Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 5359–5363, June 1993
Cell Biology

Growth inhibition by transforming growth factor β (TGF-β) type I is restored in TGF-β-resistant hepatoma cells after expression of TGF-β receptor type II cDNA

(human hepatoma/transfection/polyclonal antisera)

MITSUHIRO INAGAKI*, ARISTIDIS MOUSTAKAS†, HERBERT Y. LIN†‡, HARVEY F. LODISH†‡, AND BRIAN I. CARR*§

*Pittsburgh Transplantation Institute, University of Pittsburgh, Pittsburgh, PA 15260; †Whitehead Institute for Biomedical Research, Cambridge, MA 02142; and ‡Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

Contributed by Harvey F. Lodish, February 25, 1993

ABSTRACT **The growth of human hepatoma Hep 3B cells is potently inhibited by TGF-β_1 (ID_{50} = 0.2 ng/ml, 8 pM). A mutant cell line was derived that was not inhibited in growth by TGF-β_1 at 5 ng/ml (200 pM) and that lacked TGF-β receptor type II (TGF-βRII) gene. Transfection of the cloned cDNA for human TGF-βRII to this mutant cell line restored receptor expression as well as the inhibition in growth by TGF-β_1. In both wild-type and mutant cells stably transfected with TGF-βRII cDNA, TGF-βRII coimmunoprecipitated with TGF-β receptor type I in the presence of ligand. These experiments provide direct evidence for the role of TGF-βRII in the inhibitory effect of TGF-β on growth and suggest that TGF-βRII acts by means of a heteromeric surface complex with TGF-β receptor type I.**

TGF-β is a multifunctional protein that mediates cell proliferation, growth inhibition, differentiation, and other functions (1–5). Several transformed cell lines have lost their sensitivity to the inhibition by TGF-β on growth (6, 7), which is a possible mechanism for escape from normal regulation of growth by TGF-β and carcinogenesis. Loss of TGF-β-mediated growth regulation could result either from loss of specific receptors for TGF-β or from alterations in the postreceptor signal-transduction pathway (6, 7).

Three cell-surface proteins have been identified through their ability to bind with high affinity and be chemically crosslinked to ^{125}I-labeled TGF-β_1: types I (55 kDa), II (80 kDa), and III (280 kDa) receptors (8–19). These receptors are considered candidates for mediating signal transduction by TGF-β. Experiments using chemically mutated mink lung epithelial cells (20–22) or tumor cell lines (23) that are not inhibited in growth by TGF-β indicated that TGF-β receptors type I (TGF-βRI) and type II (TGF-βRII) might mediate the multiple effects of TGF-β. Such cell lines lack TGF-βRI and TGF-βRII but always retain the TGF-β receptor type III (TGF-βRIII). Several other naturally occurring TGF-β-resistant tumor cell lines that lack TGF-βRII also lack TGF-βRI (2, 6, 15), and in other cases they also lack TGF-βRIII (24), but absence of the TGF-β inhibitory effect on growth most consistently correlates with loss of TGF-βRI and/or TGF-βRII. These observations led to the hypothesis that TGF-β-mediated signaling involves both TGF-βRI and TGF-βRII (19, 22, 23, 25). Recently, a cDNA encoding the TGF-βRII protein has been isolated by using an expression cloning strategy (26); its cytoplasmic region, when expressed in bacteria, demonstrated serine/threonine autophosphorylation activity (26). Furthermore, another, broadly expressed TGF-β receptor (type V) has also been reported to have kinase activity (27), but its involvement in signaling by TGF-β has not been established.

To determine whether the TGF-βRII-encoding gene is important in modulating the inhibitory effects of TGF-β_1 on growth, an expression plasmid containing the human TGF-βRII cDNA was transfected into Hep 3B-TR cells (a cell line resistant to TGF-β_1 in that the inhibitory effect on growth is absent and one that lacks the TGF-βRII gene). We show that expression of recombinant TGF-βRII restores the inhibitory action of TGF-β_1 on growth and, in the presence of ligand, forms a noncovalent complex with TGF-βRI. These experiments provide direct evidence that responsiveness to TGF-β in the transfected cells correlates with ligand binding to a heteromeric receptor complex.

MATERIALS AND METHODS

Cell Culture and Growth-Inhibition Assays. Hep 3B-TR cells were established from a human hepatoma line sensitive to TGF-β (Hep 3B-TS) cells (ATCC no. HB8064) by exposure to low TGF-β_1 concentrations (0.1–1 ng/ml) (R & D Systems, Minneapolis) without any chemical mutagens (32). Cells were routinely passaged in minimal essential medium (MEM)/penicillin/streptomycin/10% fetal bovine serum (GIBCO/BRL). Growth was assayed after cells were plated at 5×10^4 cells per 35-mm plastic tissue culture dish in MEM/10% fetal bovine serum. After 24 hr, medium was replaced with the same medium but containing TGF-β_1 at various concentrations; the medium was then changed every 3 days. At various intervals, cultured cells were treated with 0.02% trypsin and suspended in 1 ml of phosphate-buffered saline (PBS)/10% calf serum. Absorbance at 660 nm was measured spectrophotometrically. Control experiments demonstrated a linear correlation between Hep 3B-TS cell density and absorbance at 660 nm measured in single-cell suspensions by hemocytometer or by spectrophotometer.

