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## INTRODUCTION

Transforming Growth Factor- $\beta$  (TGF $\beta$ ) is the most potent known inhibitor of cell cycle progression of normal mammary epithelial cells; in addition, it causes cells to deposit increased amounts of extracellular matrix which affects cell-cell and cell-substrate interactions. In general, advanced breast cancers are refractory to TGF $\beta$ -mediated growth inhibition, while the TGF $\beta$  they secrete apparently serves to enhance invasion into surrounding structures and perhaps their metastatic potential. The effects of TGF $\beta$  on cell cycle progression are transduced by two cell surface receptors, TGF $\beta$  type I (T $\beta$ R-I) and -II receptors (T $\beta$ R-II), and relayed from the membrane to the cell nucleus by three recently discovered members of the MAD family of proteins, Smad2, -3, and -4. It is our working hypothesis that TGF $\beta$ -resistance can, in principle, be caused by molecular lesions in any of these five genes, that such lesions are likely to occur during the development or progression of human breast cancer, and that they may impact on prognosis or treatment response.

This project addresses three of the fundamental research issues raised by the USAMRMC Breast Cancer Research Program. The first question is whether or not molecular lesions of the genes involved in the TGF $\beta$  signaling pathway contribute to the origin and/or progression of breast cancer. We expect changes in these genes to be relatively late events, perhaps characteristic of metastatic cancer. Secondly, we proposed to determine how molecular lesions in the TGF $\beta$  receptor and/or Smad genes affect receptor function, and how they might play a role in the development and/or progression of breast cancer. Thirdly, we intend to examine the question whether genetic lesions in TGF $\beta$  receptor and/or Smad genes are able to predict the outcome of patients with breast cancer. Because the anti-tumor effects of anti-estrogens such as tamoxifen are thought to be mediated by the auto- and paracrine induction of TGF $\beta$ , we wish to test the hypothesis that resistance of hormone-receptor positive cancers to tamoxifen is the result of inactivation of TGF $\beta$  pathway genes.

## BODY

The Statement of Work in our original proposal included the following tasks/timeline:

### **Task 1. Screening for mutations in TGF $\beta$ receptor genes in breast cancer**

- a. Identification of genetic alterations of TGF $\beta$ -receptor genes in invasive breast cancer specimens. - Months 1-24
- b. Identification of genetic alterations of TGF $\beta$ -receptor gene in sets of pre-invasive, primary invasive and metastatic (lymph node positive) breast cancers in order to determine the stage of tumor development at which these mutations occur. Months 12-36.

### **Task 2. Determination of the functional consequences of TGF $\beta$ -receptor mutations**

Cloning of TGF $\beta$ -receptor mutants into mammalian expression vectors and transfection into TGF $\beta$ -sensitive and -resistant human mammary epithelial cells to determine whether the mutations are dominant or recessive, and correlation of the site of mutations within the molecule with the way they affect the cellular phenotype. - Months 12-36

### **Task 3. To determine the potential clinical significance of genetic alterations of the T $\beta$ R genes in breast cancer**

Test the hypothesis that genetic alterations of TGF $\beta$ -receptor genes predict for resistance to anti-estrogen therapy in patients with estrogen-receptor positive tumors. Months 36-48.

This report concerns progress achieved on Tasks 1 and 2, which we will describe separately:

### **Task 1. Screening for mutations in TGF $\beta$ receptor genes in breast cancer**

Our initial studies of genes involved in TGF $\beta$  signaling focused on the T $\beta$ R-II gene. Using a chemical mismatch cleavage (CCM) assay, we were the first to identify missense mutations within the T $\beta$ R-II serine-threonine kinase domain in human cancer cell lines [ Garrigue-Antar, 1995 #717 ]. These findings raised two important questions: (1) Do such structural alterations of the T $\beta$ R genes also occur in primary tumors (particularly breast cancers) *in vivo*? and, if so (2) How do mutations in the T $\beta$ R genes affect receptor function?

Before we could embark on a large study of the T $\beta$ R genes in breast cancer specimens, several stumbling blocks had to be overcome. The first one was the fact that there was no information on the genomic organization of either the T $\beta$ R-I or -II genes. This type of information is essential for any comprehensive analysis of these genes in cases in which only genomic DNA from primary tissue specimens is available, such as from paraffin-embedded specimens. Furthermore, knowing the intron-exon boundaries would allow us to screen genomic DNA using a simpler and less laborious assay than CCM, based, for example, on single-strand conformation polymorphisms (SSCP). Because we have access to much larger numbers of paraffin-embedded than frozen breast cancer specimens at Yale, we felt that it was important to define the genomic organization of both the human T $\beta$ R-II and -I genes.

**Genomic organization of T $\beta$ R-I and -II genes.** As we reported in 1997, a human genomic library cloned into bacterial artificial chromosomes (BAC) [ Shizuya, 1992 #1128 ] was screened using a full-length T $\beta$ R-I cDNA (ALK-5 [ Franzén, 1993 #231 ] ) as probe at Genome Systems, Inc. (St. Louis, MO). We obtained a single BAC clone that contained the full-length human T $\beta$ R-I gene sequence. We determined the location of the intron-exon boundaries within the T $\beta$ R-I gene by designing a series of oligonucleotides that matched sequences located at approximately 300 bp intervals along the T $\beta$ R-I cDNA. These primers were then used for direct sequencing of purified BACH-559 DNA. We found that the T $\beta$ R-I gene consists of nine exons, with sizes ranging from 125 to more than 1,000 bp. Exon 1 contains the ATG start codon and extends at least as far upstream as the transcription start site. Most of the extracellular domain of the receptor is encoded by the first two exons. Exon 3 encodes the transmembrane domain, the juxtamembrane region, as well as the GS-domain. The serine-threonine kinase domain is encoded by exons 4 through 9. Exon 9 encodes the C-terminus of the protein, and extends at least as far as the 3' end of the published ALK5 sequence. We estimate that the human T $\beta$ R-I gene is approximately 31 kb in length. These results were recently published (1).

We have also resolved the genomic structure of the T $\beta$ R-II gene, which consists of a total of 7 exons with intervening introns (2).

**Selection of breast cancers for genomic analysis.** In collaboration with our breast pathologist, Dr. Daryl Carter, we selected a series of 36 primary stage I and -II breast carcinoma specimens for which both frozen and paraffin-embedded material is available. In 12 of these cases, we also have lymph node metastatic lesions available for analysis. We are now in the process of examining the molecular structure of the T $\beta$ R-I and -II genes in this series. Preliminary results are presented here:

Primary carcinoma material is microdissected from 5  $\mu$ m paraffin sections as previously described by Dr. Taiping Chen (3). We consider the material remaining on the slide to be representative of the patient's normal tissue, and have used this for extraction of germline DNA. Genomic DNA was extracted from tumor and normal tissues as previously described (3). Isolating genomic DNA from a single 5  $\mu$ m microdissected paraffin-embedded tumor section using

InstaGene matrix (Bio-Rad, Hercules, CA) typically yields 200 µl of DNA template solution.

**Genotyping of TGFβ signaling intermediates:** We have optimized our experimental conditions so that we can generate enough PCR product from 5 µl aliquots of template DNA for single-strand conformation polymorphism (SSCP) analysis of each of the 7 exons (10 fragments) of TBR-II, and each of the 9 exons (9 fragments) of TBR-I (Table 1). Primers used to amplify TBR-II exons were those previously described by Lu et al. (2). These included intronic primers flanking each of the intron-exon boundaries, as well as 6 internal primers to amplify the large exon 4. Each of the 9 exons of the TBR-I gene were amplified using flanking intronic primer pairs (Table 1).

**Table 1. PCR primers used in DNA amplification of TBR-I gene exons**

| Sense primer                    | Exon   | Antisense primer                |
|---------------------------------|--------|---------------------------------|
| 5'-gaggcgaggttgctggggtgaggca-3' | Exon 1 | 5'-catgtttgagaagagcaggagcgag-3' |
| 5'-ctacacaatcttctcttttcc-3'     | Exon 2 | 5'-gttttctgtagtatctagg-3'       |
| 5'-gtttatttcaactgaggcc-3'       | Exon 3 | 5'-ggagaacaattatgttac-3'        |
| 5'-gattgtgtgagtactattta-3'      | Exon 4 | 5'-ggaaaagcaaatgttacagac-3'     |
| 5'-gcccacccgaaatgtaattc-3'      | Exon 5 | 5'-ggtagaactgctatagaat-3'       |
| 5'-gcagtcattgtaattttgattc-3'    | Exon 6 | 5'-gaacgcgtattaaatagttg-3'      |
| 5'-tgtctgaaaggaggtcatcc-3'      | Exon 7 | 5'-gaacaactctgctcatgacg-3'      |
| 5'-gccttgcatagctgaataaat-3'     | Exon 8 | 5'-gcttactaagcagaagcag-3'       |
| 5'-ggaaaatggtgcattgatta-3'      | Exon 9 | 5'-gagttcaggcaaagctgtag-3'      |

PCR primers used for the amplification of individual exons of the TBR-I gene from genomic DNA. The sense primer for exon 1 and the antisense primer for exon 9 were designed based on sequences of the 5' and 3' untranslated sequences of the TBR-I coding region, respectively. All other primers are complementary to intronic sequences flanking each the exons of TBR-I.

The TBR genes are analyzed by "cold" PCR-SSCP as described by Hongyo et al. (4). Each 20 µl PCR reaction contains 500 nM of unlabeled primers. Following a 3 min hot start at 95°C, PCR is performed for 35 cycles of 95°C for 30 seconds, 55°C for 40 seconds, 72° for 30 seconds followed by a 5 min final extension at 72°C. For PCR amplification of the GC-rich exon 1 sequences we use the Advantage-GC genomic polymerase mix (Clontech Palo Alto, CA) according to the instructions supplied by the manufacturer. Aliquots of amplified PCR product (5 µl) are mixed with 15 µl loading buffer (12.5 µl 10x TBE buffer, 2 µl of 15% Ficoll, 0.1% bromophenol blue & xylene cyanol, 0.5 µl methyl mercury hydroxide), denatured by heating at 80°C for 3 minutes, and quenched on ice. The single stranded DNA fragments are then resolved using precast 20% TBE acrylamide gels on a Novex Xcell II Thermoflow apparatus (Novex, San Diego, CA). This apparatus is connected to a cooling system which allows the gel temperature to be precisely controlled throughout the run. We have found a gel temperature of 10°C to be optimal for maximal resolution of individual bands. A second advantage of this approach is that bands can be visualized immediately by staining the gel in a 1:10,000 dilution of SYBR™ Green II (Molecular Probes, Inc., Eugene, OR) for 20-30 minutes and using an Eagle Eye charged coupled device camera equipped with a SYBR™ Green band pass filter (Stratagene) for photographic documentation.

Suspect bands are cut out of the gels with a razor blade and reamplified. PCR products are then purified using the QIAquick PCR purification kit (QIAGEN, Chatsworth, CA), and subjected to DNA sequencing using a thermocycling sequencing kit (Epicentre® Technologies, Madison, WI) (usually 25-30 cycles) with either a forward or reverse primer end-labelled with [ $\gamma$ -<sup>32</sup>P]-ATP. Reaction products are denatured at 70°C for 3 minutes, resolved on 7% (w/v) denaturing polyacrylamide gels at 50°C and visualized by exposing dried gels to X-ray film overnight at 20°C.

**Genotypic analysis of the TBR-I gene in breast cancer:** At this point, we have completed a partial analysis of the TBR-I gene in this initial series of breast cancers. Our most important finding is a variant allele of the TBR-I gene with an in-frame deletion of 3 of 9 repeating GGC trinucleotides within exon 1. This deletion is easy to detect as it gives rise to a distinct SSCP pattern (**Figure 1**). Thirteen of 24 evaluable patients with BC were heterozygous carriers of this del(GGC)<sub>3</sub> TBR-I variant (54%, 95% C.I. 33-74%). This deletion results in the loss of 3 of the 9 alanine residues that constitute the hydrophobic core of the putative TBR-I signal peptide (**Figure 2A, B**) (5). Comparative hydrophobicity plots of wild type and the del (GGC)<sub>3</sub> TBR-I variant clearly show that the deletion shortens the hydrophobic core of the signal peptide (**Figure 2C**). These findings suggest that this deletion may well have functional consequences for the receptor protein, particularly its ability to be targeted to the cell membrane.

