Distribution and Modulation of the Cellular Receptor for Transforming Growth Factor-Beta

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Abstract. Scatchard analyses of the binding of transforming growth factor-beta (TGF-beta) to a wide variety of different cell types in culture revealed the universal presence of high affinity (K_d = 1–60 pM) receptors for TGF-beta on every cell type assayed, indicating a wide potential target range for TGF-beta action. There was a strong (r = +0.85) inverse relationship between the receptor affinity and the number of receptors expressed per cell, such that at low TGF-beta concentrations, essentially all cells bound a similar number of TGF-beta molecules per cell. The binding of TGF-beta to various cell types was not altered by many agents that affect the cellular response to TGF-beta, suggesting that modulation of TGF-beta binding to its receptor may not be a primary control mechanism in TGF-beta action. Similarly, in vitro transformation resulted in only relatively small changes in the cellular binding of TGF-beta, and for those cell types that exhibited ligand-induced down-regulation of the receptor, down-regulation was not extensive. Thus the strong conservation of binding observed between cell types is also seen within a given cell type under a variety of conditions, and receptor expression appears to be essentially constitutive. Finally, the biologically inactive form of TGF-beta, which constitutes >98% of autocrine TGF-beta secreted by all of the twelve different cell types assayed, was shown to be unable to bind to the receptor without prior activation in vitro. It is proposed that this may prevent premature interaction of autocrine ligand and receptor in the Golgi apparatus.

TRANSFORMING growth factor-beta (TGF-beta)[1] was originally characterized by its ability to induce reversible phenotypic transformation of non-neoplastic indicator cells (36, 41). It was proposed that transformed cells might have a selective growth advantage by secreting and responding to such growth factors in an autocrine fashion (13, 50, 52). However, TGF-beta has since been isolated and purified from a variety of normal tissues as well as neoplastic sources (43), suggesting a role for TGF-beta in normal cell physiology. The protein from all these sources is an acid-stable, disulfide-linked homodimer with an M_r of 25,000, and the sequence is highly conserved between species (14).

Evidence is accumulating that TGF-beta is an important regulator of growth and differentiation for a wide variety of both normal and transformed cell types. It stimulates the anchorage-independent growth of various normal fibroblastic cells, is mitogenic for certain fibroblasts in vitro, and stimulates wound healing, fibroblast proliferation, and angiogenesis in vivo (for a review, see reference 51). However, it inhibits the in vitro proliferation of mitogenically stimulated B and T lymphocytes (23, 24), various normal epithelial cell types (11, 35, 37, 49, 55), and of a number of human tumor lines (37, 40). This raises the possibility that the growth of certain normal cells may be regulated in a negative autocrine manner by TGF-beta, with lesions in this negative control process leading to uncontrolled cell proliferation (50). In addition to its effects on cell growth, TGF-beta is a potent inducer of differentiation for normal bronchial epithelial cells (35) and chondrocytes (48), while it inhibits the expression of differentiated function in preadipocytes (21), B and T lymphocytes (23, 24), natural killer cells (44), and myocytes (16).

A unique high affinity receptor for TGF-beta has been identified on normal rat kidney (NRK) cells (17) and other fibroblastic and epithelial cell lines (6, 17, 34, 54). The receptor is a disulfide-linked glycosylated dimer of M_r ~560,000 (15, 32), and preliminary evidence suggests that the TGF-beta receptor differs from the receptors for many other growth factors, in that it does not show ligand-induced clustering, nor does it appear to autophosphorylate (15).

In this paper we have examined the distribution and modulation of the TGF-beta receptor on a wide variety of cell types. We find the TGF-beta receptor to be universally expressed, and we demonstrate a novel inverse relationship between receptor number and affinity, such that at low TGF-beta concentrations all cell types bind essentially the same number of TGF-beta molecules per cell. This functional conservation of binding is also preserved within a given cell type

1. *Abbreviations used in this paper*: EGF, epidermal growth factor; HaSV, Harvey sarcoma virus; MoSV, Moloney sarcoma virus; NRK, normal rat kidney; PDGF, platelet-derived growth factor; TGF, transforming growth factor.

under various conditions. Thus many factors that modulate the cellular response to TGF-beta appear to have no effect on the binding of TGF-beta to its receptor, there is little or no ligand-induced receptor down-regulation, and the effects of in vitro transformation on TGF-beta binding are far less extensive than is observed for other growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (9, 53). Thus binding of TGF-beta to its receptor appears to be essentially unmodulated. Finally, we show that the biologically latent form of TGF-beta is unable to bind to the receptor, and we suggest that this may prevent premature association of ligand and receptor in the Golgi apparatus.

Materials and Methods

Cells

Pulmonary artery endothelial cells, normal human bronchial epithelial cells, mesothelial cells, and fibroblasts were all low passage explant cultures established and propagated in culture as described elsewhere (28–30, 47). Human tonsillar T lymphocytes were prepared by rosetting tonsillar mononuclear cells and activated as described previously (24). All remaining cell lines and strains were grown in DME (4.5 g/liter glucose) containing 5% FBS (Gibco, Grand Island, NY), with the exception of the NRK 49F cells which were grown in the same medium but one that contained 10% calf serum (MA Bioproducts, Walkersville, MD) in place of FBS.

Iodination of TGF-Beta

Pure TGF-beta isolated from human platelets (5) was iodinated to high specific activity (2 μCi/pmol; 80 μCi/μg), with essentially full retention of biological activity, using a modified chloramine T method (17). Typically >99% of the iodinated peptide was precipitable by TCA immediately after iodination, of which 95% was capable of specific binding to the receptor. The percentage of ^{125}I-TGF-beta capable of specific binding decreased linearly with time by ~0.35%/d thereafter, and the measured free TGF-beta concentration was routinely corrected to take this into account.