Expression Plasmids and Transfection. A recombinant plasmid was constructed (26) by inserting the ≈4.5-kbp full-length cDNA of TGF-βRII into the *Eco*RI site of the mammalian expression vector pcDNAI/neo (Invitrogen, San Diego). Recombinant plasmids, which expressed both sense and antisense TGF-βRII genes, were purified by Qiagen-pack 500 (Qiagen, Chatsworth, CA). Plasmid DNAs (20 μg), rendered linear by the *Sfi* I restriction enzyme, were transfected by electroporation at 250 V and 960 μF capacitance,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TGF-β, transforming growth factor β; TGF-βRI, TGF-βRII, and TGF-βRIII, TGF-β receptor types I, II, and III, respectively; Hep 3B-TS, human hepatoma cell line sensitive to TGF-β; Hep 3B-TR, Hep 3B hepatoma cell line resistant to TGF-β.

§To whom reprint requests should be addressed at: E1552 BST, Liver Transplantation, Department of Surgery, University of Pittsburgh, Terrace and Lothrop Streets, Pittsburgh, PA 15213.

using the Gene Pulser (Bio-Rad). Forty-eight hours after transfection, cells were treated with Geneticin (G418) (400 μg/ml) (GIBCO/BRL). G418-resistant clones were isolated by limiting dilution and were individually expanded. These clones were screened for expression of TGF-βRII and for reappearance of TGF-β sensitivity by culturing with or without TGF-β_1, at 0.5 ng/ml.

RNA and DNA Analysis. Total RNA (30 μg) was prepared from Hep 3B-TS, Hep 3B-TR, and clone 2 (Hep 3B-TR cells stably transfected with TGF-βRII cDNA) by using RNAzol (Biotecx, Houston), and RNA samples were resolved by denaturing glyoxal-agarose gel electrophoresis (28). The separated RNAs were transferred to a Zetabind nylon membrane (Whatman) and were crosslinked with UV light (254 nm). The membrane was hybridized to a ^{32}P-labeled TGF-βRII cDNA probe according to standard protocols (28) before autoradiography at −70°C. A ^{32}P-labeled oligonucleotide hybridization probe for human 28S ribosomal RNA (Oncogene Science, Manhasset, NY) was used to compare the amounts of RNA transferred onto the membrane.

For DNA analysis, 10-μg samples of DNA prepared from each cell line were digested for 3 hr with either *Hin*dIII or *Eco*RI, electrophoresed in a 1% agarose gel, and subjected to Southern transfer and hybridization with the TGF-βRII cDNA probe, according to standard protocols (28). A human β-actin probe (29) was used to verify the digestion and transfer efficiency of DNAs.

Receptor Affinity-Labeling Assays. Binding and crosslinking of ^{125}I-labeled TGF-β_1 to cells grown on six-well trays or 100-mm dishes (Fisher) was as described (26). Crosslinked proteins were resolved by 5–10% linear-gradient SDS/PAGE under reducing conditions and exposed to XAR film (Kodak) at −70°C. To test the sensitivity of TGF-βRI to the reductant dithiothreitol, cells were incubated for 5 min at 37°C in the presence of 1 mM dithiothreitol before adding ^{125}I-labeled TGF-β_1.

Immunoprecipitation of Receptors with Anti-TGF-βRII Polyclonal Antiserum. Cell-surface TGF-β receptors were labeled with ^{125}I-labeled TGF-β_1 as described above, except that saturating concentrations of radioligand (0.3 nM) were used to amplify the receptor signal. A polyclonal rabbit antiserum specific for the C-terminal 16-amino acid epitope (H2-D) of the human TGF-βRII (H.Y.L., unpublished results) was raised. Immunoglobulins (IgGs) were prepared from the crude rabbit serum by ammonium sulfate precipitation followed by DEAE chromatography (30) before use for immunoprecipitation (IP) reactions. Affinity-labeled extracts were diluted to 1-ml final volume of ice-cold IP buffer (1% Triton X-100/0.5% sodium deoxycholate/0.25% SDS/2 mM phenylmethylsulfonyl fluoride). Extracts were precleared by incubation with preimmune serum. Then, the IgG fraction was added at 30 μg/ml alone or with an equimolar amount of peptide H2-D to inhibit antibody binding to the receptor. Protein A-Sepharose (Sigma) was used to precipitate the immunocomplexes, and the pellets were thoroughly washed in the above IP buffer, dissolved in Laemmli loading buffer, and subjected to 5–10% linear gradient SDS/PAGE before autoradiography.