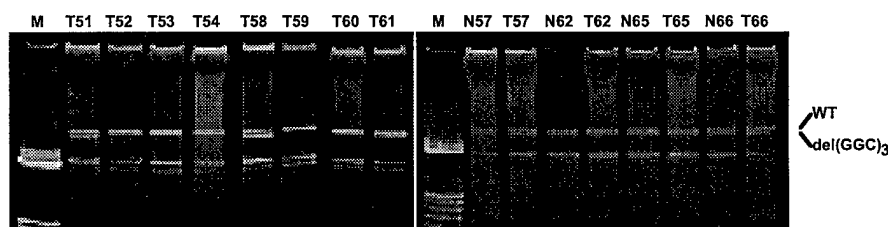
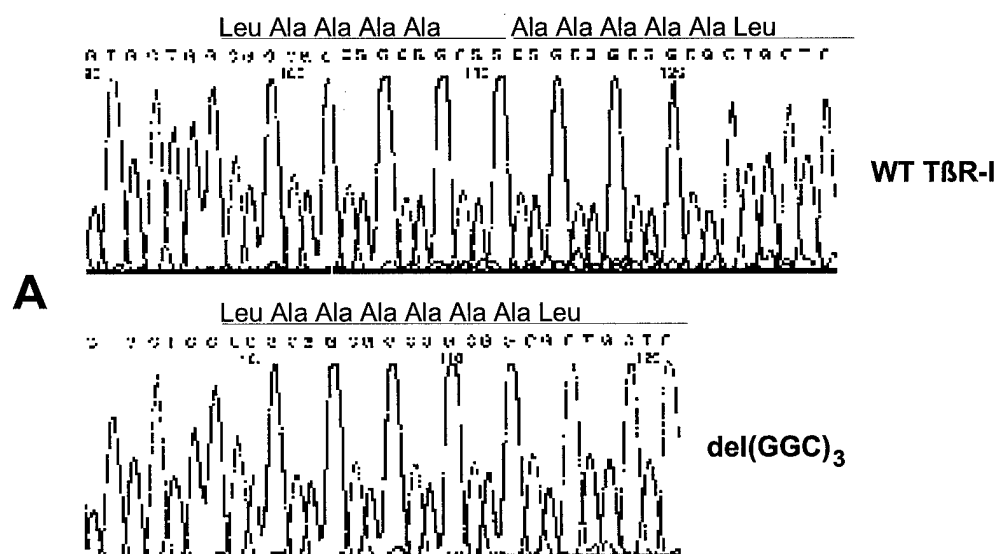
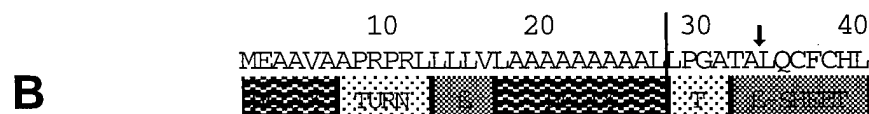
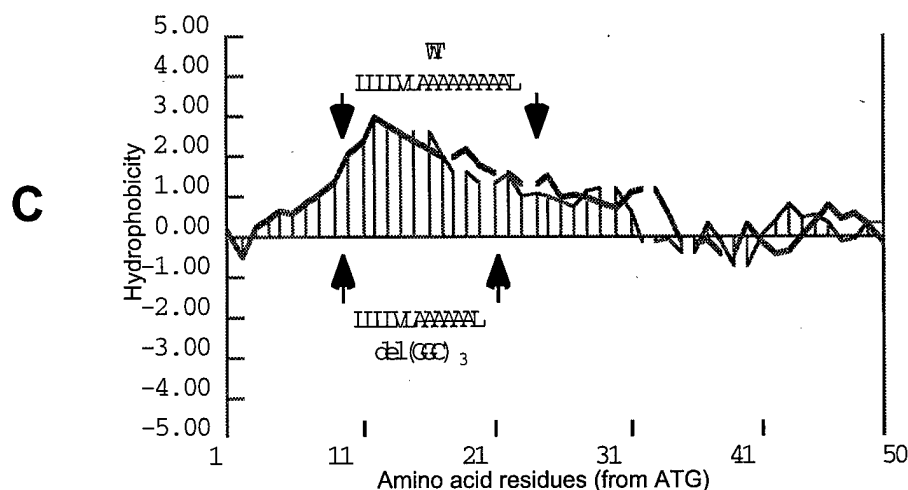


**Figure 1**



### Figure 2



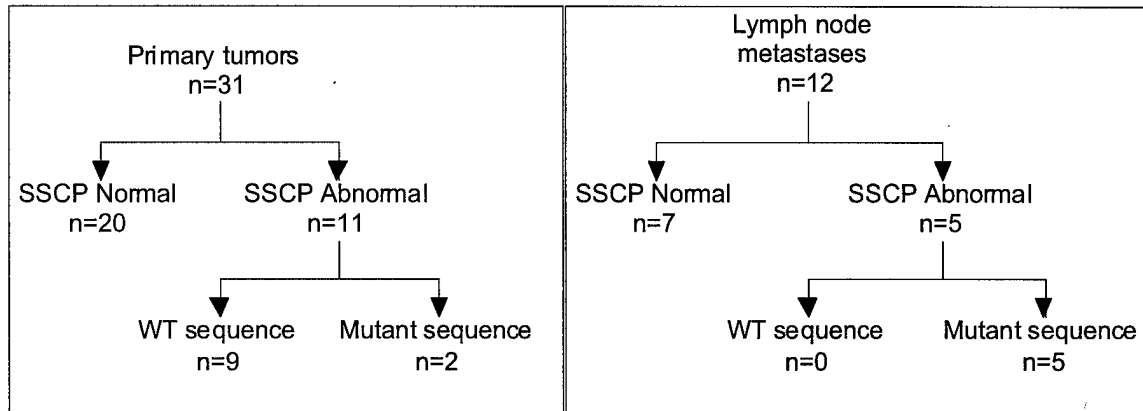


In order to determine whether there might be an association between the carrier state of the  $\text{del}(\text{GGC})_3$  TBR-I variant and the development of breast cancer, we determined the frequency of the  $\text{del}(\text{GGC})_3$  allele in a cohort of germline DNA samples from 43 independently and randomly selected individuals. Only one of these individuals was heterozygous for the  $\text{del}(\text{GGC})_3$  variant of TBR-I (2%, 95% CI: 0-12%). This translates into a highly significant increased relative risk of developing BC in carriers over control (Fisher's Exact test:  $p < 0.0001$ ) (Relative risk: 3.18; 95% C.I.: 2.32-4.36). These findings strongly argue in favor of the hypothesis that the  $\text{del}(\text{GGC})_3$  variant of TBR-I confers an increased cancer risk, presumably by decreasing the sensitivity of normal breast epithelial cells to  $\text{TGF}\beta$ .

We intend to confirm the validity of these results in a prospective case control study in collaboration with Dr. Tongzhang Zheng (Dept. of Epidemiology & Public Health) at Yale. A positive outcome of such a study would have several important implications for women's health. Particularly, the development of screening tests for carriers of the  $\text{del}(\text{GGC})_3$  allele would be extremely valuable for the identification of populations at risk. Moreover, as several second generation chemoprevention trials for breast cancer are initiated, it would be important to assess the impact of the  $\text{del}(\text{GGC})_3$  carrier state on the effect of different interventions (such as tamoxifen or other selective estrogen receptor modulators). On the other hand, we found no apparent relationship between the presence of the  $\text{del}(\text{GGC})_3$  variant of TBR-I and the presence or absence of lymph node metastases (Fisher's Exact test  $p$  value: 0.5804). Thus, the presence of the  $\text{del}(\text{GGC})_3$  variant allele is apparently not associated with greater metastatic potential as determined by lymph node involvement.

The second major finding in our survey of the TBR-I gene in breast cancer specimens was a C to A nucleotide substitution in exon 7 that predicts for a serine to tyrosine mutation at position 387. We have encountered this particular mutation in 2 of 24 (8%, 95% CI: 1-27) primary breast cancer specimens as well as in 5 of 12 (41%, 95% CI: 15-72) lymph node metastases (**Figure 3**). In addition, it should be noted that we have not found this S387Y mutation in any of 16 cervical carcinomas nor in 8 head-&-neck cancers. Thus, this missense mutation appears to be of particular importance for breast cancer.

**Figure 3. Analysis of TBR-I Gene Exon 7 by SSCP and DNA Sequencing in BC Specimens**



The S387Y mutation is likely to strongly affect the function of the TBR-I kinase for several reasons. First, amino acid 387 is positioned in the center of the catalytic core of the serine-threonine kinase domain (**Table 2**). The structure of this catalytic core is highly conserved among the TGF $\beta$  receptor family of serine-threonine kinases as well as phosphorylase B kinase gamma and the AKT-1 and -2 kinases (function unknown). Moreover, as illustrated in **Table 2**, the particular amino acid residue found at position 387 appears to vary specifically depending on the substrate of the receptor kinase. Secondly, based on the Chou and Fassman algorithm, the S387Y mutation is likely to alter the secondary structure of the TBR-I kinase by introducing two  $\beta$ -sheets flanking the central loop of the catalytic core (**Figure 4**). Therefore, our working hypothesis is that the residue at position 387 affects the interaction between the catalytic core and its specific substrate, in this case Smad2 and Smad3. Testing this hypothesis will be an important goal for the coming project year (Task 2).

**Table 2. TBR-I-homologous Serine-Threonine Kinases-Catalytic Cores**

| Protein Kinase                                                                                                                                                                                                                      | Ligand                         | Catalytic core                          | Substrate                                      |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|-----------------------------------------|------------------------------------------------|
| <ul style="list-style-type: none"> <li>TBR-I</li> <li>BMP2/4R</li> <li>BMPR-IA</li> <li>BMPR-IB</li> <li>Phosphorylase B kinase <math>\gamma</math> - muscle</li> <li>Phosphorylase B kinase <math>\gamma</math> - liver</li> </ul> | TGF $\beta$<br>BMP2/4          | GTKRYMAPEVLDD <u>S</u> INMKHFESFKRADIYA | Smad2; Smad3<br><br>Smad1<br><br>Phosphorylase |
| <ul style="list-style-type: none"> <li>TBR-II</li> </ul>                                                                                                                                                                            | TGF $\beta$                    | -----E - <u>R</u> -----D --             | TBR-I                                          |
| <ul style="list-style-type: none"> <li>ActR-IB</li> <li>TSR-I</li> </ul>                                                                                                                                                            | Activin<br>Activin/TGF $\beta$ | -----E - <u>I</u> -----D --             | Smad2; Smad3                                   |
| <ul style="list-style-type: none"> <li>TSR-IB</li> </ul>                                                                                                                                                                            | Activin/TGF $\beta$            | -----E - <u>Q</u> -----D --             | Unknown                                        |
| <ul style="list-style-type: none"> <li>ActR-II</li> <li>ActR-IIB</li> </ul>                                                                                                                                                         | Activin                        | -----E - <u>A</u> -----D --             | ActR-I                                         |
| <ul style="list-style-type: none"> <li>RAC-<math>\alpha</math> Ser/Thr kinase, AKT1</li> <li>RAC-<math>\beta</math> Ser/Thr kinase, AKT2</li> </ul>                                                                                 | Unknown                        | -----E - <u>N</u> -----D --             | Unknown                                        |

**Figure 4A. Wild Type TBR-I Serine-Threonine Kinase Domain**

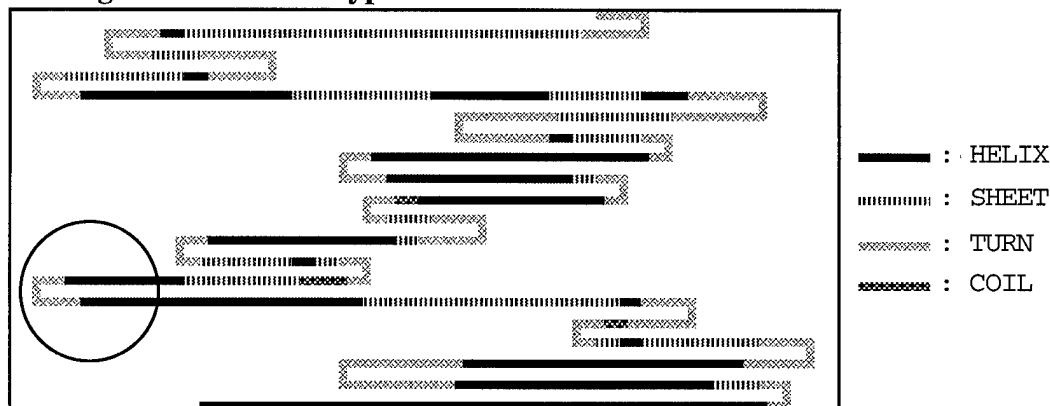
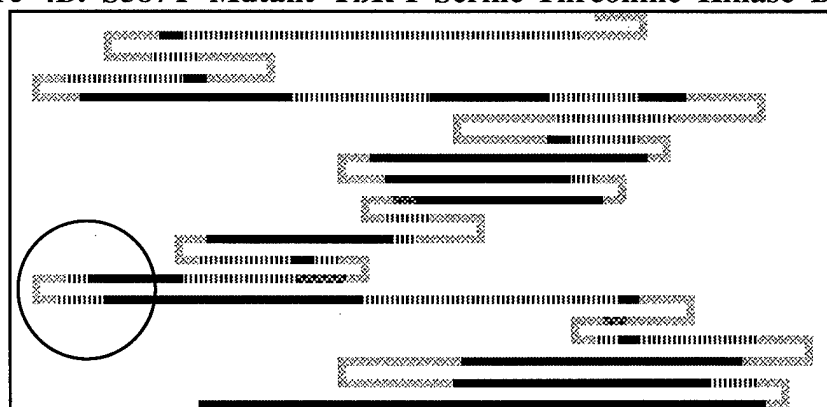


**Figure 4B. S387Y Mutant TBR-I Serine-Threonine Kinase Domain**



In summary, we have identified two particular structural alterations of the TBR-I gene that occur with high frequency in patients with breast cancer. Goals for the coming year include defining the effects of these structural alterations on receptor expression and function, and completing the analysis of both TBR-I and -II genes in primary and metastatic breast cancers.