Binding Assay

For cells that grow attached to tissue culture plates, a solid-phase monolayer assay was used, while for the T lymphocytes a suspension binding assay was used. The monolayer assay was a modification of that described by Frolik et al. (17). Cells were seeded in their normal growth medium at ~10^5 cells/well in 24-well cluster plates and allowed to grow for 24 h, such that at the time of assay they were somewhat subconfluent and still actively growing. The monolayers were then washed with 1 ml/well of binding buffer (DME containing 0.1% BSA and 25 mM Hepes, pH 7.4) and incubated in this medium for 2 h at 37°C to allow dissociation of bound endogenous TGF-beta. Control experiments showed this procedure resulted in removal of 85–95% of pre-bound TGF-beta. After washing, cells were incubated in 0.2 ml binding buffer containing 1–500 pM ^{125}I-TGF-beta for 2 h at 22°C in a 5% CO_2 atmosphere. Nonspecific binding was determined in the presence of 10 nM unlabeled TGF-beta. At the end of the incubation, the free ligand concentration was determined from the medium counts, and the cells were then washed four times in ice-cold Hanks'-buffered saline (Gibco) containing 0.1% BSA. Bound counts were solubilized by the addition of solubilization buffer (1% Triton X-100, 10% glycerol, 20 mM Hepes, pH 7.4). This solubilization protocol was used instead of the usual NaOH or formic acid–based extraction procedures because it reduced the recovery of ligand nonspecifically bound to the plastic (~30% of input counts) rather than to the cells. Typically, the level of nonspecific binding ranged from ~5% of total binding at low free ligand concentrations (1 pM) to >50% of total binding at high ligand concentrations (>200 pM), when the receptor was saturated. The cpm specifically bound generally ranged from 100 to 10,000 cpm/well over the saturation curve. Cell number was determined on wells treated identically up to the solubilization step. TGF-beta receptors on lymphocytes in suspension were assayed as described previously (24), except that the binding buffer used was MEM without bicarbonate, containing 0.1% BSA and 25 mM Hepes, pH 7.4. Binding data were analyzed according to the method of Scatchard (46).

All binding assays were performed at 22°C for comparative purposes since many of the cell types assayed detached from the culture plates during prolonged serum-free incubation at 4°C. The decrease in TCA precipitability of TGF-beta in the medium after 2 h incubation at 22°C was usually ≤3%, indicating minimal ligand degradation at this temperature. Control experiments on A549 human lung carcinoma cells confirmed that there was no ligand-induced receptor down-regulation at this temperature. Thus use of Scatchard analysis, which assumes equilibrium between free and bound ligand, is probably valid for the binding data obtained under these experimental conditions, and at very least gives useful comparative values for the K_d and receptor number per cell. Receptors could reliably be detected down to 1,000 receptors/cell for the monolayer assay and 100 receptors/cell for the suspension assay. In addition, the assay was reproducible. For example, assay of four different lots of normal human bronchial epithelial cells from different donors, using four different batches of ^{125}I-TGF-beta gave mean receptor parameters of 12 ± 2 pM for the K_d, and 10,000 ± 3,000 for the number of receptors, representing an interassay variation of <±20% for the affinity and ±30% for the receptor number. Some of this variation could be accounted for by individual differences rather than interassay variation. Thus for the A549 human lung carcinoma line, three separate assays using three different batches of iodinated TGF-beta gave a mean K_d of 15 ± 3 pM, and a receptor number of 10,000 ± 1,400 receptors/cells, corresponding to an interassay variation of only ±17% for the affinity and ±14% for the receptor number. Nevertheless, where receptor parameters were to be compared on matched pairs of cell types (e.g., transformed vs. untransformed), the cells were assayed on the same day, under identical conditions.

Collection of Conditioned Media

Cells were grown to ~80% confluence in Falcon T175 flasks in DME/5% FBS and were then washed three times at 2-h intervals with serum-free DME. The cells were then incubated for 18 h in serum-free DME and this medium was discarded. Finally, cells were incubated for 24 h in 40 ml/flask fresh serum-free DME and this medium was collected for processing. For the Myc-1 cells, 1% Nutridoma-SP (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was included with the DME and this improved cell attachment and viability. Nutridoma does not contain any assayable TGF-beta (data not shown). The cell number and viability (always >80%) at the end of the collection period were determined. At this point, conditioned media were treated in one of two ways.

Acid Dialysis. Immediately after collection, the medium was clarified by ultracentrifugation for 1 h at 100,000 g_{max}. Clarified medium was dialyzed extensively (Spectrapor dialysis tubing, 3,000 mw cutoff) against 1 M acetic acid, lyophilized, and resuspended in 1/10th vol 1 M acetic acid and redialyzed. This second dialysis step appeared to be necessary to remove residual salt and other dialyzable components that interfered with the quantitative radioreceptor assay. Typically samples were then prepared for the radioreceptor assay by lyophilizing 1–2 ml with 1/5 vol 1 M acetic acid containing 0.1% BSA and resuspending the lyophilized powder in binding buffer. The overall recovery of TGF-beta was 40%, and the final data were corrected by this factor.

Transient Acidification. BSA was added to neutral conditioned medium to a final concentration of 0.1%, before clarification of the medium by ultracentrifugation in siliconized tubes at 100,000 g_{av} for 1 h. Recovery of TGF-beta after this step was 90%. Aliquots of the medium were then acidified by addition of HCl (final pH 3.2), incubated for 1 h at 4°C, and reneutralized by the addition of 1/40 vol of 1 M Na-Hepes pH 7.0, and an appropriate volume of NaOH. In corresponding neutral aliquots, acid, base, and Hepes buffer were premixed before addition.

Quantitation of TGF-Beta in Conditioned Media by Radioreceptor Assay

TGF-beta levels in the conditioned media were determined using a two-step quantitative radioreceptor assay, so that any TGF-beta binding proteins that might be present would not score as false positives. A549 cells were seeded at 2 × 10^5 cells/well in 24-well cluster plates and grown for 18 h. Cell monolayers were washed once with binding buffer and then incubated with serial dilutions of the sample to be tested in 0.2 ml/well binding buffer (see above). For assay of neutral or transiently acidified samples, the binding buffer had NaOH and HCl added, so that the ionic strength and composition of the buffer was identical to that of the samples. Samples were allowed to bind for 2 h at 22°C, then the monolayers were washed twice with ice-cold binding buffer and 0.2 ml/well of a 50 pM stock of ^{125}TGF-beta in binding buffer was added, for a further 2-h incubation period. Monolayers were then washed with ice-cold Hanks'-buffered saline containing 0.1% BSA, and

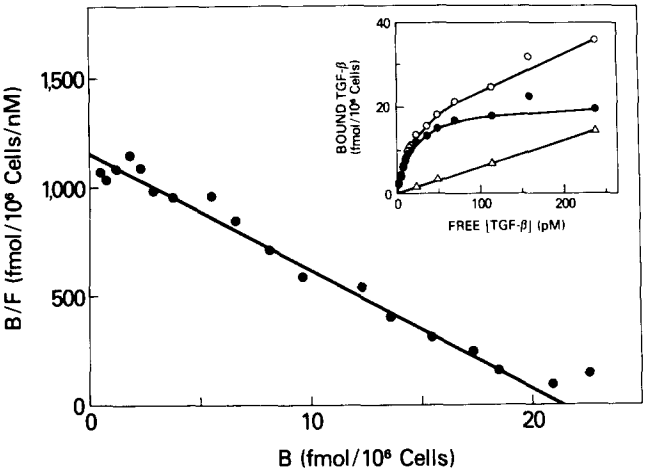

Figure 1. Scatchard plot of TGF-beta binding to the human colon carcinoma cell line HT29. Specifically bound (B) and free (F) ligand were measured as described in Materials and Methods. The data are presented in a Scatchard plot and the inset shows TGF-beta binding as a function of the free ligand concentration, where the TGF-beta specifically bound (●) is the difference beween the total (○) and nonspecific (△) binding. Each point is the mean of two determinations that generally differed by <5%.

bound counts solubilized as described above. TGF-beta present in conditioned media samples was determined by comparison with a standard curve of purified platelet TGF-beta in the range 1-1,000 pM. The detection limits of the assay were 25–50 pg/0.2 ml.