RESULTS

Growth Response to TGF-β_1. Growth of Hep 3B-TS cells was effectively inhibited by TGF-β_1; maximal inhibition of growth was achieved at 0.4 ng/ml (Figs. 1 *A* and 2). The Hep 3B-TR cells were not inhibited by TGF-β_1 at concentrations of up to 5 ng/ml (Fig. 1*B*).

After transfection with TGF-βRII cDNA, G418-resistant clones were expanded and examined for growth inhibition. Populations of G418-resistant cells that were transfected either with pcDNAI/neo/TGF-βRII (sense or antisense)

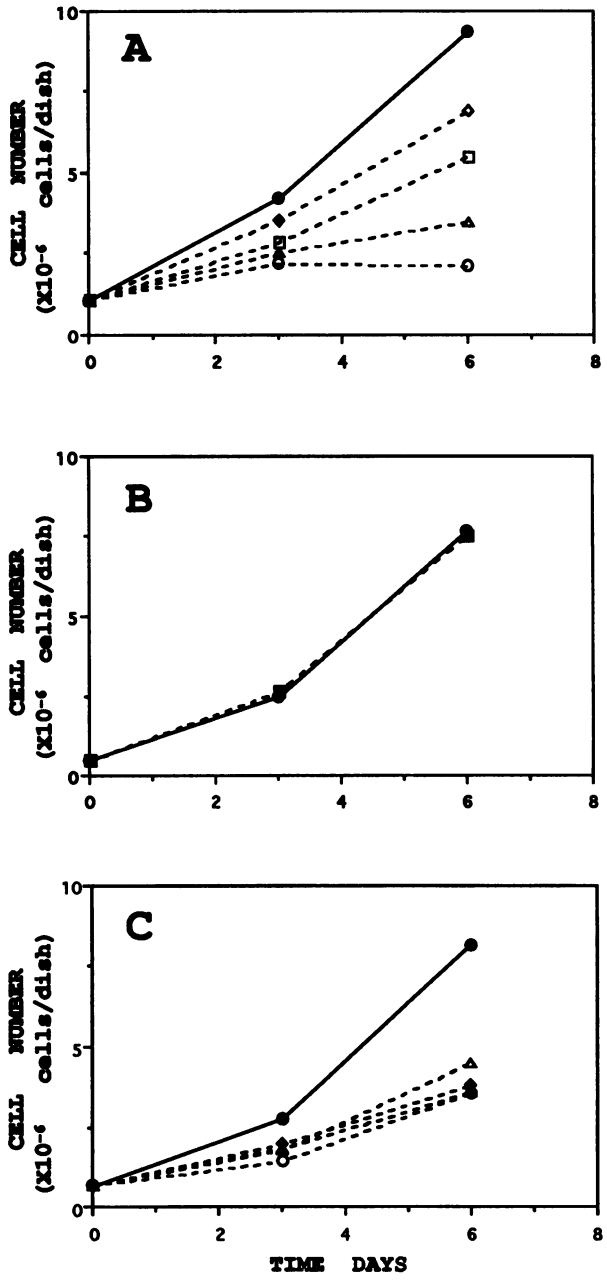

FIG. 1. Effect of TGF-β_1 on growth of Hep 3B-TS (*A*), Hep 3B-TR (*B*), and clone 2 (*C*) cells. Values were the means of duplicate dishes, and the experiments were repeated at least three times. Concentrations of TGF-β_1 are 0 ng/ml (●), 0.05 ng/ml (◇), 0.1 ng/ml (□), 0.2 ng/ml (△), 0.6 ng/ml (○), 1 ng/ml (▲), 2 ng/ml (◆), and 5 ng/ml (■) (effects of concentrations as high as 5 ng/ml are shown in *B*).

plasmid showed little, if any, inhibition by TGF-β_1, even at 2 ng/ml (data not shown). Consequently, individual G418-resistant clones were isolated by limiting dilution after transfection and expanded separately with or without TGF-β_1 at 1 ng/ml. Two selected clones were inhibited in growth by TGF-β_1 (clone 2, Fig. 1*C* and clone 24, Fig. 2), although with decreased sensitivity compared with Hep 3B-TS cells. Individual G418-resistant clones expressing the antisense TGF-βRII cDNA were not inhibited by TGF-β_1 (data not shown).

