the size and structure of the cellular receptors for TGF-β and to gain insight into possible mechanisms of signal transduction. Affinity-labeling studies indicated that the TGF-β receptor is a large membrane-bound disulfide-linked dimer. Furthermore, these data indicate, for the first time, that TGF-β is unable to induce the clustering or autophosphorylation of its cellular receptor.

The reduced rate of growth (Figure 2) and the change in

cell morphology (Figure 3) of monolayer cultures of Swiss 3T3 cells in the presence of TGF-β demonstrate that these cells are responsive to this growth factor under the conditions that were used for the experiments presented here. The bifunctional response induced by TGF-β in soft agar and monolayer cultures of Swiss 3T3 cells (Figure 2) has been observed in NRK cells and other cell types (Tucker et al., 1984b; Roberts et al., 1985). The similarity between the response of Swiss 3T3 cells and other previously characterized cell types suggests that the cells used here are an appropriate model system for studying TGF-β receptors. In addition, the shape of the dose–response curve (Figure 2) and the saturable and specific formation of complexes with ^{125}I-TGF-β (Figure 4) are characteristic of cellular receptors (Baxter & Funder, 1979) and strongly support the existence of membrane receptors for TGF-β in Swiss 3T3 cells. These data are consistent with the concept that the biological response, the saturable binding of TGF-β to cells and membranes, the single class of sites detected by binding analyses, and the M_r ~180000 cross-linked complexes all result from the presence of this receptor protein.

The identification of the M_r ~180000 band as the intact binding subunit of the TGF-β receptor is supported by its detection in intact and solubilized membranes (Figure 5) and the variability in the number and saturability of other bands. The experiments using CHAPS-solubilized receptors and a reduced extent of cross-linking do not result in the formation of smaller complexes, indicating that the M_r ~180000 complex consists only of TGF-β and its receptor. This binding subunit is probably identical with the M_r ~280000 ^{125}I-TGF-β–receptor complexes identified by Massagué and Like (1985) using 5–7% polyacrylamide gels, since, as mentioned earlier, the relative mobility of this band is dependent on the concentration of acrylamide used.

The studies presented here differ from those of Massagué (1985) and Massagué and Like (1985) in several respects. First, the investigations presented here used BS^3, which is water-soluble and therefore does not require the presence of an organic solvent, such as dimethyl sulfoxide. Such solvents can interfere with the binding of TGF-β to its receptor (data not shown) and have been shown to alter the properties of other growth factor receptors (Rubin & Earp, 1983). In addition, BS^3 is membrane-impermeable (Staros, 1982); thus, at least a portion of both subunits of the TGF-β receptor is located on the outer surface of the plasma membrane. Second, the use of a zwitterionic detergent to solubilize receptors for these studies reduces noncovalent associations of solubilized proteins, which could persist in nonionic detergents and produce artificially high estimations of the size of the receptor (Hjelmeland, 1980; Bitonti et al., 1982; Liscia et al., 1982). Third, the present investigations demonstrated the anomalous migration characteristics of affinity-labeled TGF-β receptors on SDS–PAGE; thus, previously published values for the molecular weight of the binding subunit of the TGF-β receptor should not be considered absolute. Finally, possible mechanisms of signal transduction have been explored here.

The diffuse nature of the M_r ~180000 receptor complex in polyacrylamide gels could result from it being a glycoprotein (Massagué, 1985; Fanger & Sporn, 1986), since these sometimes migrate anomalously on SDS–PAGE (Segrest et al., 1971; Hughes, 1976). Alternatively, it could be due to heterogeneity produced by the cross-linking procedure, such as intramolecular cross-linking, attachment of ligand to receptor through different lysine residues, and attachment of either one or both of the TGF-β subunits. The migration of both the M_r ~90000 and 125000 complexes and the M_r ~25000 TGF-β as distinct bands indicates that the electrophoresis system itself resulted in good resolution of most proteins.

The data presented here indicate that the receptor is a disulfide-linked dimer in its native state. This is supported by the detection of an M_r ~360000 complex after extensive cross-linking and under nonreducing conditions (Figure 6) and is consistent with data obtained by Massagué (1985). The disulfide bonds of the larger complex are very labile and can be reduced by boiling in the absence of dithiothreitol (data not shown). Similar observations have been made with the insulin receptor, where this effect has been reported to result from sodium dodecyl sulfate/heat-dependent intramolecular sulfhydryl–disulfide exchange (Boyle et al., 1985); however, others have suggested that the reduced forms exist under physiological conditions and correlate to insulin receptors with higher affinity and possibly altered function (Crettaz et al., 1984). It is not known if the dissociated subunit of the TGF-β receptor is able to bind TGF-β or generate the cellular response, but conversion between these two forms could be a mechanism for the regulation of TGF-β action. As with the reduced form of the receptor, the true size of the unreduced receptor cannot be definitively determined by SDS–PAGE alone but appears to be approximately twice that of the reduced receptor. Thus, the TGF-β receptor could be a homodimer or a heterodimer of two subunits of approximately the same size.