Antibodies to TGF-Beta

Rabbits were immunized with three doses of human platelet TGF-beta coupled to keyhole limpet hemocyanin (0.1 mg TGF-beta/dose per rabbit) in Freund's adjuvant at 3-wk intervals. The immunoglobulin fraction was purified by affinity chromatography on protein A-Sepharose (19). The purified antibodies were shown to inhibit the binding of TGF-beta to its receptor and neutralize TGF-beta biological activity (35).

Results

Distribution of TGF-Beta Receptors

The binding of TGF-beta to more than 30 different cell lines and strains were assayed and the binding data were subjected to Scatchard analysis. A typical set of binding curves and corresponding Scatchard analysis are shown for the human colon carcinoma line HT29 in Fig. 1. Specific binding was essentially saturated by 100 pM free TGF-beta and Scatchard analysis gave a linear plot characteristic of a single high affinity binding site with 13,000 receptors/cell and a dissociation constant (K_d) of 17 pM. The relatively high background of nonspecific binding (1–5% of input cpm) was observed for all cell types assayed, making it difficult to exclude the possible existence of additional lower affinity sites (K_d > 200 pM) in some cases, since data in which the nonspecific binding exceeded 50% of the total binding could not be analyzed reliably. Representative Scatchard plots for cells with low (1,000), intermediate (19,000), and high (81,000) numbers of high affinity receptors per cell are shown in Fig. 2.

The number and affinity of the receptors for TGF-beta on a wide variety of different cell types are summarized in Table I. Every cell type assayed expressed high affinity receptors for TGF-beta; these receptors were present on cells of epithelial, mesenchymal, and hematopoietic origin, on normal and tumor cells, on cells derived from adult and embryonic tissues, and on cells from different species (human, rodent, or bovine).

Relationship between Receptor Number and Affinity

The relationship between receptor affinity and number, for 34 out of the 35 cell types listed in Table I, is shown in the double log plot of Fig. 3. Linear regression analysis revealed a linear relationship between the dissociation constant K_d and the number of TGF-beta receptors per cell, such that cells with more receptors had a correspondingly lower af-

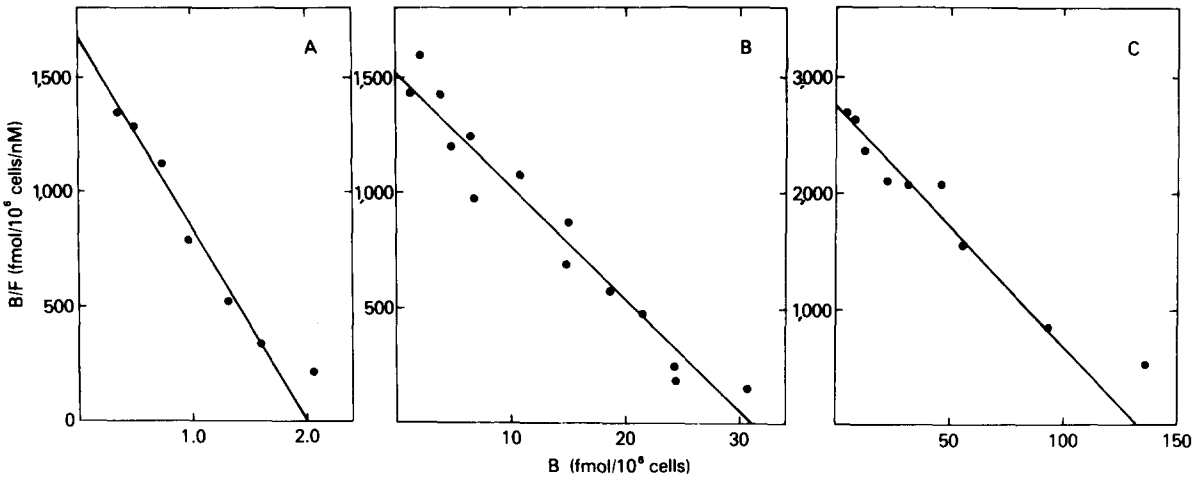

Figure 2. Representative Scatchard plots of TGF-beta binding to cells expressing low, intermediate, and high numbers of TGF-beta receptors per cell. Specifically bound (B) and free (F) ligand were determined as described in Materials and Methods. Each point is the mean of two determinations that generally differed by <5%. The cell lines represented are: (*A*) A2058, human melanoma line (1,000 receptors/cell); (*B*) passage 3 adult human bronchial fibroblasts (19,000 receptors/cell); (*C*) Swiss 3T3 mouse embryonic fibroblast line (81,000 receptors/cell).

Table I. Distribution and Properties of the TGF-Beta Receptor on a Wide Spectrum of Cell Types

Cell type		TGF-Beta receptor parameters	
		K_d	No./cell
		pM	
Normal fibroblastic			
HEL 299	Human embryonic lung	28	7,000
WI 38	Human embryonic lung	27	17,000
Flow 2000	Human embryonic lung	13	10,000
LL 47	Human adult lung	40	18,000
–	Human adult lung (P3)	19	19,000
NRK-49F	Rat kidney	40	25,000
RAT-1	Rat embryonic	22	14,000
FR-3T3	Rat embryonic	28	25,000
C3H10T1/2	Mouse embryonic	60	20,000
NIH 3T3	Mouse embryonic	26	36,000
SWISS 3T3	Mouse embryonic	45	81,000
AKR-2B	Mouse embryonic	16	9,000
–	Mouse adult	47	41,000
Normal endothelial			
–	Bovine pulmonary artery (P2)	26	9,000
Normal mesothelial			
–	Human mesothelial (P1)	10	20,000
Normal epithelial			
NHBE 555	Human bronchial (P3)	10	10,000
NRK-52E	Rat kidney	5	9,000
MMCE c17	Rat	3	1,500
Normal hematopoietic			
–	Human tonsillar T lymphocytes (Concanavalin A-stimulated)	2	600
Human tumor			
A673	Rhabdomyosarcoma	10	3,000
A204	Rhabdomyosarcoma	41	3,000
RD	Rhabdomyosarcoma	23	7,000
HT1080	Fibrosarcoma	6	7,000
DND10B67	Mesothelioma	8	6,000
VAMT-1	Mesothelioma	2	3,000
A2058	Melanoma	1	1,000
FmX-II	Melanoma	38	14,500
A549	Lung adenocarcinoma	13	10,000
Calu I	Lung carcinoma (squamous)	7	8,000
HUT 292	Lung carcinoma (mucoepidermoid)	1	2,000
SW 900	Lung carcinoma (undifferentiated)	4	4,000
T24	Bladder carcinoma	9	9,000
A431	Cervical carcinoma	22	12,000
HT 29	Colon carcinoma	17	13,000
A2380	Pancreatic carcinoma	29	27,000