RNA and DNA Analysis of the TGF-βRII-Encoding Gene. The Hep 3B-TS cell line expressed TGF-βRII mRNA (Fig. 3, lane 1), whereas the Hep 3B-TR cells expressed none (Fig. 3, lane 2). Clone 2 (Fig. 3, lane 3) and clone 24 (data not shown)

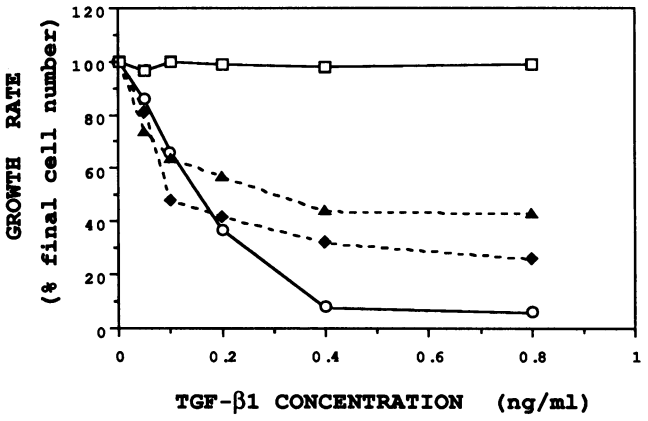

FIG. 2. Effect of TGF-β_1 on growth of cell clones during a 6-day culture. ○, Hep 3B-TS cells; □, Hep 3B-TR; ▲, clone 2; and ◆, clone 24.

did express TGF-βRII message. We did not detect TGF-βRII mRNA expression in any of the four G418 and TGF-β-resistant clones tested that were derived after transfection with the sense TGF-βRII vector (data not shown).

Southern analysis showed that the Hep 3B-TS cells contained TGF-βRII genomic sequences, whereas the Hep 3B-TR cells did not (Fig. 4). Transfection of TGF-βRII cDNA into Hep 3B-TR cells resulted in appearance of the recombinant TGF-βRII-specific DNA sequences. With either *Hin*dIII or *Eco*RI restriction enzymes, Hep 3B-TS cells and clones 2 and 24 showed different patterns on Southern blots. Thus, the TGF-β-sensitive phenotype of clones 2 and 24 was not due to cell contamination by Hep 3B-TS cells. The different Southern patterns in clones 2 and 24 resulted from independent integration events of the TGF-βRII cDNA in the host cell genome, thus proving the clonal origin of these two cell lines.

Chemical Crosslinking of ^{125}I-Labeled TGF-β_1 to Cell-Surface Receptors. Hep 3B-TS cells express three detectable TGF-β-binding proteins, TGF-βRI (55 kDa), TGF-βRII (80 kDa), and TGF-βRIII (280 kDa) (Fig. 5*A*, lanes 1 and 2). TGF-βRI and TGF-βRII were more abundantly expressed than TGF-βRIII. Binding to each of these proteins was saturable, as indicated by their disappearance in the presence of 1 nM (20-fold excess) unlabeled TGF-β_1 (data not shown). Hep 3B-TR cells expressed no detectable TGF-βRII or TGF-βRI. However, increased levels of TGF-βRIII were found, as well as several additional binding proteins of undetermined origin, two of which (named DDT-R, 50 and 42

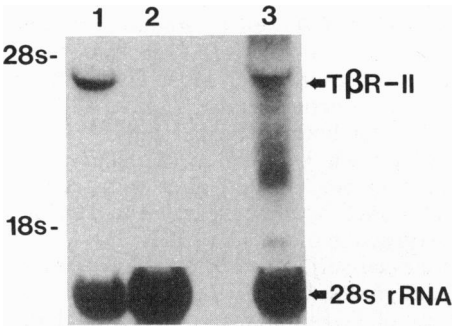

FIG. 3. Expression of TGF-βRII mRNA, determined by RNA hybridization. Each lane contained 30 μg of total RNA prepared from Hep 3B-TS cells (lane 1), Hep 3B-TR cells (lane 2), or clone 2 (lane 3). Equivalent RNA loading and transfer were confirmed by subsequent probing with a human 28S ribosomal RNA oligonucleotide. Positions of TGF-βRII (labeled TβR-II) and 28S RNAs are indicated by arrows.