## **Task 2. Determination of the functional consequences of TGF $\beta$ -receptor mutations**

As indicated above, we were the first to identify missense mutants (R537P and E526Q) of the TBR-II gene in the TGF $\beta$ -resistant human head-&-neck SCC cell lines A253 and SqCC/Y1. Because we anticipate that we will encounter mutations in the TBR-I and -II genes in human breast cancers as well, we needed to develop an experimental strategy that would allow us to precisely determine the consequences of individual mutations for receptor function.

In order to prove that the TBR-II gene mutations were solely responsible for the TGF $\beta$ -resistant phenotype and to determine more precisely how they affected receptor function, we introduced the two mutations into a full-length mouse wild type TBR-II cDNA. We used a mammalian expression vector in which wild type and mutant TBR-II cDNAs were placed under transcriptional control of a CMV promoter. We studied the effects of the two mutations on receptor function in a series of transient transfection assays using T47D breast carcinoma cells as target. This is an ideal cell line to use for this type of experiments, as transfection of wild type TBR-II restores all responses to TGF $\beta$  (6). Two separate luciferase reporter gene constructs, pCAL2 and 3TP-Lux, were used to assess the dual types of responses to TGF $\beta$ : Repression of the cyclin A promoter activity (pCAL2) has been shown to correlate extremely well with the ability of epithelial

cells to respond to TGF $\beta$ -mediated cell cycle arrest, and activation of the PAI-1 promoter (3TP-Lux) reflects TGF $\beta$ -induced PAI-1 production (7, 8). As shown in **Figure 5A**, expression of wild type T $\beta$ R-II in T $\beta$ R-II-deficient T47D cells resulted in an approximately 50% reduction in cyclin A promoter activity compared to cells transfected with an inert control vector. In contrast, cyclin A promoter activity was not repressed in cells transfected with either the E526Q or the R537P receptor mutants (**Figure 5A**). Repression of pCAL2 was independent of the addition of exogenous TGF $\beta$ , presumably because, at high levels of expression, the T $\beta$ R-II and -I receptors can spontaneously dimerize and activate the signaling pathway (9-12). Alternatively, it is possible that bioactive TGF $\beta$ 2 produced by T47D cells is sufficient to stimulate their TGF $\beta$  receptors in an autocrine manner (13). Although attempts to block endogenous TGF $\beta$ 2 activity by using a neutralizing anti-TGF $\beta$ 2 antibody (R & D Systems) did not affect the results of the transfection experiments (data not shown), endogenous TGF $\beta$ 2 may bind and activate the receptors intracellularly, as has been noted for platelet-derived- and hepatocyte growth factor (14).

In cells transfected with wild type T $\beta$ R-II, the activity of the PAI-1 promoter (3TP-Lux) was increased approximately 6-fold over controls, while no increase was observed in cells transfected with either receptor mutant (**Figure 5B**). Thus, both mutations abolish TGF $\beta$ -dependent cell cycle arrest as well its effects on ECM-associated proteins indicating that the amino acid residues at positions 526 and 537 are critical for normal TGF $\beta$  signaling with respect to both cell cycle regulation and extracellular matrix protein induction.

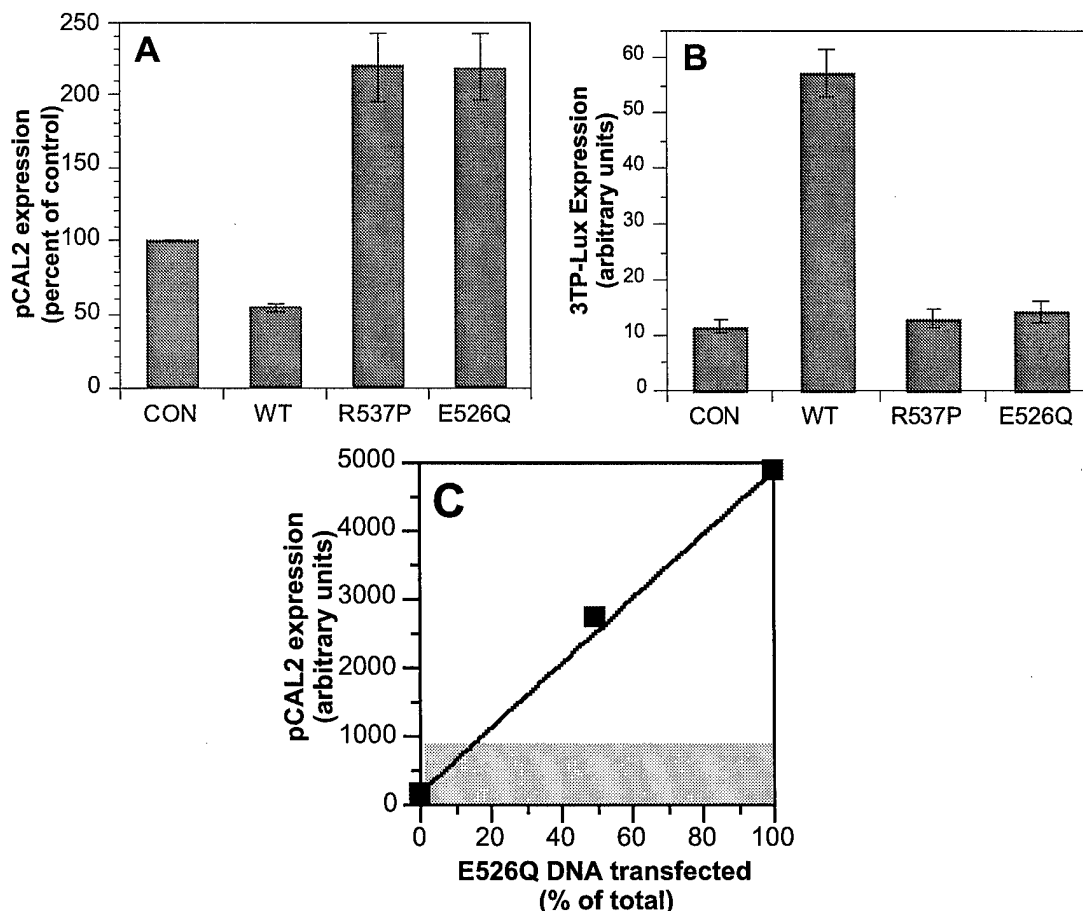


**Figure 5. Functional analysis of T $\beta$ R-II mutants**

Interestingly, both the E526Q and R537P mutants caused a two-fold increase in cyclin A promoter activity over control levels (**Figure 5A**). This observation raised the possibility that both mutants have a strong dominant-negative effect. Besides several other missense mutants within the

juxtamembrane and kinase domains (15-18), C-terminally truncated TBR-II receptor proteins are particularly potent dominant-negatives that completely abrogate all of the responses to TGF $\beta$  when transfected into normal endothelial and bronchial cells (19-22).

Dominant-negative mutants may interfere with TGF $\beta$  signaling by competing with wild type TBR-II for heterodimerization with TBR-I molecules or by sequestering co-expressed wild type TBR-II molecules in homodimeric complexes. However, if this interpretation is correct, this would imply that T47D do, in fact, express a low level of wild type TBR-II, even though the protein is not detectable by ligand binding assays, TBR-II-specific mRNA is undetectable by Northern blot analysis, and the cells are unresponsive to TGF $\beta$  (6). In order to test whether the TBR-II mutants were dominant negative, we co-transfected them into T47D cells together with the wild type construct. As shown in **Figure 5C**, the pCAL2 activity we measured in cells transfected with a 1:1 ratio of E526Q mutant and wild type TBR-II cDNA was exactly what one would expect if the mutant cDNA has no repressive activity of its own and does not affect repression of pCAL2 by the wild type receptor. Similar results were obtained with the R537P mutant (not shown). Thus, based on these results, it is very unlikely that the induction of pCAL2 activity in cells expressing mutant receptors is due to a dominant negative effect.

The increase in cyclin A promoter activity induced by the two TBR-II mutants suggests the possibility that the proliferation of tumor cells that express such mutants might be stimulated by TGF $\beta$ . Such a seemingly paradoxical response to this growth inhibitor has been reported in a number of different tumor systems. For example, highly metastatic human colorectal carcinoma sublines are frequently stimulated by TGF $\beta$  (23, 24). This switch in responsiveness to TGF $\beta$  has been attributed to the use of an alternative TGF $\beta$  signaling pathway in which two particular myelin basic protein kinases that are normally inhibited become activated (25). It is conceivable that activation of this alternative signaling pathway results in the induction of cyclin A expression and, consequently, in stimulation of cell proliferation. These questions will best be addressed by generating T47D cell lines that are stably transfected with the TBR-II mutants.

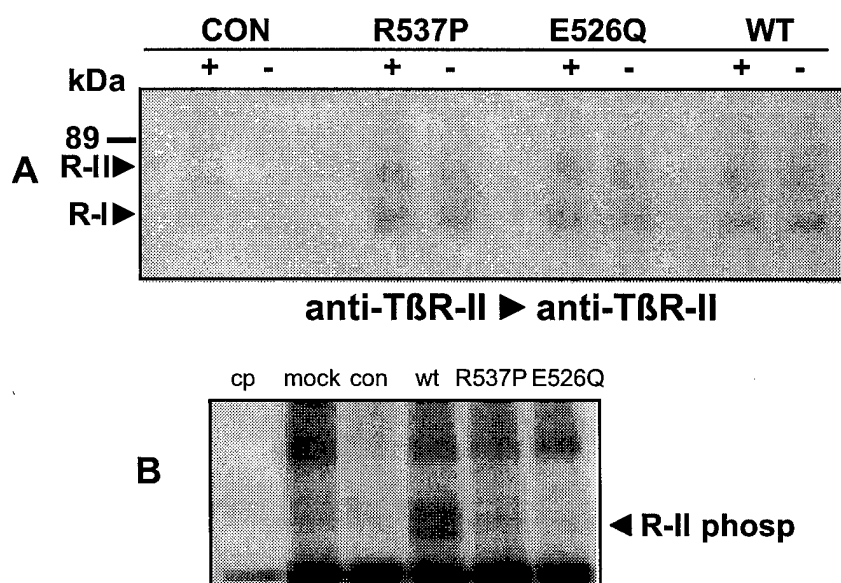


**Figure 6. Expression and *in vitro* kinase activity of TBR-II mutants**

Moreover, a second band of approximately 55 kDa representing co-immunoprecipitated TBR-I, was also seen in all three cases. No receptor protein was detected in control transfected cells. These results indicate that the two TBR-II mutations did not affect receptor protein expression nor the

In order to rule out that the observed differences in reporter gene activity were simply due to variations in levels of expression of wild type and mutant TBR-IIs in transfected cells, parallel dishes of [ $^{35}$ S]-methionine and -cysteine labeled cells were subjected to double immunoprecipitation with anti-TBR-II antiserum (**Figure 6A**). Discreet bands of approximately equal intensity at the level of 65 kDa corresponding to the TBR-II receptor were detected in extracts from both wild type- and mutant TBR-II-transfected cells.

ability of the receptor mutants to form heteromeric complexes with endogenous TBR-I receptors.

Interestingly, this receptor phenotype is clearly distinct from that described for a closely related TBR-II mutant in which a proline residue at position 525 is replaced by a leucine (18). This P525L TBR-II mutant receptor is capable of binding ligand and of forming a complex with TBR-I but fails to transphosphorylate TBR-I (18). However, P525L differs from the E526Q and R537P mutants in that its kinase activity does not appear to be impaired as demonstrated by normal levels of TBR-II autophosphorylation (18). In addition, the P525L mutant is clearly dominant negative, whereas our two mutants are not (18). Thus, mutations that affect two neighboring amino acids (525 and 526) display very different phenotypes, thereby underscoring the functional importance of the C-terminal portion of the kinase domain. This is also underscored by the fact that the arginine residue at position 528 is highly conserved among all protein kinases and forms an ion pair with an equally conserved glutamic acid residue in subdomain VIII that is essential for kinase function (26, 27). Thus, it is likely that mutations involving codon 528 identified in gastric carcinomas (2, 28, 29) also severely disrupt TBR-II receptor function.

To determine the effects of the two missense mutations on receptor kinase activity, we assessed the *in vitro* kinase activity of the two cloned mutants in immunoprecipitates from transiently transfected T47D cells (30). As shown in **Figure 6B**, a phosphorylated protein of ~65 kDa corresponding to TBR-II was easily detectable in cells transfected with wild type TBR-II, while such phosphoproteins were barely detectable in cells transfected with either of the two TBR-II mutants. Thus, both the E526Q and the R537P mutations have a dramatic negative impact on the receptor kinase activity. The finding that the E526Q mutation results in a dramatic reduction in the TBR-II receptor's kinase activity (**Figure 6**) is in accord with our previous observation that immunoprecipitated receptor preparations from SqCC/Y1 cells were largely devoid of *in vitro* kinase activity (30).