The binding of ^{125}I-TGF-beta to various cell types at 22°C was measured as described in Materials and Methods, and binding data were analyzed by the method of Scatchard (46). The passage number of low passage cultures is indicated. From repeated assays of the same cell type on different days and with different batches of iodinated ligand, the interassay variation was shown to be $<\pm 20\%$ for the K_d of all cell types and $<\pm 15\%$ for receptor number on cell lines, with $<\pm 30\%$ variation for low passage cultures of the same cell type from different individuals.

finity (higher K_d). Only the human rhabdomyosarcoma line A204 did not obey this relationship and showed an unusual combination of low affinity and low number of receptors. Inclusion of this cell line in the linear regression analysis reduced the correlation coefficient from +0.85 to +0.77. The unusual binding parameters of this cell line may explain why

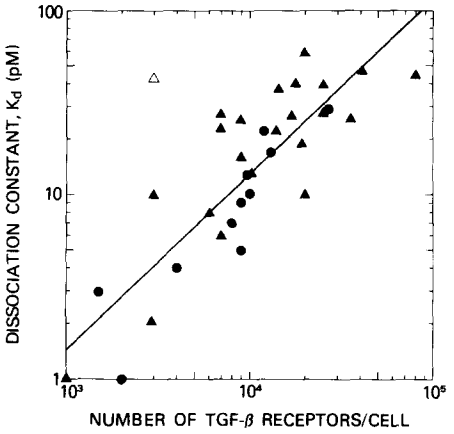

Figure 3. Relationship between receptor affinity and the number of cellular receptors for TGF-beta on normal and tumor cells. The relationship between the number of TGF-beta receptors per cell and the dissociation constant, K_d, was determined in a double log plot using the data in Table I for the complete set of normal and tumor cells of epithelial (●) and mesenchymal or neuroectodermal (▲) origin. Linear regression analysis revealed an essentially linear relationship between the two parameters with a correlation coefficient of +0.85. Only the human rhabdomyosarcoma line A204 (△) did not obey the relationship and was not included in the regression analysis (see Results).

it appeared to have no receptors in a one-point binding assay (54). T lymphocytes were omitted from the linear regression analysis because they have a small surface area relative to that of the typical cell in tissue culture.

One prediction from this relationship is that at low TGF-beta concentrations, all cells will bind approximately the same number of TGF-beta molecules per cell. The data in Fig. 4 show that in the range of concentrations for which TGF-beta is normally physiologically active (0.1–10 pM), a representative selection of human cell lines with widely differing TGF-beta binding parameters do indeed bind approximately the same number of TGF-beta molecules per cell at any given TGF-beta concentration. Again, the rhabdomyosarcoma line A204 is a notable exception, binding at least 10-fold less TGF-beta at any given ambient TGF-beta

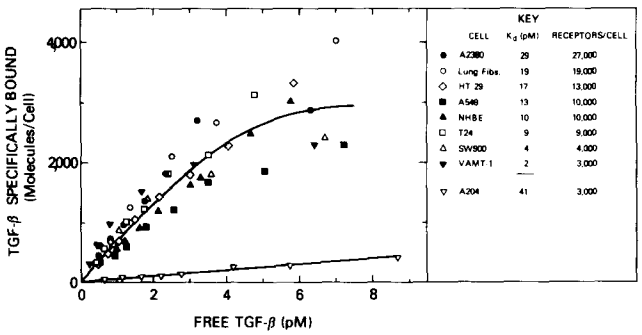

Figure 4. All cell types (except A204) bind essentially the same number of TGF-beta molecules per cell at low TGF-beta concentrations. The specific binding of TGF-beta to its cellular receptors was determined for a series of human cells with widely differing receptor properties (see key) as described in Materials and Methods. Each point is the mean of two determinations that differed by <5%.

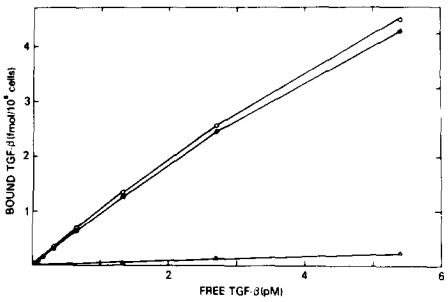

Figure 5. Binding of low concentrations of TGF-beta to A549 human lung adenocarcinoma cells: lack of evidence for "super-high" affinity sites. The sensitivity of the standard binding assay was increased 6–8-fold by seeding cells at 5×10^5 cells/well in 6-well cluster dishes and using high specific activity (4 μCi/pmol) ^{125}I-TGF-beta. Each point is the mean of two determinations differing by <10%. The amount of ligand specifically bound (●) is the difference between the total (○) and nonspecific (△) binding, and ranged from 125 cpm/well to 6,000 cpm/well over the range of free TGF-beta concentrations from 50 fM to 5 pM.

concentration. The very different binding properties of this one line make it unlikely that the similar levels of binding seen in the other cell types are merely an assay artifact, since assay conditions were essentially identical for all cell types.