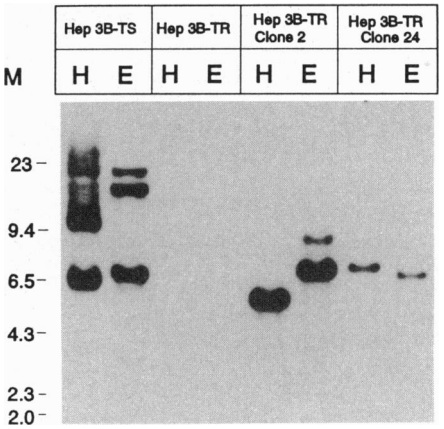

FIG. 4. DNA analysis by Southern hybridization. Ten micrograms of DNA prepared from Hep 3B-TS cells, Hep 3B-TR cells, clone 2, or clone 24 were digested by either *Hin*dIII (H) or *Eco*RI (E) for 3 hr at 37°C before electrophoresis, and the filter was hybridized with the full-length human TGF-βRII cDNA. Control hybridization of the filter with an actin DNA probe (data not shown) revealed equal intensities of bands in all four DNAs, indicating equal loading and transfer of DNA in all lanes.

kDa) were the most abundant species (Fig. 5*A*, lane 3). These small binding proteins are not TGF-βRI because TGF-β binding to TGF-βRI is abrogated by treatment with dithiothreitol (25) (Fig. 5*A*, lane 2), whereas dithiothreitol treatment had no effect on the DTT-R proteins (Fig. 5*A*, lane 4). Furthermore, binding of ^{125}I-labeled TGF-β_1 to cell-surface TGF-βRI and TGF-βRII in Hep 3B-TS cells was not competed against by 100-fold excess TGF-β_2; yet binding to the DTT-R species in Hep 3B-TR was readily competitive and to the same extent as was binding of ^{125}I-labeled TGF-β_1 to TGF-βRIII (data not shown). After transfection of TGF-βRII cDNA into Hep 3B-TR cells, expression of TGF-βRII was barely detectable at the cell surface by chemical crosslinking of ^{125}I-labeled TGF-β_1 in either clone 2 (Fig. 5*A*, lanes 5 and 6) or clone 24 (data not shown), even though the growth of these two transfected clones was inhibited by TGF-β_1, although less than that of the parental Hep 3B-TS cells.

Immunoprecipitation with Anti-TGF-βRII Polyclonal Serum. Anti-TGF-βRII polyclonal serum immunoprecipitated labeled TGF-βRII from Hep 3B-TS and clone 2 cells incubated with ^{125}I-labeled TGF-β_1 and then subjected to crosslinking (Fig. 5*B*, lanes 1 and 5). Immunoprecipitable receptors could not be detected in similarly labeled Hep 3B-TR cells, even after prolonged autoradiography (Fig. 5*B*, lane 3). The levels of immunoprecipitated TGF-βRII from clone 2 were at least 10-fold lower than those in Hep 3B-TS cells. Both precipitates contained a labeled species with identical electrophoretic mobility as TGF-βRI (Fig. 5*B*, lanes 1 and 5) and characteristic sensitivity to dithiothreitol (data not shown). As a control, peptide H2-D, against which the antiserum was raised, suppressed the immunoprecipitation of both TGF-βRII and TGF-βRI (Fig. 5*B*, lanes 2 and 6). The efficient coimmunoprecipitation of TGF-βRI with TGF-βRII, even with 0.25% SDS during immunoprecipitation, suggests a strong interaction of the two receptors on the cell surface when they bind ligand. Under these conditions very small amounts of labeled TGF-βRIII coimmunoprecipitated, indicating a much weaker interaction between ligand-bound cell-surface TGF-βRII and TGF-βRIII, as we have consistently seen in a variety of other cell lines (A.M., unpublished work). Because the coimmunoprecipitated receptors resolve on the reducing SDS/PAGE, the receptor complexes are not linked covalently. Boiling samples in 1% SDS before addition of the anti-TGF-βRII serum abolishes immunoprecipitation