Mutations of the TBR-II gene associated with human neoplasms appear to cluster in two particular hotspots: Mutations in the 5' half of the gene result in the synthesis of truncated and presumably soluble TBR-II exodomains (31). Such truncated receptors have been shown to inhibit TGF $\beta$  signaling in a dominant-negative manner (32, 33). The second group of mutations appear to be centered on the C-terminal portion of the serine-threonine kinase domain, particularly subdomains X and XI (2, 28-30, 34). Knaus et al. (34) demonstrated recently that a D404G mutation found in a case of cutaneous T-cell lymphoma caused TGF $\beta$ -resistance by inhibiting cell surface expression of TGF $\beta$  receptors in a dominant-negative manner. However, up to this point, the impact of these various C-terminal missense mutations on receptor kinase activity or signaling function of the receptor protein itself had not been addressed. The current study demonstrates that two different mutations within the C-terminus of the TBR-II receptor that we had identified in human cancer cells abolish the signaling function of the receptor because they cause a near complete loss of serine-threonine kinase activity. These results indicate that these mutant receptors are responsible for the resistance of the tumor cells to TGF $\beta$ , and provide strong evidence for a role of these mutants in the pathogenesis of the head-&-neck tumors they were found in.

In summary, the human breast carcinoma cell line, T47D, is an ideal target cell line to test the function of TBR-II gene mutants. Both missense mutations in the TBR-II gene that we had previously identified in human head-&-neck cancer cells largely abolish the capability of the TGF $\beta$  receptor system to transduce TGF $\beta$ 's effects on cell cycle progression as well as induction of ECM-associated proteins. In addition, our study indicates that these TBR-II mutants inhibit TBR-I function in a dominant-negative fashion.

## CONCLUSIONS

1. The intron-exon organization of both the T $\beta$ R-I and -II genes have been resolved. This has allowed us to embark on a large scale screening of primary breast carcinoma specimens for the presence of structural alterations of both genes.
2. Thus far, we have identified two specific structural alterations of the T $\beta$ R-I gene that are particularly common in breast cancer.
3. We have developed experimental assays using T47D human breast carcinoma cells to test the function of specific T $\beta$ R-II gene mutants. These assays have allowed us to assess the effects of individual mutations on the ability of TGF $\beta$  to inhibit cell cycle progression and to induce extracellular matrix-related target genes.

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Reiss M, Barcellos-Hoff MH: The role of Transforming Growth Factor- $\beta$  in breast cancer-A working hypothesis. *Breast Cancer Res. Treatm.* 45:81-95, 1997

Vellucci VF, Reiss M: Cloning and genomic organization of the human Transforming Growth Factor- $\beta$  type I receptor gene. *Genomics* 46:278-283, 1997

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## **APPENDICES**

Reprints

## *Hypothesis*

# **Transforming growth factor- $\beta$ in breast cancer: A working hypothesis**

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**Key words:** breast cancer, TGF $\beta$ , activation, resistance, receptors, tamoxifen, chemoprevention

## **Summary**

Transforming Growth Factor- $\beta$  (TGF $\beta$ ) is the most potent known inhibitor of the progression of normal mammary epithelial cells through the cell cycle. During the early stages of breast cancer development, the transformed epithelial cells appear to still be sensitive to TGF $\beta$ -mediated growth arrest, and TGF $\beta$  can act as an anti-tumor promoter. In contrast, advanced breast cancers are mostly refractory to TGF $\beta$ -mediated growth inhibition and produce large amounts of TGF $\beta$ , which may enhance tumor cell invasion and metastasis by its effects on extracellular matrix. We postulate that this seemingly paradoxical switch in the responsiveness of tumor cells to TGF $\beta$  during progression is the consequence of the activation of the latent TGF $\beta$  that is produced and deposited into the tumor microenvironment, thereby driving the clonal expansion of TGF $\beta$ -resistant tumor cells. While tumor cells themselves may activate TGF $\beta$ , recent observations suggest that environmental tumor promoters or carcinogens, such as ionizing radiation, can cause stromal fibroblasts to activate TGF $\beta$  by epigenetic mechanisms. As the biological effects of the anti-estrogen tamoxifen may well be mediated by TGF $\beta$ , this model has a number of important implications for the clinical uses of tamoxifen in the prevention and treatment of breast cancer. In addition, it suggests a number of novel approaches to the treatment of advanced breast cancer.

## **Introduction**

Because the components of the molecular machinery that controls the cell cycle are often mutated in human neoplasia, cancer may be considered a disorder of the cell cycle [1]. However, whether or not a given cell actually enters the cycle and proceeds through cell division is critically dependent on the input it receives from growth factors and growth inhibitors in the extracellular milieu. One of these factors, Transforming Growth Factor- $\beta$  (TGF $\beta$ ), is

the most potent physiological inhibitor of cell cycle progression of normal epithelial cells, such as those in the mammary gland [2, 3]. During mammary gland development, TGF $\beta$  selectively inhibits ductal elongation by causing the disappearance of the proliferating stem cell layer and rapid involution of ductal end buds, while alveolar morphogenesis is not affected [4, 5]. Moreover, transgenic expression of TGF $\beta$ 1 targeted to the mammary epithelium inhibits the normal development of the ductal and lobular epithelium in a dose-dependent manner [6,

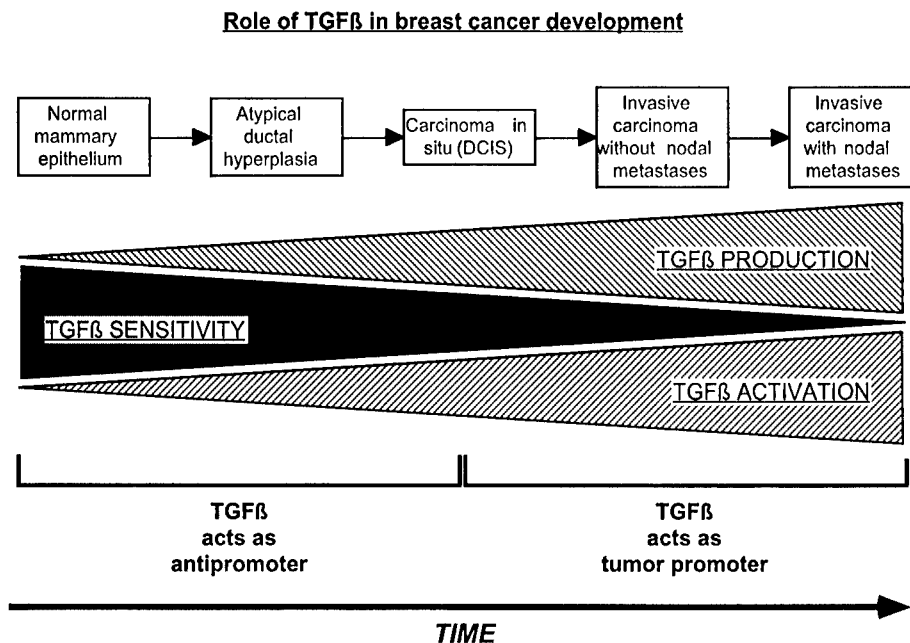


Figure 1. Proposed role of TGF $\beta$  in mammary carcinogenesis. Human breast cancers at successive stages of tumor progression appear to be associated with increasing production of TGF $\beta$ , suggesting that this growth factor is providing a selective advantage. In parallel, one sees the preferential outgrowth of TGF $\beta$ -resistant tumor cell populations, which is driven by the activation of the TGF $\beta$  present in the tumor microenvironment.

7]. In the adult mammary gland, TGF $\beta$  appears to control the massive cell death and restructuring that takes place during post-lactational involution [8]. Thus, TGF $\beta$  is a critical regulator of the temporal and spatial patterns of epithelial cell proliferation and regression that take place during mammary gland development and during and after lactation.

Based on these physiological effects, it is not surprising that TGF $\beta$  has also been implicated in mammary carcinogenesis. One view that has been proposed is that TGF $\beta$  functions primarily as a growth inhibitor for breast cancer cells, and mediates the cytostatic and chemopreventive actions of anti-estrogens, such as tamoxifen (reviewed in [9]). An alternative view is that breast cancer cells produce TGF $\beta$  which somehow promotes tumor progression, while the tumor cells themselves are refractory to TGF $\beta$ -mediated cell cycle arrest (reviewed in [10]).

We would like to propose that these divergent views are both compatible with the notion that the role of TGF $\beta$  undergoes a shift during breast cancer

progression from being predominantly an anti-promoter during the early stages of neoplasia to becoming conducive to cancer invasion, and perhaps metastasis, by advanced tumors (Figure 1). We propose that this shift is brought about by the increasing production and release of TGF $\beta$  by the tumor cells, the activation of latent TGF $\beta$  within the microenvironment, and the clonal expansion of tumor cells that are resistant to TGF $\beta$  on the basis of inactivation of genes encoding TGF $\beta$  receptors or perhaps other elements of the signaling pathway. This working hypothesis reconciles the two seemingly contrary views of TGF $\beta$ 's role in breast cancer and has important consequences for chemoprevention as well as therapy of the disease.

#### **Production of TGF $\beta$ by breast cancer cells increases during neoplastic progression**

The TGF $\beta$ s (TGF $\beta$ 1–3) comprise a family of highly conserved dimeric 25 kDa polypeptides that are ubiquitously expressed in normal mammalian tis-

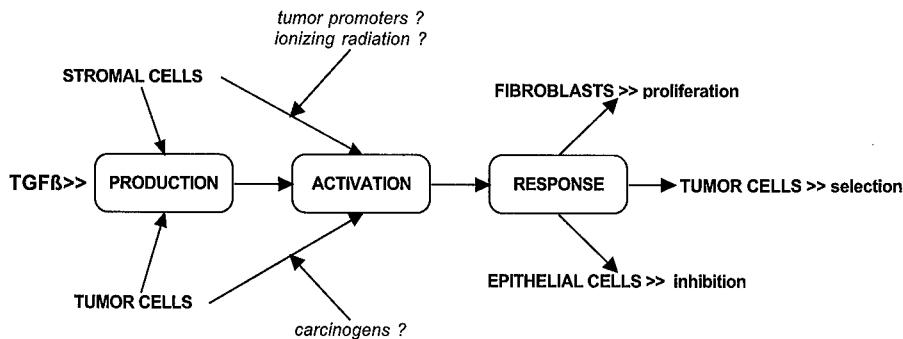


Figure 2. Putative relationship between TGF $\beta$  production, activation, and biological effects in invasive breast cancer. Either the epithelial tumor cells themselves or surrounding stromal fibroblasts can be the source of tumor associated TGF $\beta$ . Similarly, activation of latent TGF $\beta$  can be caused by genetic mutations acquired by the tumor cells, or by epigenetic events that affect the surrounding stromal cells, such as, for example, exposure to carcinogens, tumor promoters, or ionizing radiation. Activated TGF $\beta$  acts as a mitogen for normal fibroblasts, and may be responsible for the desmoplastic reaction often seen in breast carcinoma, while it provides a negative selective force that favors the expansion of TGF $\beta$ -resistant epithelial tumor cell clones.

sues [11]. TGF $\beta$ s exert two major biological effects on epithelial cells. First, picomolar amounts of TGF $\beta$  are able to arrest human mammary epithelial cells (HME) at the G<sub>1</sub>/S boundary, resulting in complete inhibition of DNA replication and clonal growth [2, 3]. Secondly, TGF $\beta$  elicits a series of cellular responses that include, for example, the induction of fibronectin and other protein components of extracellular matrix, as well as plasminogen activator inhibitor type 1 [12, 13]. This second set of responses results in a net accumulation of extracellular matrix.

That neoplastic transformation and progression of human breast epithelial cells might be associated with an increased constitutive production of TGF $\beta$  was first suggested by tissue culture models. For example, 184 HME cells and oncogene-transformed sublines are exceedingly sensitive to TGF $\beta$ -mediated growth arrest, and secrete barely detectable amounts of TGF $\beta$  [3]. In contrast, most cell lines derived from invasive human breast carcinomas secrete much larger amounts of TGF $\beta$ , mostly TGF $\beta$ 2 [3, 14, 15]. In primary human breast cancers, TGF $\beta$  is localized in and around the epithelial tumor cells, while the surrounding stromal cells are negative [16–18]. Even more striking is the observation that the production of TGF $\beta$  by primary breast cancers appears to increase with advancing stages of tumor progression. For example, Walker and Dearing [16] reported that 45% of carcinomas *in situ* and 66% of

invasive carcinomas contained immunodetectable amounts of TGF $\beta$ , whereas there was no staining of adjacent normal epithelium. Moreover, the strongest staining was observed in invasive carcinomas with associated lymph node metastases. These results have been confirmed independently by several other studies [17–19]. Interestingly, most of the TGF $\beta$  is deposited at the advancing edges of the tumors in areas of active growth, suggesting a possible role in tumor cell invasion [18]. This increased production of TGF $\beta$  in human breast cancer associated with tumor progression suggests that it may be acting as a tumor promoter rather than as an inhibitor of tumor growth.