Relationship between Ligand Binding and Biological Response

For a number of cell types, the half-maximal biological effect of TGF-beta is observed at a much lower concentration of TGF-beta than that required to half-saturate the receptor. Thus TGF-beta inhibits DNA synthesis in normal human bronchial epithelial cells half-maximally at 0.4 pM (35), while the receptor has a K_d of 10 pM, and similarly, the receptor on the lung adenocarcinoma line has a K_d of 13 pM, whereas TGF-beta inhibits the growth of these cells half-maximally at 0.4 pM (40). The possible existence of previously undetected "super-high" affinity binding sites that might mediate this biological response was tested for in A549 cells, using higher specific activity ^{125}I-TGF-beta (4 μCi/pmol) and increased numbers of cells, to increase the sensitivity of the assay ~6–8-fold. The results in Fig. 5 show no indication of a saturable higher affinity site in the range of free TGF-beta concentrations from 50 fM to 5 pM. Extrapolation of the Scatchard curve obtained from this binding data shows the binding detected corresponds to a receptor with a K_d of ~18 pM, and 11,000 sites/cell (data not shown), which is the same class of binding sites as is detected under less sensitive conditions. Since the more sensitive conditions would allow detection of <200 receptors/cell, if a higher affinity receptor is present it must represent <2% of the total receptor population. While this possibility cannot be ruled out, it seems likely that the receptor identified by these binding assays is the one that mediates the biological response, and that maximum biological response to TGF-beta may be elicited at 10–50% receptor occupancy. However, it should be noted that the time-scale and culture conditions are often very different for the binding and biological assays, and that this may be a source of some of the discrepancy. Full biological response at fractional receptor occupancy has also been observed for EGF and insulin (1, 25).

Modulation of the TGF-Beta Receptor by Heterologous Agents

Many of the agents that modulate EGF/TGF-alpha action act by altering the affinity or number of available EGF receptors (3, 8, 22, 31, 45). The possibility that various factors that enhance or antagonize TGF-beta action might similarly act through effects on the TGF-beta receptor was explored. The data in Table II show that none of the agents tested, including other growth factors, retinoic acid, phorbol esters, or epinephrine, had an appreciable effect on TGF-beta binding, even through they all modulate the response of the various target cells to TGF-beta.

Effects of Transformation on TGF-Beta Receptor Properties

The effects of in vitro transformation by various agents on the specific binding of TGF-beta to its cellular receptor were determined for matched pairs of normal and in vitro transformed cells (Fig. 6). Transformation appeared to affect the TGF-beta receptor in one of two ways; retroviral infection and transfection with certain viral oncogenes caused a decrease in binding of TGF-beta to its receptor, whereas transformation by the DNA virus SV-40 and chemical transformation resulted in increased binding of TGF-beta. Scatchard analyses of these data (Table III) revealed that the effect was primarily on the number of TGF-beta receptors per cell, with little significant change in the receptor affinity, except in the case of the ethylnitrosourea-treated MMCE cells where a 3.7-fold decrease in receptor affinity was observed on transformation. However, in no case was TGF-beta binding altered by more than 2–3-fold, which is in marked contrast to the dramatic decrease in or total loss of assayable TGF-alpha/EGF or PDGF receptors seen on transformation (9, 53).

The rates of secretion of TGF-beta by a number of different human tumor cells, in vitro transformed rodent cells and their untransformed counterparts were determined, and compared with the number of TGF-beta receptors expressed by these cells (Table IV). It is apparent that there is no simple correlation between levels of TGF-beta secretion, and the number of receptors on the cells. For example, of the three rhabdomyosarcoma lines assayed, the RD line which secreted 2.5 times more TGF-beta than the A673 line and over 26 times more than the A204 line on a per cell basis nevertheless expressed more than twice as many receptors as either of these two lines. Thus the different numbers of receptors expressed on the various cell types are probably not due to varying degrees of receptor down-regulation in response to endogenously secreted TGF-beta.

Similarly, the data in Table V show that growth of Moloney sarcoma virus (MoSV) transformed NRK cells (536-MSV) for 24 or 48 h in the presence of 100 μg/ml of the immunoglobulin fraction of an anti-TGF-beta antiserum (sufficient to neutralize binding and biological activity of 300 pM TGF-beta) had essentially no effect on the level of specific binding of TGF-beta to the cells at the end of this period. It is possible that endogenous TGF-beta produced by these cells could be causing receptor down-regulation from an immunologically inaccessible site, or that receptor resynthesis could take longer than 48 h. However, it seems likely that the decreased receptor number seen in these and other cell types on transformation is not a result of increased TGF-beta secretion

Table II. Effect of Agents That Modulate Cellular Response to TGF-Beta on the Binding of TGF-Beta to Its Cellular Receptor

Cell	Agent	Biological effect (ref.)	Effect on TGF-Beta Binding
NRK	EGF	Required for TGF-beta to induce colony formation in soft agar (4)	<20% Decrease
NRK	PDGF	Required for TGF-beta to induce colony formation in soft agar (4)	NE
NRK	Retinoic acid	Sensitizes cells to TGF-beta-induced colony formation (8× decrease in ED_{50} for TGF-beta) (39)	<10% Decrease
NRK	TPA	Inhibits TGF-beta-induced colony formation by up to 50%*	<10% Decrease
Myc-1	Retinoic acid	Inhibits colony formation in response to PDGF + TGF-beta (but not in response to EGF) (42)	NE
NHBE	Epinephrine	Inhibits TGF-beta-induced squamous differentiation (35)	NE

Cells were grown for 1–4 d in their normal growth media (see Materials and Methods), in 24-well cluster plates with or without addition of the agents listed above, and ^{125}I-TGF-beta binding was determined over a range of concentrations from 1–200 pM and normalized for differences in cell number for the different treatments. Concentrations of the reagents were as follows: EGF, 1 nM; retinoic acid, 1 μM; TPA, 100 ng/ml; epinephrine, 1.6 μM. Retinoic acid and TPA were added in dimethylsulfoxide (final concentration, 0.1%). Control experiments confirmed that dimethylsulfoxide itself had no effect on TGF-beta binding. For the experiment comparing TGF-beta binding to NRK cells in the presence and absence of PDGF, cells were grown either in DME containing 10% platelet-poor calf plasma (for the no PDGF condition) or in DME containing 10% autologous calf serum (+ PDGF condition). The 10% calf serum was estimated to contain ~3 ng/ml PDGF since addition of this amount of PDGF to the autologous platelet-poor plasma restored the full response of the NRK cells to EGF and TGF-beta that was seen in the presence of the calf serum (4). Abbreviations used are as follows: NRK, normal rat kidney fibroblast; Myc-1, Fischer rat 3T3 fibroblast transfected with c-*myc* oncogene; NHBE, normal human bronchial epithelial cells; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NE, no effect.
* A. B. Roberts, personal communication.