In marked contrast to a number of other growth factors (James & Bradshaw, 1984), TGF-β does not appear to induce clustering or autophosphorylation of its cellular receptor. None of the numerous different combinations of conditions that were tested resulted in the detection of TGF-β-induced phosphorylation, even though receptors were active, as determined by the ability of cells to respond in monolayer cultures (Figures 2 and 3) and the ability of the membrane preparations to bind and be cross-linked to TGF-β (Figure 5). It is possible, however, that a kinase activity is present that either is very weak, is inactivated during the isolation of membranes, or requires conditions that differ from those for phosphorylation of EGF receptors. It is also possible that a kinase activity is present that is directed toward a cellular substrate other than the TGF-β receptor, although other phosphoproteins do not appear to be affected by TGF-β in Swiss 3T3 membranes (Figure 8) or intact NRK cells [see Frolik et al. (1984)].

Several relevant points should be considered with respect to these data. First, receptors for insulin and somatomedin C are usually precipitated by antibodies or lectins to enrich ligand-dependent kinase activity (Kasuga et al., 1982; Jacobs et al., 1983). When antibodies to the TGF-β receptor become available, the kinase activity of the receptor can be examined in a more sensitive system. Second, the *erb B* gene product was originally reported to be devoid of detectable kinase activity (Graf & Beug, 1983; Privalsky et al., 1983) despite the suggestion that it should contain a kinase activity due to its sequence homology to the EGF receptor and other tyrosine protein kinases (Yamamoto et al., 1983; Downward et al., 1984; Privalsky et al., 1984). Very recently, three groups have reported evidence, using *erb B* antibodies and genetically engineered viruses, that the *erb B* gene product does indeed possess a kinase activity (Decker, 1985; Gilmore et al., 1985; Kris et al., 1985). Finally, TGF-β often inhibits, rather than stimulates, the growth of cells in culture (Tucker et al., 1984b; Roberts et al., 1985). Thus, it may be inappropriate to compare it with growth factors that stimulate growth. Instead, the actions of TGF-β receptors may resemble those of non-

mitogenic polypeptides. IGF-II receptors, for example, appear to lack both kinase and mitogenic activities [see Mottola & Czech (1984)].

The absence of TGF-β receptor kinase activity implies that another mechanism exists for the transduction of the hormone signal. Cyclic AMP, which mediates the actions of a number of bioactive peptides (Ross & Gilman, 1980), is not effected by TGF-β in normal human bronchial epithelial cells (Masui et al., 1986). Recent evidence from this laboratory indicates that some of the effects of TGF-β are mediated through other receptor systems. In NRK cells, for which EGF and TGF-β are required for colony formation in soft agar (Anzano et al., 1983), TGF-β can specifically modulate EGF receptors in a manner that parallels the effects of TGF-β on cell growth (Assoian, 1985). In this respect, the actions of TGF-β may resemble those of glucocorticoids, which exert permissive control over other cellular processes (Granner, 1979). It seems unlikely that there is a direct effect on the actions of other receptors at the cell surface, since TGF-β receptors do not become cross-linked to EGF receptors or any other protein at 37 °C (Figure 7) and the presence of TGF-β had no noticeable effect on EGF-induced phosphorylation in Swiss 3T3 cell membranes (Figure 8).

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Selective Radiolabeling and Isolation of the Hydrophobic Membrane-Binding Domain of Human Erythrocyte Acetylcholinesterase†

William L. Roberts and Terrone L. Rosenberry*
Department of Pharmacology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106
Received October 25, 1985; Revised Manuscript Received January 14, 1986

ABSTRACT: The hydrophobic, membrane-binding domain of purified human erythrocyte acetylcholinesterase was labeled with the photoactivated reagent 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine. The radiolabel was incorporated when the enzyme was prepared in detergent-free aggregates, in detergent micelles, or in phospholipid liposomes, but the highest percentage of labeling occurred in the detergent-free aggregates. Papain digestion of the enzyme released the hydrophobic domain, and polyacrylamide gel electrophoresis in sodium dodecyl sulfate or gel exclusion chromatography demonstrated that the label was localized exclusively in the cleaved hydrophobic domain fragment. This fragment was purified in a three-step procedure. Digestion was conducted with papain attached to Sepharose CL-4B, and the supernatant was adsorbed to acridinium affinity resin to remove the hydrophilic enzyme fragment. The nonretained fragment associated with Triton X-100 micelles was then chromatographed on Sepharose CL-6B, and finally detergent was removed by chromatography on Sephadex LH-60 in an ethanol–formic acid solvent. The fragment exhibited an apparent molecular weight of 3100 on the Sephadex LH-60 column when compared with peptide standards. However, amino acid analysis of the purified fragment revealed only 1 mol each of histidine and glycine per mole of fragment in contrast to the 25–30 mol of amino acids expected on the basis of the molecular weight estimate. This result suggests a novel non-amino acid structure for the hydrophobic domain of human erythrocyte acetylcholinesterase.

Acetylcholinesterase (AChE,[1] EC 3.1.1.7) occurs in a variety of forms in vertebrate tissues. These forms can be classified as either asymmetric if they contain a collagen-like tail or globular if they lack such a tail structure [see Massoulié & Bon (1982) and Rosenberry (1985)]. The globular AChEs include a class of membrane-bound forms which are the predominant AChEs in mammalian brain and in muscle cells outside of the neuromuscular junction. These forms possess a hydrophobic domain that anchors the enzyme in the cell

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[1] Abbreviations: AChE, acetylcholinesterase; RBC AChE, human erythrocyte acetylcholinesterase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; G_n, a globular AChE form with *n* catalytic subunits; PIPLC, phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus*; kDa, kilodalton(s); HPLC, high-pressure liquid chromatography.

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