There are several different ways by which tumor-derived TGF $\beta$  might promote tumor progression. One possibility is that TGF $\beta$  affects cell-cell and/or cell-substrate interactions, resulting in a greater propensity for invasion and/or metastasis. For example, human breast cancer cells that had been exposed to TGF $\beta$  *in vitro* or had been transfected with a TGF $\beta$ 1 expression vector were significantly more tumorigenic when they were injected into nude mice than control cells [20, 21]. Another possibility is that tumor-derived TGF $\beta$ 1 acts as an immune suppressor, and allows the tumor cells to escape from immune surveillance [22–24].

Table 1. Effects of TGF $\beta$  on proliferation of normal and transformed epithelial cells of the mammary gland—summary of published studies

| Species | Cell line(s)                                  | Transforming agent or tumor type | Assay used | IC <sub>50</sub> (pM) | Maximal inhibition | Concentration (pM) | Ref. |
|---------|-----------------------------------------------|----------------------------------|------------|-----------------------|--------------------|--------------------|------|
| Man     | Primary human mammary epithelial cells (HMEC) | None                             | ADG        | 8–36                  | 80–100%            | 120                | [2]  |
| Man     | 184A1 HMEC (immortalized)                     | Benzo(a)pyrene                   | ADG        | 10                    | 70–100%            | 40                 | [2]  |
| Man     | 184B5 HMEC (immortalized)                     | Benzo(a)pyrene                   | ADG        | 10→800                | 20–100%            | 40–800             | [2]  |
| Man     | Primary human mammary epithelial cells        | None                             | ADG        | 3.2                   | 75%                | 100                | [3]  |
| Man     | A1N4 HMEC (immortalized)                      | Benzo(a)pyrene                   | ADG        | 2.6                   | 44.3%              | 100                | [3]  |
| Man     | A1N4T HMEC (immortalized)                     | SV40 (large T)                   | ADG        | 1.2                   | 86%                | 100                | [3]  |
| Man     | A1N4M HMEC (immortalized)                     | <i>v-mos</i>                     | ADG        | 0.7                   | 63%                | 100                | [3]  |
| Man     | A1N4H HMEC (weakly tumorigenic)               | <i>v-Ha-ras</i>                  | ADG        | 1.1                   | 78.3%              | ≥10                | [3]  |
| Man     | A1N4MH HMEC (weakly tumorigenic)              | <i>v-mos</i> + <i>v-Ha-ras</i>   | ADG        | 1.5                   | 82.7%              | 100                | [3]  |
| Man     | A1N4TH HMEC (highly tumorigenic)              | SV40 (large T) + <i>v-Ha-ras</i> | ADG        | 2.9                   | 33.7%              | 100                | [3]  |
| Man     | MCF-7A                                        | Adenocarcinoma (ER positive)     | AIG        | NA                    | 86%                | 100                | [47] |
| Man     | MCF-7B                                        | Adenocarcinoma (ER positive)     | AIG        | NA                    | 36%                | 100                | [47] |
| Man     | LY2                                           | Adenocarcinoma (ER positive)     | AIG        | NA                    | 28%                | 100                | [47] |
| Man     | MDA-MB-231                                    | Adenocarcinoma (ER negative)     | AIG        | NA                    | 93%                | 100                | [47] |
| Man     | MCF-7                                         | Adenocarcinoma (ER positive)     | ADG        | NA                    | 86.5%              | 100                | [14] |
| Man     | MCF-7L                                        | Adenocarcinoma (ER positive)     | ADG        | >100                  | 10%                | 100                | [14] |
| Man     | T47D                                          | Adenocarcinoma (ER positive)     | ADG        | >100                  | No inhibition      | 1000               | [14] |
| Man     | ZR75-1                                        | Adenocarcinoma (ER positive)     | ADG        | >100                  | 20%                | 100                | [14] |
| Man     | MDA 330                                       | Adenocarcinoma (ER negative)     | ADG        | >100                  | 10%                | 100                | [14] |
| Man     | BT20                                          | Adenocarcinoma (ER negative)     | ADG        | >100                  | 30%                | 100                | [14] |
| Man     | MDA-MB-231                                    | Adenocarcinoma (ER negative)     | ADG        | 100                   | 50%                | 100                | [14] |

### Activation of TGF $\beta$ in the tumor microenvironment

As indicated above, the malignant progression of breast cancer appears to be associated with the increased production and secretion of TGF $\beta$  by the tumor cells themselves. However, the active form of TGF $\beta$ 1 is derived from a 390-amino acid precursor which is processed into a homodimer of the mature 112 amino acid carboxy-terminal TGF $\beta$ 1 peptide in a non-covalent association with a dimer of the processed N-terminal pro-segment, called the latency-associated peptide (LAP). This latent TGF $\beta$  complex is secreted but unable to bind to TGF $\beta$  receptors unless the biologically active mature TGF $\beta$  is dissociated from the LAP [25]. Since TGF $\beta$  recep-

tors are apparently ubiquitously expressed [26] and latent TGF $\beta$  is abundant in all tissues [11], release from the latent complex is the key control of TGF $\beta$ 's biological activity. This so-called activation is considered the critical event that regulates TGF $\beta$  function *in vivo* [27]. TGF $\beta$  activation in normal adult tissue *in vivo* appears to be the principal switch that initiates the response to damage and orchestrates the acute inflammatory reaction. Thus, TGF $\beta$  has been shown to play a prominent role in wounding, ischemia/reperfusion injury, the response to ionizing radiation, and other causes of acute inflammation (reviewed in [28]). Under these physiological conditions, the effects of TGF $\beta$  activation are rapidly limited by the production of antagonists, such as decorin. Consequently, elevated

Table 1. Continued

| Species | Cell line(s)  | Transforming agent or tumor type      | Assay used | IC <sub>50</sub> (pM) | Maximal inhibition | Concentration (pM) | Ref. |
|---------|---------------|---------------------------------------|------------|-----------------------|--------------------|--------------------|------|
| Man     | HS578T        | Adenocarcinoma (ER negative)          | ADG        | 100                   | 50%                | 100                | [14] |
| Man     | MCF-7         | Adenocarcinoma (estrogen dependent)   | TI         | 5                     | 85%                | 1000               | [49] |
| Man     | MCF-7         | Adenocarcinoma (estrogen independent) | TI         | 200                   | 58%                | 400                | [49] |
| Man     | MDA-MB-231    | Adenocarcinoma (ER negative)          | TI         | 400–1000              | 65%                | 1000               | [49] |
| Man     | SK-BR-3       | Adenocarcinoma (ER negative)          | AIG        | 4                     | 75%                | 40                 | [48] |
| Man     | SK-BR-3       | Adenocarcinoma (ER negative)          | AIG        | 20                    | 75%                | 200                | [48] |
| Man     | Hs578T        | Adenocarcinoma (ER negative)          | AIG        | 4                     | 75%                | 40                 | [48] |
| Man     | MDA-MB-468    | Adenocarcinoma (ER negative)          | AIG        | 20                    | 100%               | 400                | [48] |
| Man     | MDA-MB-468-S4 | Adenocarcinoma (ER negative)          | AIG        | > 400                 | No inhibition      | 400                | [48] |
| Man     | MCF-7         | Adenocarcinoma (ER positive)          | ADG        | 40                    | 55%                | 80                 | [15] |
| Man     | T47D          | Adenocarcinoma (ER positive)          | ADG        | > 80                  | No inhibition      | 80                 | [15] |

Summary of published studies illustrating the differential sensitivity to the anti-proliferative effect of TGF $\beta$  between non-neoplastic HMEC and human breast cancer cells *in vitro* as well as *in vivo*. The average IC<sub>50</sub> of TGF $\beta$  for breast cancer cells is considerably higher and the maximally achievable inhibition of growth consistently lower than for normal HMEC. This difference is of the same order of magnitude as that found between other types of primary epithelial cells and the carcinomas derived from them (see [10] for review). Immortalization of primary HMEC is not associated with the acquisition of TGF $\beta$ -resistance, but the development of tumorigenic (i.e. invasive) properties is, suggesting that there is a linkage between these two phenotypic characteristics of breast carcinomas. ADG: Anchorage-dependent growth; ER: Estrogen receptor; AIG: Anchorage-independent growth; NA: Not available; TI: Thymidine incorporation.

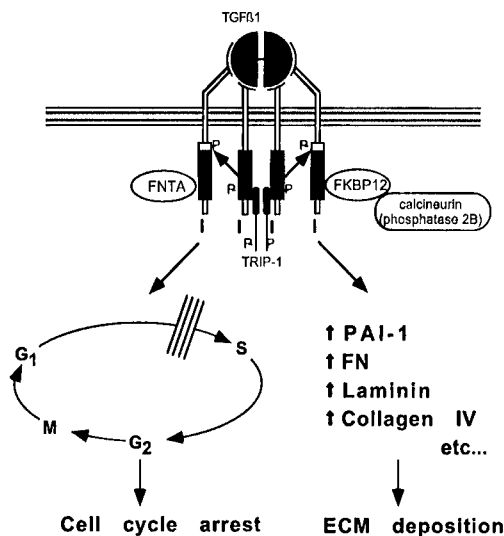
**Current model of the TGF $\beta$  signaling pathway**

**Figure 3.** Current model of TGF $\beta$  signaling pathway. TGF $\beta$ 1 binds to T $\beta$ R-II homodimers followed by the recruitment of one (or more) T $\beta$ R-I receptor molecules into a stable ternary complex with T $\beta$ R-II, which results in the phosphorylation of the GS domain of T $\beta$ R-I by the T $\beta$ R-II kinase. The  $\alpha$ -subunit of the ras farnesyltransferase (FNTA) and FKBP-12 are associated with T $\beta$ R-I. FKBP-12 functions as a bridge molecule between T $\beta$ R-I and the Ca<sup>2+</sup>/calmodulin-dependent protein phosphatase 2B (calcineurin), while FNTA is involved in activation of the ras pathway. The function of the T $\beta$ R-II-associated protein TRIP-I is unknown. TGF $\beta$  induces the expression of cyclin-dependent kinase inhibitors, such as p15<sup>INK4B</sup> and p21<sup>WAF-1</sup>, whereas it inhibits expression of cyclins A and E, as well as cdk4, presumably leading to cell cycle arrest in mid- to late G<sub>1</sub>. In addition, TGF $\beta$  induces the expression of genes that encode extracellular matrix proteins as well as plasminogen activator inhibitor-1.

expression of latent complex by itself (as seen in tumor tissue) is unlikely to have major biological consequences unless it is accompanied by increased activation (Figure 2) [29].

It is possible that tumor cells themselves cause the activation of the TGF $\beta$  that they produce. *In vitro*, a significant fraction of the TGF $\beta$  that breast carcinoma cells secrete into the culture medium is in its activated form [3, 14, 15]. Whether breast carcinoma cells also cause activation of TGF $\beta$  *in vivo* is not clear because none of the published immunohistochemical studies employed antibodies that specifically recognize the activated form of the TGF $\beta$ . However, advanced breast cancers often produce increased amounts of urokinase-type plasminogen activator (uPA) [30–32], which catalyzes

the conversion of plasminogen to plasmin. Because plasmin is believed to activate latent TGF $\beta$ , the increased levels of uPA in tumors may cause TGF $\beta$  activation [33].

The recent observation that stromal cells are the principal source of breast cancer-associated uPA is compatible with the notion that fibroblasts and not tumor cells induce TGF $\beta$  activation [34]. Alternatively, environmental agents may cause TGF $\beta$  activation by stromal cells within the tumor microenvironment, such as occurs during the acute response to tissue injury. For example, we have shown recently that ionizing radiation leads to a rapid and global remodeling of the microenvironment in the virgin mouse mammary gland [35]. Within 24 hr of exposure to doses of 5 Gy or less, we observed loss of collagens type I and III in the peri-epithelial stroma and *de novo* expression of collagen type III in the adipose stroma [35]. In addition, hyaluronic acid was lost in the irradiated mammary gland and the glycoprotein tenascin was induced at the stromal/epithelial interface (M.H. Barcellos-Hoff, unpublished data). Both tenascin and hyaluronic acid have been implicated in epithelial migration during tissue remodeling and wound healing [36, 37]. Moreover, tenascin is developmentally regulated in mouse mammary gland and is normally turned off in adult tissue [38]; however it is re-expressed in malignant breast tumors of both mice and humans, suggesting that its anti-adhesive characteristics may be conducive to tumor growth and invasion [38–40].