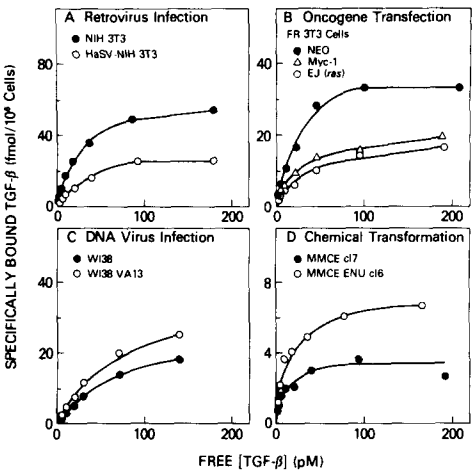

Figure 6. The effect of transformation by various agents on specific binding of TGF-beta to its cellular receptors. The specific binding of TGF-beta to its cellular receptors was determined as described in Materials and Methods for a number of matched pairs of normal and transformed cell types in culture. In each case, binding to the normal cell is represented by closed circles (●) and binding to the corresponding transformed cells by open symbols (○) (△). HaSV is the Harvey sarcoma virus. NEO, Myc-1, and EJ are Fischer rat 3T3 lines transfected respectively with a neomycin marker alone, or with this marker plus the c-*myc* or H-*ras* oncogene from the EJ human bladder carcinoma line. WI38 VA13 is an SV-40 transformant of the human embryonic lung fibroblast WI38 and MMCE ENU is an ethylnitrosourea-induced transformant of a rat epithelial cell. Each point is the mean of two determinations.

causing receptor down-regulation, as had been suggested previously (2), but due to some other effect of transformation. Particle size analysis using a Coulter counter showed that the median cell volume of Harvey sarcoma virus (HaSV)-transformed NIH 3T3 cells (2.0×10^3 μm^3) was greater than that of the normal parent (1.5×10^3 μm^3), eliminating the possibility for these cells that the decreased number of receptors on the transformed cell was due to a transformation-induced change in cell size. Thus the mechanism of the small transformation-induced changes in TGF-beta binding remains obscure.

Ligand-induced Down-Regulation of the TGF-Beta Receptor in Transformed Cells

A549 cells were exposed to 20 pM added human platelet TGF-beta for 2 h at 37°C to allow any ligand-induced receptor internalization to occur. After extensive washing over a further 2-h period at 37°C to dissociate any cell surface–associated TGF-beta that had not been internalized (control experiments showed this procedure removed 93% of TGF-beta on the cell surface), the residual binding capacity of these cells for TGF-beta was determined at 22°C, a temperature at which no further down-regulation occurs (data not shown). This residual binding capacity was then compared with that of cells that had not been previously exposed to TGF-beta (Table VI). It can be seen that pre-exposure of the A549 cells to exogenous TGF-beta results in down-regulation of a substantial fraction (~70%) of the receptors that were occupied during this pre-incubation period. However, not all tumor-derived cells can down-regulate their receptors; the human rhabdomyosarcoma line RD and the MoSV-transformed NRK line 536-MSV did not show any receptor

Table III. Effect of Transformation on TGF-Beta Receptor Properties

Cell Type		TGF-Beta Receptor Parameters	
		K_d	No./cell
		pM	
Retrovirus infection			
NIH 3T3	Mouse fibroblast	23	36,000
HaSV-NIH 3T3	HaSV infected	31	17,000
NRK	Rat kidney fibroblast	36	31,000
536-MSV	MoSV infected	36	21,000
Oncogene transfection			
	Fischer rat 3T3 cells		
NEO	Neomycin transfectant	28	25,000
MYC-1	*Myc* transfectant	18	12,000
EJ	H-*ras* transfectant	24	12,000
DNA virus infection			
WI38	Human embryonic lung fib.	27	17,000
WI38VA13-2RA	SV-40 infected	36	21,000
Chemical transformation			
MMCE Cl#7	Rat epithelial cell	3	1,500
MMCE ENU Cl#6	Ethylnitrosourea-treated	11	4,100
C3H10T1/2	Mouse fibroblast	60	20,000*
MCA Cl#6	Methylcholanthracene-treated	80	32,000*

The specific binding of TGF-beta to its cellular receptors was determined as described in Materials and Methods for matched pairs of non-neoplastic and transformed cell types in culture, and the binding data were subjected to Scatchard analysis. The two cell lines marked with an asterisk showed some indication of a class of higher affinity receptors that were unaffected by transformation. Interassay variation was typically <±20% for both binding parameters.

down-regulation induced by TGF-beta, and even the A549 cells when exposed to saturating concentrations of TGF-beta (400 pM) only down-regulated the same number of receptors (~3,000/cell) as were down-regulated by exposure to low levels of TGF-beta (data not shown). We have demonstrated similar partial down-regulation for nontransformed cells (17). Thus even cells that can down-regulate their receptor do not do so extensively. Given that full biological response can be observed at partial receptor occupancy, down-regulation of <50% of the total receptors may not affect the ability of the cell to respond to the ligand, unless the biological response is mediated by a subpopulation of receptors that are indistinguishable from the rest in terms of their equilibrium binding characteristics, and it is this subpopulation that is down-regulated.

Table V. Effect of Prolonged Exposure to Anti-TGF-Beta Antibody on Growth of a MoSV-Transformed NRK Cell Line (536 MSV) and Its Ability to Bind TGF-Beta

Growth conditions	No. cells per well × 10^5	% Control	TGF-beta specifically bound	% Control
			fmol/10^6 cells	
No additions	1.25 ± 0.01	"100"	14.3 ± 0.3	"100"
+ Anti-TGF-beta IgG	1.26 ± 0.01	101	14.5 ± 0.3	101
+ Normal rabbit IgG	1.24 ± 0.04	99	14.3 ± 0.2	100

536 MSV cells were seeded at 2 × 10^4 cells/well in DME containing 5% FBS, and allowed to grow for 24 h. Growth medium was then removed and replaced with the same medium containing either 100 μg/ml of the immunoglobulin (IgG) fraction of anti-TGF-beta antiserum or normal rabbit serum. A control with no IgG added was also included. After a further 24-h incubation, TGF-beta binding was assayed by addition of 50 pM ^{125}I-TGF-beta with or without 10 nM unlabeled TGF-beta, as described in Materials and Methods. The results are the mean ± SD of three determinations. Essentially similar results were obtained if the cells were exposed to antibody for 48 h rather than 24 h.