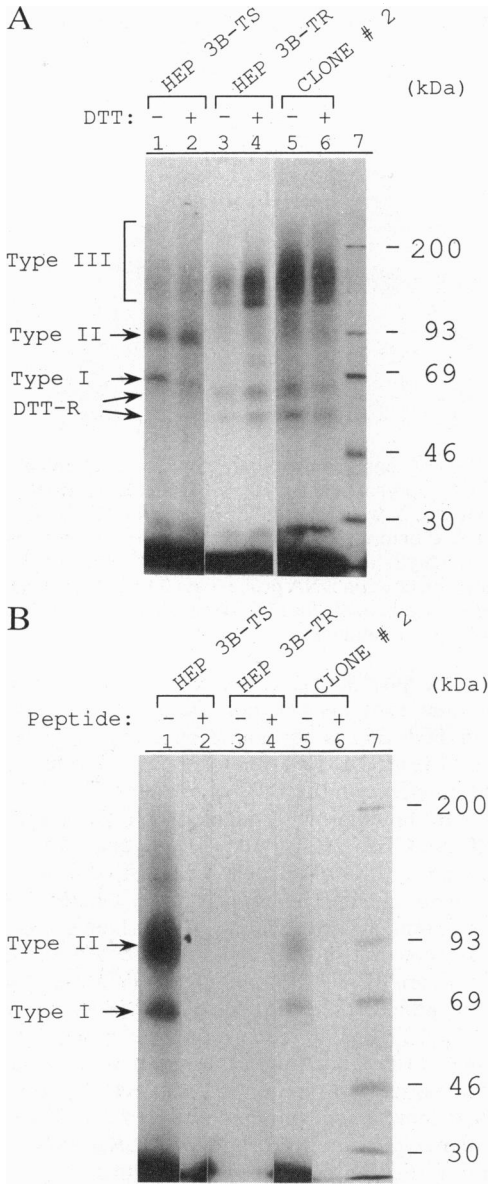

FIG. 5. (*A*) Chemical crosslinking of ^{125}I-labeled TGF-β_1 to Hep 3B-TS, Hep 3B-TR, and transfected clone 2. Hep 3B-TS (lanes 1 and 2), Hep 3B-TR (lanes 3 and 4), clone 2 (lanes 5 and 6) cells were incubated with 50 pM of ^{125}I-labeled TGF-β_1 and crosslinked with disuccinimidyl suberate before analysis on SDS/PAGE. In lanes 2, 4, and 6 cells were treated with 1 mM dithiothreitol for 5 min at 37°C before binding of ^{125}I-labeled TGF-β_1. Autoradiograms were exposed for 12 days except for lanes 3 and 4, which were exposed for 6 days. The different TGF-β receptor subtypes and the additional dithiothreitol (DTT)-resistant (DTT-R) binding proteins in Hep 3B-TR are indicated, as are molecular mass markers (lane 7). (*B*) Immunoprecipitation with anti-TGF-βRII serum. Hep 3B-TS (lanes 1 and 2), Hep 3B-TR (lanes 3 and 4), and clone 2 (lanes 5 and 6) were affinity-labeled with 0.3 nM ^{125}I-labeled TGF-β_1. Detergent extracts from these cells were immunoprecipitated with anti-TGF-βRII serum in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of equimolar amounts of immunogenic peptide H2-D. Autoradiography was done for 12 days. The two TGF-β receptor subtypes and molecular mass markers (lane 7) are also indicated.

of TGF-βRI but does not abolish that of TGF-βRII (data not shown). These results indicate that the coimmunoprecipitation of TGF-βRI with TGF-βRII is from interactions between the two receptor types in the presence of TGF-β, although the possibility that the antiserum recognized native but not denatured TGF-βRI cannot be fully excluded.

DISCUSSION

Characterization of a TGF-β-Resistant Hepatoma Cell Line. Hep 3B-TR cells were established by exposing Hep 3B-TS cells to stepwise increases in TGF-β_1 concentration (0.1–1 ng/ml) without the use of any chemical mutagen. These cells show resistance to the inhibitory action of TGF-β_1 on growth at concentrations as high as 5 ng/ml. The resistant cell line expressed a distinctive pattern of cell-surface TGF-β-binding proteins, as determined by chemical crosslinking of ^{125}I-labeled TGF-β_1. No apparent band(s) comigrating with TGF-βRII were detectable despite the presence of a faint smear in that region of the gel that resulted from high TGF-βRIII levels. Hep 3B-TR also had decreased levels of cell-surface binding by TGF-βRI and increased TGF-βRIII binding. An additional set of TGF-β-binding proteins was detected in this cell, among which two low-molecular-mass proteins (DTT-R, 50 and 42 kDa) were the most abundant. We confirmed that these were not identical to TGF-βRI, based on their different electrophoretic mobilities, their different sensitivities to dithiothreitol, and their different binding affinities for ^{125}I-labeled TGF-β_1 in the presence of excess amounts of competitor TGF-β_2. Specifically, a brief preincubation of cells with 1 mM dithiothreitol abolished the ability of TGF-βRI to bind ^{125}I-labeled TGF-β_1 without perturbing binding to TGF-βRII or TGF-βRIII. TGF-β_2 effectively abolished ^{125}I-TGF-β_1 binding to these two proteins, similar to its action on TGF-βRIII and characteristically different from that on TGF-βRI and TGF-βRII.

DNA analysis showed a loss of the TGF-βRII gene in Hep 3B-TR cells and consequent loss of TGF-βRII mRNA. Our data suggested that the loss of the TGF-βRII gene is the mechanism by which this hepatoma cell line lost its sensitivity to the inhibitory actions of TGF-β_1 on growth. This is a different situation, compared with other TGF-β-resistant cells, such as chemically induced lung epithelial cell mutants (22) or naturally occurring solid tumor cell lines (23), which lose responsiveness to TGF-β by acquiring point mutations in TGF-βRII protein (25).