It is striking that the proteins that are induced by radiation are all known to be regulated by TGF $\beta$ . Using antibodies that discriminate between the active and latent forms of TGF $\beta$  [41], we found that latent TGF $\beta$  was abundant in the normal mammary gland, but that active TGF $\beta$  was restricted to epithelial structures, where it was faintly detectable. However, at 1 hr after radiation exposure, latent TGF $\beta$  immunoreactivity was strikingly diminished in the adipose stroma, while active TGF $\beta$  immunoreactivity was induced in the previously negative adipose stroma and dramatically increased within the epithelium and peri-epithelial stroma. These data suggest that, either directly or indirectly, ionizing radiation induces TGF $\beta$  activation. This pattern persisted in the epithelium for 24 hr, and did not re-

vert to pre-irradiation conditions in the stroma for at least 7 days. Furthermore, TGF $\beta$  activation was detected at doses as low as 0.1 Gy and failed to show a threshold effect (E.J. Ehrhart and M.H. Barcellos-Hoff, submitted). Besides ionizing radiation, chemical tumor promoters have also been shown to activate TGF $\beta$ . For example, phorbol ester treatment of carcinogen-initiated skin rapidly induces TGF $\beta$  mRNA and protein immunoreactivity [42, 43] that most likely reflects the production of active TGF $\beta$ , since the antibody used in these studies reacts specifically with active TGF $\beta$  [41, 44]. Similarly, phenobarbital induces active TGF $\beta$  immunoreactivity in normal liver tissue, resulting in the selective clonal expansion of transformed hepatocytes that express decreased levels of TGF $\beta$  receptors [45, 46].

The observations that radiation and chemical agents can induce the activation of a cytokine that is instrumental in restraining growth may appear paradoxical. This paradox may be resolved by assuming that TGF $\beta$  activation causes a selective expansion of cells in which mutations confer resistance to TGF $\beta$ , as is observed, for example, in diethylnitrosamine-induced liver tumors [46]. Conversely, one would predict that transformation in which TGF $\beta$  activation does not occur would not be associated with TGF $\beta$ -resistance of the associated tumors. Thus, carcinogenic agents such as radiation appear to have a dual role: Besides their classic carcinogenic effect of inducing mutations in the target cell DNA, such agents also fundamentally alter the microenvironment in which the damaged epithelial cells reside (e.g. by causing the activation of latent TGF $\beta$ ), thereby promoting the selective outgrowth of mutant cells that display a particular phenotype (in this case, TGF $\beta$ -resistance). The recognition of these epigenetic effects of radiation may have important implications for the therapeutic use of ionizing radiation, as will be discussed below.

### Effects of TGF $\beta$ on breast carcinoma cells

*In vitro*, mammary epithelial cell lines range from being exquisitely sensitive to being completely refractory to TGF $\beta$ -mediated growth inhibition (Table 1). For instance, spontaneously immortalized

HMEC (A1N4 cells) are nearly as sensitive to TGF $\beta$  as primary HMEC, even when stably transfected with single viral oncogenes [3]. In contrast, only highly tumorigenic variants of A1N4, obtained by transfection with the combination of v-Ha-ras and SV40 large T antigen, are significantly less responsive to TGF $\beta$  than their non-tumorigenic precursors [3]. Moreover, compared with HMEC, most cell lines derived from invasive human breast carcinomas are much less sensitive to the anti-proliferative effects of TGF $\beta$ , depending, to some extent, on whether cells are grown in monolayer or in soft-agar. By and large, TGF $\beta$ s appear to be more potent inhibitors of anchorage-independent growth than of growth in monolayer culture [14, 47, 48], perhaps as a consequence of their effects on cell-matrix interactions rather than a direct effect on cell cycle progression.

Several studies have suggested that estrogen-receptor (ER)-negative breast cancer cell lines are relatively more sensitive to TGF $\beta$  than ER-positive ones [47, 48], although there is considerable variability between studies. For example, clonal growth in monolayer culture of the ER-negative line MDA-MB-231 was found to be strongly inhibited by TGF $\beta$  in two studies [47, 48], but not in a third [14]. Another possibility is that TGF $\beta$ -sensitivity is primarily a function of the estrogen-dependence of the breast cancer cells, rather than the expression of hormone receptors. For example, estrogen-dependent MCF-7 breast cancer cells are quite sensitive to TGF $\beta$ -mediated growth inhibition, whereas estrogen-independent sublines are refractory to TGF $\beta$  [49]. In any event, even if modest differences in TGF $\beta$  sensitivity exist among breast carcinoma cell lines, the average IC<sub>50</sub> of TGF $\beta$  for tumor cells is considerably higher and the maximally achievable inhibition of growth consistently lower than for primary HMEC (Table 1).

*In vivo*, transgenic mice that produce a constitutively active form of TGF $\beta$ 1 have been found to be resistant to 7,12-dimethylbenz[a]anthracene-induced mammary tumor formation [50]. Furthermore, cross-breeding of such mice with a strain that overexpressed the epithelial mitogen TGF $\alpha$ , in which mammary tumors develop at a high rate, also resulted in marked reduction in the incidence of

mammary tumors [50]. On the other hand, treatment of mice bearing MDA-MB-231 human breast cancer xenografts with TGF $\beta$  did not result in any suppression of tumor growth *in vivo* [51]. In aggregate, these studies provide compelling evidence that overexpression of TGF $\beta$ 1 can markedly suppress *de novo* mammary tumor development, but that this effect is lost once invasive carcinomas have arisen.

### Mechanisms of escape from TGF $\beta$ control

In order to understand the mechanisms that cause tumor cells to become refractory to TGF $\beta$ -mediated cell cycle arrest, it is necessary to examine the molecular components of the TGF $\beta$  signaling pathway. The biological effects of TGF $\beta$ 1 are transduced by two interacting and interdependent receptor subtypes, type I (T $\beta$ R-I) and type II (T $\beta$ R-II) (Figure 3) [52, 53]. Both are highly conserved transmembrane serine-threonine receptor kinases [54–56]. Binding of TGF $\beta$ 1 to T $\beta$ R-II homodimers is followed by the recruitment of T $\beta$ R-I receptor molecules into a stable heterotetrameric complex with T $\beta$ R-II (Figure 3) [57]. This triggers the phosphorylation of the juxtamembrane (GS) domain of T $\beta$ R-I by the T $\beta$ R-II kinase [58, 59]. The activated T $\beta$ R-I kinase presumably phosphorylates downstream elements in the signaling cascade [59].

A number of proteins which physically interact with T $\beta$ R-I [60] or with the T $\beta$ R-II receptor [61], may be required for signaling. For example, FKBP-12 functions as a bridge molecule between T $\beta$ R-I and the Ca<sup>2+</sup>/calmodulin-dependent protein phosphatase 2B (calcineurin) [60, 62]. Moreover, the  $\alpha$ -subunit of the *ras* farnesyltransferase (FNTA), which is also associated with T $\beta$ R-I, is phosphorylated and released upon TGF $\beta$  stimulation, indicating that activation of *ras* and the mitogen-activated protein kinase cascade may play a role in mediating the cellular responses to TGF $\beta$  [63–66]. Further downstream, TGF $\beta$  induces the expression of the cyclin-dependent kinase inhibitors p15<sup>INK4B</sup> and p21<sup>WAF-1</sup> [67, 68], whereas expression of cyclins A and E as well as cdk4 is inhibited [69, 70].

Thus far, molecular alterations of only two cell cycle control genes have been associated with loss of responsiveness to TGF $\beta$ . We and others [71, 72] have shown that cells that express mutant forms of the p53 tumor suppressor protein lose sensitivity to TGF $\beta$ . Secondly, Okamoto et al. [73] noted TGF $\beta$ -resistance in esophageal epithelial cells overexpressing cyclin D1. The p53 gene is often mutated in human breast cancers and cyclin D1 levels are elevated in one third to one half of all breast cancers [74–76]. In a tumor microenvironment that contains activated TGF $\beta$ , even a partial loss of responsiveness due to expression of mutant p53 or an excess of cyclin D1 in the tumor cells may well confer a selective proliferative advantage.

The main mechanism that results in complete abrogation of TGF $\beta$  responsiveness is inactivation of either one of the two T $\beta$ R receptors [58, 77]. There appears to be a direct quantitative relationship between the level of T $\beta$ R-II expression in tumor cell lines and TGF $\beta$ -responsiveness. For example, somatic cell fusion of two different, TGF $\beta$ -refractory, T $\beta$ R-II-negative carcinoma cell lines gave rise to hybrids that re-expressed T $\beta$ R-II protein and regained sensitivity to TGF $\beta$  [78]. Several TGF $\beta$ -resistant human breast cancer cell lines fail to express T $\beta$ R-II mRNA transcripts [52, 79]. Moreover, transfection with a T $\beta$ R-II expression vector results in the restoration of TGF $\beta$ -responsiveness *in vitro* and, most importantly, in suppression of tumorigenicity *in vivo* [79, 80]. Besides T $\beta$ R-II, genetic defects of the T $\beta$ R-I receptor can also result in TGF $\beta$  resistance: Kim et al. [81] recently identified a human prostatic carcinoma cell line that was refractory to TGF $\beta$  because of loss of T $\beta$ R-I expression as a consequence of gene rearrangement. The relationship between loss of T $\beta$ R expression and TGF $\beta$  resistance appears to hold up *in vivo* as well. For example, Kadin et al. [82] demonstrated that the loss of T $\beta$ R-II transcripts parallels the progressive loss of TGF $\beta$  responsiveness in cells obtained from serial biopsies of a single patient with progressive Ki-1<sup>+</sup> cutaneous T-cell lymphoma. Similarly, malignant CD4<sup>+</sup> lymphocytes obtained from patients with Sézary syndrome are also refractory to TGF $\beta$  and fail to express T $\beta$ R-II cell surface receptors [83].

The importance of loss of T $\beta$ R-II expression in

primary human solid tumors remains to be established. We showed recently that T $\beta$ R-II transcripts are absent in approximately 25% of primary esophageal carcinomas [84]. Moreover, T $\beta$ R-II receptor levels appear to be reduced in high-grade prostatic carcinomas [84]. On the other hand, primary malignant melanomas also express normal T $\beta$ R-II mRNA levels [85], while T $\beta$ R-II transcripts appear to be increased in pancreatic carcinomas compared to adjacent normal tissue [86]. Thus far, each of the primary breast carcinomas that we screened expressed T $\beta$ R-II transcripts (L. Garrigue-Antar and M. Reiss, unpublished observations). Thus, although changes in T $\beta$ R-II gene expression do occur in human tumors, there is no evidence as yet that this occurs in primary breast cancer.

Besides loss of T $\beta$ R-II expression, we recently identified several mutants of the T $\beta$ R-II receptor gene in human squamous head-&-neck carcinoma cell lines that account for their resistance to TGF $\beta$ 1-mediated cell cycle arrest [87]. Mutational inactivation of the T $\beta$ R-II gene also occurs frequently in colorectal carcinoma cell lines as well as in primary colorectal and gastric cancers from patients with an inherited DNA mismatch repair deficiency [88–91]. Although mutations within the T $\beta$ R-I gene have not yet been described, it is likely that they occur in human cancers as well.

At this point, structural information about TGF $\beta$  genes in primary human breast cancers is extremely limited. Ke et al. [92] were unable to detect any T $\beta$ R-II microsatellite mutations in a small series of sporadic breast carcinomas. However, in our own preliminary analysis of primary breast cancer specimens, we have encountered several T $\beta$ R-II missense mutations which are likely to affect receptor kinase function (L. Garrigue-Antar and M. Reiss, unpublished observations). Thus, genetic alterations of the T $\beta$ R-II gene do occur during human mammary carcinogenesis, but the actual frequency of such events still remains to be established.

### Clinical implications

The experimental data summarized above indicate that the malignant progression of breast cancer is

associated with the increased autocrine production, secretion, and activation of TGF $\beta$ . While, in the early stages of breast cancer development, mammary epithelial cells are sensitive to growth inhibition by TGF $\beta$ , TGF $\beta$ -resistance and the accompanying higher levels of TGF $\beta$  production by the tumor cells probably represent late events in breast cancer progression, associated with a greater invasive and/or metastatic potential (Figure 1).

This model has a number of important implications with respect to the chemoprevention and treatment of breast cancer. For example, therapeutic maneuvers designed to induce and/or activate TGF $\beta$  would be expected to have their greatest therapeutic benefit in the setting of a breast carcinoma or a preneoplastic lesion that is still sensitive to its anti-proliferative effect. On the other hand, treatments that induce the production and/or activation of TGF $\beta$  may be associated with the risk of enhancing growth or invasiveness of tumors that have arisen in a microenvironment that contains activated TGF $\beta$ . In these cases, targeting TGF $\beta$  itself might be a more effective therapeutic strategy, as has been proposed for the treatment of chronic inflammatory conditions and of malignant gliomas [24, 93].