The Latent Form of TGF-Beta Does Not Bind to the Receptor

A latent form of TGF-beta that is only biologically active after acidification has been demonstrated in the neutral condi-

Table IV. Lack of Correlation between Levels of TGF-Beta Secretion and Number of TGF-Beta Receptors on Normal and Transformed Cells

Cell type		[TGF-beta] in conditioned medium	Rate of TGF-beta secretion	TGF-beta receptors
		pM	*pg/10^6 cells per 24 h*	*No./cell*
Normal human				
NHBE	(Bronchial epithelial)	2.0	275	10,000
Human tumor				
A549	(Lung adenocarcinoma)	33.0	625	10,000
A2380	(Pancreatic carcinoma)	0.003	0.5	27,000
A2058	(Melanoma)	1.8	50	1,000
A204	(Rhabdomyosarcoma)	0.3	40	3,000
A673	(Rhabdomyosarcoma)	35.0	390	3,000
RD	(Rhabdomyosarcoma)	28.0	990	7,000
Rodent				
Fischer rat 3T3 cells				
Neo	(Neomycin transfectant)	0.8	240	25,000
Myc-1	(*Myc* transfectant)	9.0	230	12,000
EJ	(H-*ras* transfectant)	63.0	555	12,000
MMCE cl#7	(Rat epithelial)	8.0	245	1,500
MMCE ENU	(Chemical transformant)	11.0	430	4,000

TGF-beta concentrations were determined for acid-dialyzed, concentrated conditioned media using a quantitative radioreceptor assay as described in Materials and Methods. The number of TGF-beta receptors per cell was determined from Scatchard analysis of binding data as in Table I.

Table VI. Down-Regulation of TGF-Beta Receptors on A549 Human Lung Carcinoma Cells

Experiment		Conditions	TGF-beta specifically bound	No. occupied receptors per cell
			fmol/10⁶ cells	
A	No. receptors occupied in presence of 20 pM TGF-beta	Cells exposed to 20 pM ^{125}I-TGF-beta for 2 h at 22°C	6.68 ± 0.06	4,020 ± 40
B	Total No. of assayable receptors	Cells exposed to 500 pM ^{125}I-TGF-beta (saturating) for 2 h at 22°C	11.88 ± 1.19	7,150 ± 720
C	Total No. of assayable receptors after pre-exposure to 20 pM TGF-beta	Cells pre-exposed to 20 pM unlabeled TGF-beta for 2 h at 37°C, washed for 2 h at 37°C, and exposed to 500 pM ^{125}I-TGF-beta for 2 h at 22°C	6.60 ± 0.81	3,970 ± 490
D	No. of receptors remaining occupied after pre-exposure to 20 pM TGF-beta and washing	Cells pre-exposed to 20 pM ^{125}I-TGF-beta for 2 h at 37°C and then washed for 2 h at 37°C	0.44 ± 0.03	270 ± 20
E	No. of receptors down-regulated by exposure to 20 pM TGF-beta	B − (C + D)	4.84	2,910

A549 cells were seeded at 1.5 × 10^5 cells/well in 24-well cluster plates and allowed to grow for 24 h. The extent of down-regulation of the TGF-beta receptor in response to pre-exposure of the cells to 20 pM unlabeled TGF-beta was then determined as detailed in the table. The determination of the number of receptors remaining occupied after pre-exposure to 20 pM TGF-beta and washing (step D above) allowed elimination of the possibility that apparent down-regulation was simply due to persistence of bound TGF-beta from the preincubation period.

tioned media of a number of different cell types (26, 27, 38). We therefore examined the possibility that this biologically latent form might be unable to bind to the receptor. Conditioned medium was collected from A549 cells under neutral conditions and assayed by a competitive radioreceptor assay, with and without transient acidification to pH 3.2 with HCl, as described in Materials and Methods. The results are shown in Fig. 7. The neutral conditioned medium did not compete for binding to the TGF-beta receptor at any concentration tested. In contrast, transient acidification followed by reneutralization resulted in high levels of competing activity. Addition of an excess of anti-TGF-beta antibody neutralized at least 83% of the competing activity, suggesting that the majority of the competing activity was due to TGF-beta or a closely related protein. The latent form was biologically inactive as determined by its inability to induce the growth of NRK cells in soft agar in the presence of EGF without prior acidification (data not shown).

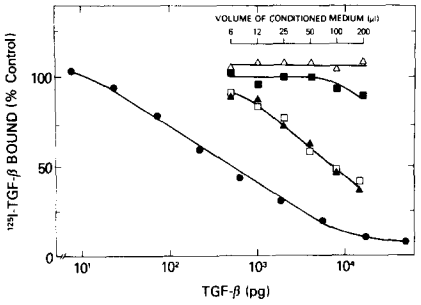

Figure 7. Determination of TGF-beta concentration in neutral and acidified conditioned media from A549 cells using a quantitative radioreceptor assay. Neutral serum-free conditioned medium was collected from A549 cells and aliquots were prepared for assay with or without transient acidification and reneutralization as described in Materials and Methods. The concentration of TGF-beta in the conditioned media samples was then determined in a quantitative radioreceptor assay, using A549 cells as the indicator cell, as detailed in Materials and Methods. Bound counts were normalized as a percent of the counts bound in control wells (10,480 ± 240 cpm) after subtraction of counts bound in the presence of an excess (10 nM) of unlabeled TGF-beta (2,670 cpm). The samples were as follows: human platelet TGF-beta standard (●); neutral A549 conditioned medium (△); transiently acidified A549 conditioned medium, with no further treatment (▲), or preincubated at 4°C overnight with 100 μg/ml of the immunoglobulin fraction of normal rabbit serum (□) or anti-TGF-beta antiserum (■).

Similar assays on the neutral conditioned media of ten other transformed or tumor-derived cell lines ($B16F_1$, mouse melanoma; A375 and A2058, human melanomas; A673 and RD, human rhabdomyosarcomas; HT1080, human fibrosarcoma; A431, HT29, and HUT292, respectively, human vulvar, colon, and lung carcinomas; and 536-MSV, an MoSV-transformed NRK cell) and one normal cell type (normal human bronchial epithelial cells), showed that all these cells also secrete TGF-beta in a latent form (data not shown). Thus it appears to be a common property of all cells to secrete TGF-beta in a form that cannot bind to the receptor without prior activation. This could partly explain the lack of receptor down-regulation by endogenously secreted TGF-beta in cell types, such as A549 cells, that retain the ability to down-regulate their receptors in response to exogenously added platelet TGF-beta.

Discussion

In this paper, the distribution and modulation of the cellular receptor for TGF-beta on a wide variety of cell types were examined and the results, which are considered in detail below, give an insight into possible mechanisms of control of TGF-beta action.

The data show universal expression of a single class of high affinity receptors for TGF-beta on all cell types assayed to date, from both normal and neoplastic sources. This makes the distribution of the TGF-beta receptor even more

widespread than the EGF receptor (10), which is not found on hematopoietic cells, and suggests that all cell types may be potential targets for TGF-beta action. Partial occupancy (10–50%) of this receptor class appears to be sufficient to give full biological response, since there was no evidence for the existence of yet higher affinity receptor classes. However, due to the high nonspecific binding of the ligand, the existence of lower (K_d < 200 pM) affinity receptors could not be ruled out in some cases, and further studies will be necessary to determine how the binding sites identified by these equilibrium binding studies relate to the three structural classes of receptor identified by Cheifetz et al. in chemical cross-linking studies (12).