Inhibitory Effect of TGF-β_1 on Growth Is Restored After Expression of TGF-βRII in Hep 3B-TR Cells. Here we demonstrate the reestablishment of growth inhibition by TGF-β_1 in Hep 3B-TR cells after transfection with an expression vector for TGF-βRII cDNA. In different G418-resistant clones the expression of TGF-βRII mRNA and the TGF-βRII cell-surface protein correlated perfectly with the response to TGF-β_1 of inhibiting growth. Specifically, after transfection of TGF-βRII cDNA into Hep 3B-TR cells, we established two clones (2 and 24) the growth of which was potently inhibited by TGF-β_1. RNA analysis showed that clones 2 and 24 expressed TGF-βRII mRNA. Chemical crosslinking of ^{125}I-labeled TGF-β_1 followed by immunoprecipitation with antiserum against TGF-βRII showed that the TGF-βRII protein had also reappeared. Most G418-resistant clones derived from transfection with the sense TGF-βRII vector tested negative for both TGF-βRII mRNA expression and inhibition of growth by TGF-β_1. Similarly, none of the G418-resistant clones isolated after transfection with the antisense TGF-βRII vector responded to TGF-β_1. We conclude that expression of the TGF-βRII cDNA in Hep 3B-TR cells restored cell-surface receptor levels adequate for the modulation of growth by TGF-β_1.

The pattern of TGF-β-binding proteins in clones 2 and 24 was virtually indistinguishable from that of the parental Hep 3B-TR cell line, when assayed by chemical crosslinking of ^{125}I-labeled TGF-β_1. The inability to detect TGF-βRII on the cell surface of clones 2 and 24 by this technique is from expression of a low number of receptors, as proven by immunoprecipitation with an antiserum against TGF-βRII. Clones 2 and 24, but not parental Hep 3B-TR cells, exhibited

a surface protein of mobility identical to TGF-βRII but at decreased levels compared with those in Hep 3B-TS cells. The lower levels of TGF-βRII on the cell surface correlate with the decreased sensitivity of the transfected clones to TGF-β_1 compared with the parental Hep 3B-TS cell line. Use of specific antiserum to identify TGF-β receptors, as exemplified here for TGF-βRII, greatly improves the sensitivity of detection. Even for cells in which TGF-β receptors cannot be visualized by the conventional binding-crosslinking technique (Fig. 5*A*), immunoprecipitation allows their clear detection (Fig. 5*B*). This technical advance may prove useful in reevaluating the expression patterns of TGF-β receptors in various cells that, as assayed by binding-crosslinking, appeared to lack such surface molecules. This technique is especially important in cases of TGF-β-responsive cells with very low cell-surface receptor numbers. Our experiments with clones 2 and 24 reinforce the hypothesis that very low levels of cell-surface receptors are adequate for TGF-β-mediated growth arrest, as has been seen in many other cell types (1, 18, 31).

TGF-βRII and TGF-βRI Form a Cell-Surface Complex with Bound TGF-β. Coimmunoprecipitation of TGF-βRI and TGF-βRII by antiserum against TGF-βRII in Hep 3B-TS cells and clone 2 indicates the formation of a complex between TGF-βRI and TGF-βRII on the cell surface. The facts that TGF-β1 is a disulfide-linked homodimer and that the coimmunoprecipitated receptors were revealed after chemical crosslinking to TGF-β_1 led to the model of two receptor species held in physical proximity via binding to each of the TGF-β_1 monomers in the dimer. TGF-βRI expression was not detectable by immunoprecipitation with anti-TGF-βRII serum in Hep 3B-TR cells that lack TGF-βRII, further supporting the idea that these two receptors are complexed together. Similar results have been reported (25) in mink lung cell mutants transfected with the same recombinant TGF-βRII cDNA. Thus, we conclude that TGF-βRI and TGF-βRII form a complex that may mediate the signal transduced by TGF-β_1 in this cell system. However, that TGF-βRII alone might mediate the signal by TGF-β is not excluded (14). Different types of signals could be generated by homodimers of TGF-βRI or TGF-βRII and heterooligomers of TGF-βRI and TGF-βRII. Hence, different cells might require different types of such intracellular signals for TGF-β-mediated inhibition of growth. Additionally, distinct receptor combinations may be used by a single cell type to mediate distinct growth-inhibitory and gene-regulatory effects in response to TGF-β.

The present results highlight the importance of growth regulation by TGF-β in the process of hepatic tumor development. Although Hep 3B-TS is a tumor cell line, it responds to TGF-β and expresses all three receptors for TGF-β. Our experiments clearly show that resistance to TGF-β can be achieved by *in vitro* selection of this cell type. Addressing the question of tumorigenicity and the differing biology of the parental, resistant, and transfected cell lines now becomes imperative.