Particularly intriguing is the possibility that the clinical and chemopreventive effects of tamoxifen may be mediated by TGF $\beta$ . Treatment of human ER-positive breast cancer cell lines with tamoxifen *in vitro* induces the production and secretion of TGF $\beta$  by these tumor cells and slows down their growth [47]. This observation suggested that the antitumor effects of tamoxifen on established invasive and metastatic cancer might be mediated by TGF $\beta$ , and that this was dependent on the expression of functional ER. Paradoxically, ER-negative breast cancer cell lines appear to be more sensitive to TGF $\beta$ -mediated growth inhibition than ER-positive ones, at least *in vitro* [14]. Moreover, some breast cancer cell lines respond to tamoxifen in spite of the fact that they are refractory to TGF $\beta$ -mediated growth arrest because they do not express TGF $\beta$  receptors [15, 80, 94]. These *in vitro* studies may be confounded by the low responsiveness of all cell lines derived from established breast carcinomas to TGF $\beta$  compared to normal HMEC, and by

the fact that *in vitro* assays fail to take into account the role of stromal cells in mediating tumor growth *in vivo*. In this light, it is interesting that a number of recent clinico-pathological studies have demonstrated that tamoxifen induces the production of TGF $\beta$  isoforms *in vivo*: Thus, several studies have demonstrated elevations of plasma levels of TGF $\beta$ 2 in response to tamoxifen treatment, and these elevations appear to correlate with tumor regression [95, 96]. In addition, a number of investigators have examined the effects of tamoxifen treatment on the expression of TGF $\beta$  isoforms in breast cancer specimens. Although some of these studies suggested that TGF $\beta$ 1 was induced in tumor stroma in response to tamoxifen [97], others have failed to confirm these results [98, 99]. However, several recent reports seem to indicate that the predominant TGF $\beta$  isoform induced in breast cancer-associated stromal fibroblasts following tamoxifen treatment is TGF $\beta$ 2 [99, 100]. Although none of these studies formally prove that TGF $\beta$  mediates the effects of tamoxifen on breast tumor growth, they strongly suggest a relationship between the induction of TGF $\beta$ 2 in tumor stroma with elevations of TGF $\beta$ 2 plasma levels, and tumor regression.

The key point here is that, *in vivo*, tamoxifen-responsiveness may be dependent not so much on whether or not the breast cancer cells express hormone-receptors, but rather on whether or not they are still sensitive to TGF $\beta$ -mediated growth arrest. Although the majority of ER-positive breast cancers respond to tamoxifen, a significant number do not. For example, approximately 30% of women with ER-positive tumors who are treated with tamoxifen in the adjuvant setting eventually suffer recurrences, indicating that these tumors are tamoxifen-resistant [101]. Furthermore, 40–45% of ER-positive metastatic tumors are also clinically resistant to anti-estrogen therapy [102]. Tamoxifen-resistance may be the result of a decreased expression of ER [103], mutations in the ER or PgR genes [104], or the increased conversion of tamoxifen to inactive metabolites [105]. However, in a significant proportion of cases, none of these mechanisms is operative [103]. We would like to propose that some ER-positive tumors are resistant to tamoxifen because the tumor cells no longer respond to TGF $\beta$ -

mediated cell cycle arrest. This may also explain why primary breast cancers that are clinically resistant to tamoxifen express the highest levels of TGF $\beta$ 1 [106]. Moreover, tumors that express low levels of uPA are more likely to respond to tamoxifen than those that express high uPA, in which TGF $\beta$  is presumably activated [107]. Consequently, uncovering the molecular basis of TGF $\beta$  resistance should allow us to better identify patients who are likely to benefit from anti-estrogen therapy, as well as those for whom tamoxifen may be detrimental.

Based on our model, one would predict that treatments that activate TGF $\beta$  would be most effective when applied early on in breast cancer progression. For example, in addition to causing cell kill by inducing DNA damage, the particularly strong beneficial effects of ionizing radiation in the treatment of *in situ* breast cancer may be mediated by TGF $\beta$  activation and TGF $\beta$ -mediated apoptosis [108, 109]. Similarly, there is good rationale for the ongoing clinical studies of tamoxifen in the treatment of *in situ* breast cancer. Because the pharmacological induction of TGF $\beta$  expression and activation might also be able to prevent primary breast cancer [110], tamoxifen is currently being evaluated as a chemopreventive agent in large scale clinical trials [111, 112]. Similarly, the chemopreventive actions of certain retinoids may be mediated by the activation of TGF $\beta$  in stromal elements [113, 114]. Monoterpenes, such as d-limonene, also represent potentially useful chemopreventive agents in breast cancer [115]. In rodent models, these agents have been shown to increase the expression of mannose 6 phosphate/insulin-like growth factor-II receptors (M6P/IGF-IIR) and TGF $\beta$ 1 by mammary carcinoma cells [115]. Expression of M6P/IGF-IIR facilitates the activation of TGF $\beta$ , thereby restoring the autocrine growth inhibitory loop and preventing the outgrowth of transformed cell clones.

Conversely, in the treatment of advanced TGF $\beta$ -resistant breast cancers, tamoxifen may be ineffective and perhaps may even promote tumor growth. Similarly, resistance of tumor cells to TGF $\beta$  may negate some of the cytotoxic effects of radiation treatment, and radiation-dependent activation of TGF $\beta$  in the stroma may even be detrimental if it makes the microenvironment more conducive to tumor

progression and contributes to normal tissue damage leading to fibrosis [116]. This is perhaps the reason that radiation appears to be more effective in providing local control after resection of small, mammographically detected tumors than of larger, palpable lesions. In these types of settings, one should consider therapeutic strategies aimed at inhibiting the activity of TGF $\beta$ . In fact, radiation-induced TGF $\beta$  may even be detrimental in the case of a TGF $\beta$ -sensitive breast tumor, as TGF $\beta$ -dependent cell cycle arrest may allow for repair of DNA damage. Two different approaches to prevent inflammation-associated fibrosis by inactivating TGF $\beta$  have been successfully tested in experimental models [28, 93]. These include the use of neutralizing antibodies to TGF $\beta$  [117] and the administration of the TGF $\beta$ -binding protein, decorin [118]. Based on the idea that tumor-derived TGF $\beta$  allows tumors to escape from immune surveillance, a recent study showed that regression of experimental brain tumors in rats could be achieved by expressing antisense TGF $\beta$ 2 mRNA by enhancing immune rejection [24]. Similar approaches might be developed for the treatment of advanced breast cancers that are associated with TGF $\beta$  activation.

In summary, the detection of activated TGF $\beta$  in breast cancer stroma and/or the identification of molecular lesions in the TGF $\beta$  signaling system may provide new clinical tools to distinguish tumors in which augmentation of TGF $\beta$  production and/or activation might be therapeutically beneficial from those in which this would not be the case. This is particularly pertinent for the identification of patients who are likely not to benefit from anti-estrogen or radiation therapy. Conversely, the development of agents aimed at inactivating TGF $\beta$  is likely to benefit the treatment of advanced breast cancer, as has been suggested for the treatment of chronic inflammatory conditions associated with fibrosis [93].

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# Functional Characterization of Transforming Growth Factor $\beta$ Type II Receptor Mutants in Human Cancer<sup>1</sup>

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## ABSTRACT

We recently identified missense mutations at amino acid residues 526 and 537 located within the highly conserved subdomain XI of the transforming growth factor  $\beta$  type II receptor (T $\beta$ R-II) serine-threonine kinase in two human squamous carcinoma cell lines. These cell lines are resistant to transforming growth factor  $\beta$ -mediated inhibition of growth. Moreover, treatment with transforming growth factor  $\beta$  fails to increase the levels of type 1 plasminogen activator inhibitor and fibronectin synthesis. To test the effects of the mutations on receptor function, mutant T $\beta$ R-II cDNAs were expressed in T $\beta$ R-II-deficient T47D cells. Cyclin A promoter activity was reduced by 50% in cells expressing wild-type T $\beta$ R-II but increased 2-fold in cells transfected with either of the two mutant receptors. Conversely, plasminogen activator inhibitor type 1 promoter activity was increased 6-fold in cells transfected with wild-type receptor but not with either of the two mutant receptors. Moreover, the activity of both mutant serine-threonine kinases was strongly reduced compared to that of the wild-type receptor. Thus, the amino acid residues at positions 526 and 537 seem to be essential for kinase function and signaling activity of the T $\beta$ R-II.

## INTRODUCTION

TGF- $\beta$ <sup>4</sup> is a 25-kDa dimeric polypeptide that is ubiquitously present in the extracellular milieu of adult mammals (1), where it seems to play a key role in orchestrating the cellular responses to tissue injury (2). For example, when TGF- $\beta$  becomes activated in response to wounding or inflammation, it regulates the deposition of the ECM by mesenchymal cells and controls the proliferation of the epithelial cells (3, 4). Thus, one of the main functions of TGF- $\beta$  is to enable the cellular microenvironment to regulate epithelial cell proliferation by exerting G<sub>1</sub>-S-phase cell cycle checkpoint control. This is illustrated by the fact that, *in vitro*, TGF- $\beta$  is one of the most potent inhibitors of cell cycle progression of normal epidermal and oral keratinocytes (5, 6). In contrast, SCC cell lines derived from head and neck tumors are usually refractory to TGF- $\beta$ -mediated inhibition of DNA replication (6-8), as are cell lines derived from many other types of malignancies (reviewed in Ref. 8). These observations led us to propose the hypothesis that the escape from TGF- $\beta$ -mediated growth control is caused by inactivation of the TGF- $\beta$  signaling pathway and represents an important step in the malignant transformation of epithelial cells (8, 9).

The TGF- $\beta$  signal is transduced by a unique mechanism that involves a pair of transmembrane serine-threonine kinase receptors

(10). Free ligand binds primarily to the T $\beta$ R-II. Two T $\beta$ R-I molecules are then recruited into a ternary complex with two T $\beta$ R-II molecules and a single ligand dimer. The formation of this ternary complex allows the T $\beta$ R-II kinase to phosphorylate specific serine residues located within the highly conserved so-called GS domain immediately upstream of the T $\beta$ R-I serine-threonine kinase domain. This activation of the T $\beta$ R-I kinase is the necessary first step in further transducing the signal downstream (10). Postreceptor events in TGF- $\beta$  signaling are mediated by homologues of the *Drosophila* mother-against-dpp protein (11, 12). In response to TGF- $\beta$  binding, two of these proteins, Smad2 and Smad3, become transiently associated with the activated T $\beta$ R-I receptor and are phosphorylated, presumably by the T $\beta$ R-I kinase (12, 13). After their activation, Smad2 and Smad3 accumulate in the nucleus in heteromeric complexes with a third member of the MAD superfamily, Smad4 (14). Whereas the NH<sub>2</sub>-terminal domains of Smad3 and Smad4 are primarily involved in dimerization, the COOH-terminal domains seem to be involved in the regulation of gene transcription (15-20). TGF- $\beta$ 1 effectuates cell cycle arrest by increasing the levels of expression of cyclin-dependent kinase inhibitors such as p15, p21, and p27 (21-23), as well as by repressing the tyrosine phosphatase Cdc25A, Cdk4, cyclin A, and cyclin E genes (24-27). Besides inhibiting cell cycle progression, TGF- $\beta$  also induces the expression of a number of protein components of the ECM (for example, FN, laminin, and collagen IV) as well as proteins that inhibit ECM degradation, such as PAI-1 (28). Gene losses, rearrangements, mutations, and truncations of the T $\beta$ R-I, T $\beta$ R-II, Smad2, Smad3, and Smad4 genes have all been described in human cancer cell lines or in primary tumor specimens (29-35). These studies have provided strong support for the hypothesis that inactivation of the TGF- $\beta$  signaling pathway contributes to malignant progression.

Recently, we identified missense mutations at codons 526 and 537 of the T $\beta$ R-II gene in two head and neck carcinoma SCC cell lines, A253 and SqCC/Y1 (29). Similar missense mutations at codons 528 and 537 have since been reported in a small number of primary colorectal carcinomas (31, 32, 36), and closely related mutations within the COOH terminus of T $\beta$ R-II have been noted in cutaneous T-cell lymphoma cells (30). The clonal selection of tumor cells bearing these T $\beta$ R-II mutations strongly suggests that they contributed directly to neoplastic transformation. However, to prove this hypothesis, one needs to demonstrate that individual mutations have a negative impact on receptor function.

The mutation we found in A253 cells predicts for an amino acid change from arginine→proline at position 537 (R537P), whereas the SqCC/Y1 cell mutation causes a glutamic acid→glutamine change at position 526 (E526Q). Both mutations fall within subdomain XI of the receptor serine-threonine kinase (37) and involve amino acid residues that are highly conserved among members of the T $\beta$ R-II gene subfamily (29). In addition, both mutations predict for significant changes in the secondary structure of the cytoplasmic tail of the protein (29). Thus, it is likely that both mutations would affect receptor function. We now report that introducing either one of the two mutations into a wild-type T $\beta$ R-II gene completely abolishes the transduction of all cellular responses to TGF- $\beta$  by dramatically reducing receptor serine-threonine kinase activity.

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<sup>4</sup> The abbreviations used are: TGF- $\beta$ , transforming growth factor  $\beta$ ; SCC, squamous cell carcinoma; T $\beta$ R-II, type II TGF- $\beta$  receptor; T $\beta$ R-I, type I TGF- $\beta$  receptor; ECM, extracellular matrix; PAI-1, plasminogen activator inhibitor type 1; TBS-T, Tris-buffered saline with Triton-X100; FN, fibronectin; CMV, cytomegalovirus; IP, immunoprecipitation;  $\alpha$ R-II, anti-T $\beta$ R-II receptor antibody.

## MATERIALS AND METHODS

**Cell Culture.** The human epidermal keratinocyte cell line HaCat was obtained from Dr. Norbert Fusenig (German Cancer Research Center, Heidelberg, Germany). SCC cell lines A253 (38), SqCC/Y1 (39), and FaDu (40) were derived from primary SCCs of the head and neck. These cell lines were all maintained in enriched MCDB153-LB<sup>++</sup> medium as described previously (7). T47D human breast carcinoma cells (41) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum, penicillin, and streptomycin.