The breadth of the present study allowed us to demonstrate a novel inverse relationship between the affinity of the TGF-beta receptor and the number of receptors expressed per cell on the various normal and tumor cell types assayed (Fig. 3), which has not been observed for any other growth factor receptor. This relationship suggests a remarkable conservation of the degree of ligand binding, with all cell types binding approximately the same number of TGF-beta molecules per cell at physiological TGF-beta concentrations. Presumably TGF-beta binding triggers a response that must be very tightly controlled in its degree.

Given the bidirectional effects of TGF-beta on cell growth (40, 55), it was possible that normal cells might become transformed either by loss of functional receptors for TGF-beta in the instances where TGF-beta is growth inhibitory, or by overexpression of TGF-beta receptors in cell types where TGF-beta stimulates growth (50). However, in no case was a tumor-derived cell line found to have lost all receptors, nor were there tumor cells expressing exceptionally high numbers of receptors. The human melanoma line A2058 had only 1,000 receptors/cell but was strongly growth-inhibited by TGF-beta (40), indicating that only a very low number of receptors are required to mediate a biological effect, and that a low number of TGF-beta receptors per se does not prevent the cell responding. However, the human rhabdomyosarcoma line A204 expressed an unusual combination of low affinity and low number of receptors such that it bound 10-fold less TGF-beta per cell than other cell types assayed. Since these cells do not respond to TGF-beta (data not shown), they may be functionally receptorless, and it is possible that this feature contributes to the uncontrolled proliferation of these cells. With the exception of this case, there is no simple relationship between binding parameters and the type or direction of the biological response of tumor cells to TGF-beta. Thus, unlike the more limited data of Tucker et al. (54), the present results do not indicate any correlation between receptor numbers on tumor-derived cell lines that grow well in soft agar (A673, A204, RD, Calu I, HT 29, FmX II, HT1080) and those that grow poorly (A549, DND10B67, T24, A431), nor between receptor parameters and the direction of effect of TGF-beta, be it stimulatory (DND10B67), inhibitory (A549, HT1080, A2058), or no effect at all (Calu I, FmX II, HT29, and A673).

Not only is the degree of TGF-beta binding highly conserved between cell types, but TGF-beta binding to any given cell type is essentially unaffected by agents that affect the biological action of TGF-beta in that cell. This suggests that modulation of binding may not be an important control mechanism in the action of this growth factor, but rather that the cellular response may be modulated at subsequent steps. This is in direct contrast to the situation with TGF-alpha and EGF, since a wide number of agents, including PDGF, fibroblast growth factor, TGF-beta, phorbol esters, and retinoic acid, change the number or affinity of the TGF-alpha/EGF receptor, thereby altering the cellular response to these growth factors (3, 8, 22, 31, 45). Equally, TGF-beta causes only minimal (<50%) down-regulation of its receptor in most cell types, and the TGF-beta receptor is relatively unaffected (<2–3-fold changes) by cellular transformation. Again this is marked contrast to the dramatic decrease or total disappearance of assayable receptors for TGF-alpha/EGF or PDGF that occurs on transformation of many cell types (9, 53), and which, for PDGF, has been attributed to receptor down-regulation due to increased secretion of PDGF-like proteins (9, 18, 20). While this work was in progress, Massagué and Kelly (33) published data suggesting that the lack of apparent down-regulation of the TGF-beta receptor in BALB/c 3T3 fibroblasts is due to rapid recycling of the receptor after ligand-induced internalization, or rapid replenishment of surface receptors from a large intracellular pool. Presumably similar mechanisms may operate in other cell types, and may account for the lack of correlation between levels of secreted TGF-beta and the number of receptors expressed by cells analyzed in the present work. Thus, at least in cell culture, expression of the TGF-beta receptor appears to be constitutive as well as universal.

Extractable TGF-beta has been found in all normal and tumor tissues assayed to date (43). Since the receptor seems to be universally expressed, this would suggest that all cells are potentially in a continual state of autocrine or paracrine regulation by TGF-beta. However, we have confirmed and extended the finding (26, 27, 38) that cells appear to secrete TGF-beta in a biologically inactive form, and we have demonstrated that this latent TGF-beta cannot bind to the receptor. This allows some target specificity to be introduced into the system, since only cells capable of activating the latent form of TGF-beta will be responsive. Thus, activation of latent TGF-beta to a form that can bind to the receptor is probably an important extracellular regulatory point in TGF-beta action. This raises the possibility that the tumorigenic lesion in some carcinoma cells may be loss of the ability to activate latent autocrine TGF-beta, such that these cells can no longer limit their own growth; the evidence to date suggests that epithelial cells are always inhibited by the peptide (11, 35, 37, 40, 49, 55). Preliminary experiments (Wakefield, L. M., and T. Masui, unpublished observations) suggest that normal human bronchial epithelial cells can activate and respond to the TGF-beta that they secrete, while the human lung adenocarcinoma line A549 has lost the ability to activate latent TGF-beta.

It has been suggested that some positive autocrine growth factors may interact with their receptors in the Golgi apparatus or secretory vesicles, thus generating an intracellular autocrine loop (7, 20). One important aspect of the secretion of TGF-beta in a latent form that cannot bind to the receptor may be that it would force the TGF-beta autocrine regulatory loop to operate extracellularly, by preventing premature interaction of the ligand with its receptor in the Golgi apparatus. This would potentially make growth regulation by TGF-beta subject to modulation by agents in the extracellular space, giving increased opportunity for communication with

surrounding cells. Since interaction of active TGF-beta with the receptor does not appear to be modulated by any of the agents tested, this type of communication between cells would probably have to be at the level of activation of the latent form of TGF-beta. The nature of the latent form and the in vivo mechanism of activation that is mimicked by acidification in vitro are currently being investigated (Wakefield, L. M., D. M. Smith, K. C. Flanders, and M. B. Sporn, manuscript in preparation).

In conclusion, we have shown that the TGF-beta receptor is universally expressed and is strongly functionally conserved, such that all cell types bind the same number of TGF-beta molecules per cell at physiological TGF-beta concentrations. Binding to the receptor is not modulated by agents affecting TGF-beta action and, compared with the receptors for other growth factors, TGF-beta binding is only slightly affected by the ligand itself and by transformation, confirming the pronounced conservation of binding. Finally we have shown that the biologically inactive form of TGF-beta secreted by various cell types is unable to bind to the receptor without prior activation, and we suggest that this may prevent premature association of ligand and receptor in the Golgi apparatus or secretory vesicles.

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