We thank Drs. P. Knaus and Y. I. Henis for helpful discussions and Y. I. Henis for purification of the antiserum. A.M. is an Anna Fuller Fund postdoctoral fellow (Grant 719). This work was supported, in part, by National Institutes of Health Grant CA44602 (B.I.C.) and HC41484 (H.F.L.).

1. Roberts, A. B. & Sporn, M. B. (1990) in *Peptide Growth Factors and Their Receptors*, eds. Sporn, M. B. & Roberts, A. B. (Springer, Heidelberg), pp. 419–472.
2. Massague, J. (1990) *Annu. Rev. Cell Biol.* **6**, 597–641.
3. Moses, H. L., Yang, E. L. & Pietenpol, J. A. (1990) *Cell* **63**, 245–247.
4. Faust, N., Mead, J. E., Gruppuso, P. A. & Braun, L. (1990) *Ann. N.Y. Acad. Sci.* **593**, 231–242.
5. Carr, B. I., Hayashi, I., Branum, E. L. & Moses, H. L. (1986) *Cancer Res.* **44**, 2230–2234.
6. Kimchi, A., Wang, X.-F., Weinberg, R. A., Cheifetz, S. & Massague, J. (1988) *Science* **240**, 196–199.
7. Hoosein, N. M., McKnight, M. K., Levine, A. E., Mulder, K. M., Childress, K. E., Brattain, D. E. & Brattain, M. G. (1989) *Exp. Cell Res.* **181**, 442–453.
8. Frolik, C. A., Wakefield, L. M., Smith, D. M. & Sporn, M. B. (1984) *J. Biol. Chem.* **259**, 10995–11000.
9. Tucker, R. F., Branum, E. L., Shipley, G. D., Ryan, R. J. & Moses, H. L. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6757–6761.
10. Cheifetz, S., Like, B. & Massague, J. (1986) *J. Biol. Chem.* **261**, 9972–9978.
11. Cheifetz, S., Weatherbee, J. A., Tsang, M. L.-S., Anderson, J. K., Mole, J. E., Lucas, R. & Massague, J. (1987) *Cell* **48**, 409–415.
12. Cheifetz, S., Andres, J. L. & Massague, J. (1988) *J. Biol. Chem.* **263**, 16984–16991.
13. Fanger, B. O., Wakefield, L. M. & Sporn, M. B. (1986) *Biochemistry* **25**, 3083–3091.
14. Lin, H. Y. & Lodish, H. F. (1993) *Trends Cell Biol.* **3**, 14–19.
15. Massague, J., Boyd, F. T., Andres, J. L. & Cheifetz, S. (1990) *Ann. N.Y. Acad. Sci.* **593**, 59–72.
16. Massague, J. (1992) *Cell* **69**, 1067–1070.
17. Segarini, P. R. & Seyedin, S. M. (1988) *J. Biol. Chem.* **263**, 8366–8370.
18. Segarini, P. R., Rosen, D. M. & Seyedin, S. M. (1989) *Mol. Endocrinol.* **3**, 261–272.
19. Segarini, P. R. (1990) *Ann. N.Y. Acad. Sci.* **593**, 73–90.
20. Boyd, F. T. & Massague, J. (1989) *J. Biol. Chem.* **264**, 2272–2278.
21. Laiho, M., Weis, F. M. B. & Massague, J. (1990) *J. Biol. Chem.* **265**, 18518–18524.
22. Laiho, M., Weis, F. M. B., Boyd, F. T., Ignotz, R. A. & Massague, J. (1991) *J. Biol. Chem.* **266**, 9108–9112.
23. Geiser, A. G., Burmester, J. K., Webbink, R., Roberts, A. B. & Sporn, M. B. (1992) *J. Biol. Chem.* **267**, 2588–2593.
24. Siepl, C., Malipiero, U. V. & Fontana, A. (1991) *J. Immunol.* **146**, 3063–3067.
25. Wrana, J. L., Attisano, L., Caramo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.-F. & Massague, J. (1992) *Cell* **71**, 1003–1014.
26. Lin, H. Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R. A. & Lodish, H. F. (1992) *Cell* **68**, 775–785, and erratum (1992) **70**, 1069.
27. O'Grady, P., Lin, Q., Huang, S. S. & Huang, J. S. (1992) *J. Biol. Chem.* **267**, 21033–21037.
28. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Plainview, NY), 2nd Ed.
29. Gunning, P., Leavitt, J., Muscat, G., Ng, S.-Y. & Kedes, L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4831–4835.
30. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab., Plainview, NY).
31. Ohta, M., Greenberger, J. S., Anklesaria, P., Bassols, A. & Massague, J. (1987) *Nature (London)* **329**, 539–541.
32. Hasegawa, K., Carr, B. I., Wang, Z. & Whitson, R. M. (1992) *Proc. Am. Assoc. Cancer. Res.* **33**, 427.