**PAI-1 Expression.** To measure PAI-1 production *in vivo*, cells were grown to confluence in 6-well tissue culture dishes and treated with 100 pM TGF- $\beta$ 1 (Austral Biologicals, San Ramon, CA) for 18 h. After collecting the supernatants, cell lysates were prepared in TBS-T buffer [20 mM Tris, 0.5 M NaCl, 1% Triton X-100 (pH 8.5)]. PAI-1 concentrations in conditioned media and clarified cell extracts were then determined separately using an ELISA kit (American Diagnostica, Greenwich, CT) according to the protocol supplied by the manufacturer.

**Measurement of FN Synthesis.** For measurements of FN synthesis, cells were plated in 60-mm tissue culture dishes. Twenty-four h later, the culture medium was replaced with fresh medium, and cells were treated with TGF- $\beta$ 1 (100 pM). During the final 4 h of an 18-h incubation, cells were exposed to 150  $\mu$ Ci/ml [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine (50 Ci/mmol; DuPont New England Nuclear, Boston, MA). Levels of radiolabeled FN were determined by a one-step purification using gelatin-Sepharose as described by Laiho *et al.* (42). Briefly, 500  $\mu$ l of conditioned medium or cell lysate were incubated in the presence of 100  $\mu$ l of gelatin-Sepharose (Pharmacia Biotech) and 3  $\mu$ l of Triton X-100 (Sigma Chemical Co., St. Louis, MO) on a rotating mixer for 24 h at 4°C. After three washes with TBS-T, the Sepharose beads were collected by centrifugation at 1500 rpm for 5 min at 4°C and resuspended in SDS-PAGE sample buffer containing  $\beta$ -mercaptoethanol, and the FN was eluted by boiling for 10 min. Proteins were then resolved by electrophoresis on a 6% (w/v) SDS-polyacrylamide gel, and radiolabeled bands were visualized by autoradiography of the dried Autofluor (National Diagnostics)-treated gels at -70°C using Kodak XAR film.

**Site-directed Mutagenesis.** Specific mutations were introduced into mammalian expression vector p122 (a generous gift of Dr. R. Derynck, University of California, San Francisco, CA) that contains a full-length wild-type mouse T $\beta$ R-II cDNA under the transcriptional control of a CMV promoter (43). It was legitimate to use the mouse T $\beta$ R-II sequence, because the amino acid sequence homology between the murine and human receptors is 91% overall, with 100% homology in subdomain XI of the serine-threonine kinase domain where both of the mutations are localized (44). For site-directed mutagenesis, two overlapping PCR fragments were generated using oligonucleotide primers carrying the desired mutation in the antisense and sense orientation, respectively. Gel-purified reaction products were then combined and reamplified using flanking wild-type primers. After removing the flanking sequences by *HindIII/XbaI* digestion, the newly generated 470-nucleotide *HindIII/XbaI* fragment was ligated into the p122 backbone that had been prepared by partial *HindIII/XbaI* digestion. The presence of the specific desired mutation was confirmed by restriction digestion and DNA sequencing.

**Reporter Gene Assays.** The signaling function of mutant  $\alpha$ R-IIs was assessed in transient transfection assays into T47D breast carcinoma cells. This cell line is convenient, because it is refractory to TGF- $\beta$  and fails to express detectable levels of T $\beta$ R-II mRNA or protein, and all responses to TGF- $\beta$  can be restored by reexpressing wild-type T $\beta$ R-II (45). Two separate reporter gene constructs were used to assess the different types of responses to TGF- $\beta$ : (a) pCAL2 (a generous gift of Dr. R. Derynck), which contains cyclin A gene promoter sequences driving the expression of firefly luciferase cDNA (43); and (b) p3TP-Lux (a generous gift from Dr. M. Centrella, Department of Plastic Surgery, Yale University), which contains the TGF- $\beta$  response element from the PAI-1 gene promoter and three tetradecanoylphorbol acetate response elements driving a firefly luciferase gene (46).

For transfections, T47D cells were plated at  $1.4 \times 10^5$  cells/well in 6-well cluster dishes and allowed to adhere overnight at 37°C. Before transfection, 10  $\mu$ l of Lipofectin (Life Technologies, Inc.) were diluted into 700  $\mu$ l of Opti-MEM (Life Technologies, Inc.) and incubated at 20°C for 45 min. Lipofectin was then combined with plasmid DNA (1  $\mu$ g T $\beta$ R-II plasmid DNA and 2  $\mu$ g

reporter plasmid/300  $\mu$ l Opti-MEM) and incubated for an additional 15 min at 20°C. After washing the cell monolayers twice with PBS, the DNA/Lipofectin mixture was added to each well in a dropwise fashion, and the cells were incubated at 37°C. Four h later, 2 ml of Opti-MEM were added to each plate, and 18 h later, the medium was completely replaced with 3 ml of fresh RPMI 1640 (Life Technologies, Inc.) supplemented with 0.2% fetal bovine serum, with or without 200 pM TGF- $\beta$ 1. Forty eight h posttransfection, cells were harvested by scraping them with a rubber policeman into a reporter lysis buffer (Promega Corp., Madison, WI). To control for variations in transfection efficiency, we used the Dual-Luciferase Reporter Assay System (Promega) that involves cotransfecting a small amount (0.005  $\mu$ g) of pRL-CMV, a plasmid expressing a *Renilla* luciferase reporter gene (Promega). Firefly and *Renilla* luciferase activities can be detected separately in the same cell lysates because of their different substrate specificities using the protocol provided by the manufacturer (Promega). Cell lysate (20  $\mu$ l) was mixed with 100  $\mu$ l of the appropriate luciferase assay reagent, and photon emission was measured for 10 s using a Series 20 Barthold Luminometer (Turner Designs, Sunnyvale, CA).

**IP and *in Vitro* Kinase Assay.** After incubation of cultured cells in the presence of 150  $\mu$ Ci/ml [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine (50 Ci/mmol; DuPont New England Nuclear) for 4 h, cell lysates were prepared from TGF- $\beta$ 1-treated cells (400 pM; 30 min at 4°C) using IP buffer [1% (v/v) Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA (pH 8), 1 mM EGTA (pH 8), 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 4  $\mu$ g/ml leupeptin]. Lysate containing 1 mg of total protein was incubated with 1  $\mu$ g of polyclonal rabbit antibody directed against the T $\beta$ R-II receptor ( $\alpha$ R-II, R220) (Santa Cruz Biotechnology) for 2 h at 4°C on a rotating shaker, followed by the addition of 40  $\mu$ l of presaturated protein A/G-agarose conjugate (Santa Cruz Biotechnology) for 30 min at 4°C. Pellets were centrifuged and washed twice with IP buffer, and complexes were eluted and dissociated by boiling for 5 min in IP buffer in the presence of 1% SDS. After clarification of the samples, supernatants were diluted 1:10 with IP buffer, reprecipitated with  $\alpha$ R-II antiserum, and incubated with protein A/G-agarose. After three washes with IP buffer, the agarose pellets with receptor complexes were resuspended in 2 $\times$  SDS-PAGE sample buffer, boiled for 5 min, and resolved by electrophoresis on a 7.5% (w/v) SDS-polyacrylamide gel. Gels were stained, destained, and dried, and the radiolabeled proteins were visualized by autoradiography using Kodak XAR film without enhancing screens. Controls included IPs performed using  $\alpha$ R-II antibody that had been presaturated with a 10-fold molar excess of the recombinant target peptide.

For *in vitro* kinase assays, unlabeled cell lysates containing 1 mg of total protein were incubated with 1  $\mu$ g of  $\alpha$ R-II antibody for 2 h at 4°C on a rotating shaker, followed by the addition of 40  $\mu$ l of presaturated protein A/G-agarose conjugate (Santa Cruz Biotechnology) for 30 min at 4°C. After centrifugation and washes, the pellets were incubated in 30  $\mu$ l of kinase buffer [20 mM HEPES (pH 7.7), 75 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA (pH 8), 0.05% (v/v) Triton X-100, 0.5 mM DTT, 20 mM  $\beta$ -glycerol phosphate, 100  $\mu$ M sodium orthovanadate, 4  $\mu$ g/ml leupeptin, and 100  $\mu$ g/ml phenylmethylsulfonyl fluoride] containing 20  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (specific activity, 0.25 Ci/M) for 20 min at 30°C. Immune complexes were eluted and dissociated by boiling in IP buffer with 1% (w/v) SDS for 5 min, followed by reprecipitation with the  $\alpha$ R-II antibody. Precipitated proteins were resolved by SDS-PAGE [7.5% (w/v)], and the phosphorylated species were visualized by autoradiography. Controls included IPs performed using  $\alpha$ R-II antibody that had been presaturated with a 10-fold molar excess of the recombinant target peptide.

## RESULTS AND DISCUSSION

In a previous study, we identified two human head and neck SCC cell lines (A253 and SqCC/Y1) that were homozygous carriers of two different missense mutants (R537P and E526Q) of the T $\beta$ R-II gene (29). We had shown previously that TGF- $\beta$  fails to inhibit DNA synthesis in these tumor cell lines (7). The fact that this resistance to inhibition of DNA replication results in continued cell proliferation in the presence of TGF- $\beta$  is illustrated in Fig. 1; whereas proliferation of nonneoplastic HaCat keratinocytes was inhibited by approximately 75% at concentrations of TGF- $\beta$ 1 of  $\geq 25$  pM, the anchorage-dependent growth of A253 and SqCC/Y1 cells was not affected by TGF- $\beta$ 1,

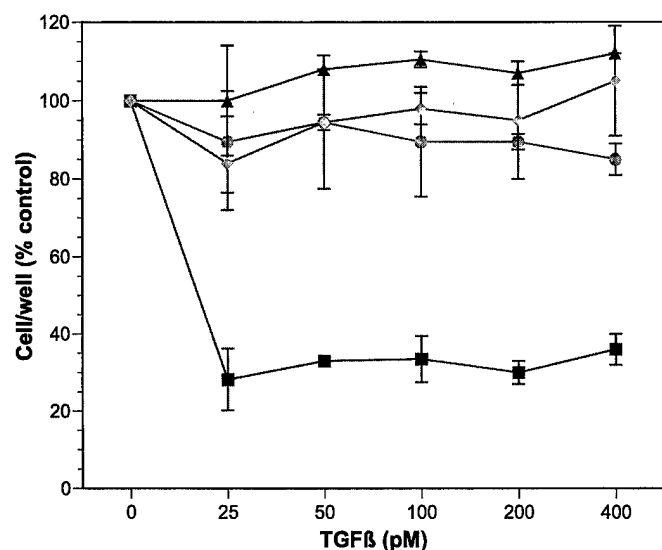


Fig. 1. Effect of TGF- $\beta$ 1 on cell proliferation of keratinocytes and SCC cell lines *in vitro*. Cells ( $10^4$  cells/well) were plated in 24-well cluster dishes, followed by the addition of TGF- $\beta$ 1 24 h later at the indicated concentrations. Medium and TGF- $\beta$ 1 were replaced after 3 days, and cell numbers in duplicate wells were determined after 6 days using a model ZM Coulter particle counter (Coulter Electronics, Ltd., Luton Beds, United Kingdom). ■, HaCat; ●, A253; ▲, FaDu; ◆, SqCC/Y1. The means  $\pm$  SE of three independent experiments are shown.

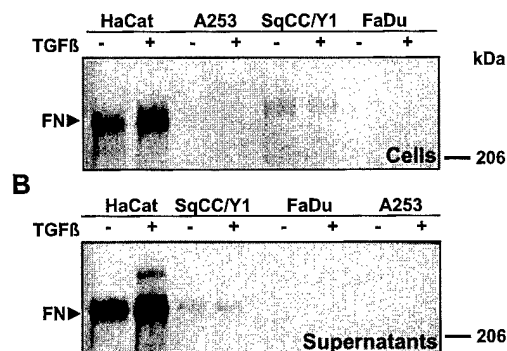
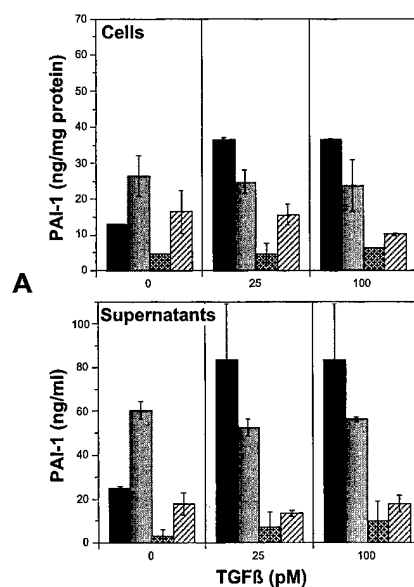
even at concentrations as high as 400 pM. The proliferation of FaDu cells, which fail to express the downstream effector of TGF- $\beta$  action, Smad4 (47), was similarly unaffected by treatment with TGF- $\beta$ 1.

Besides inhibiting cell cycle progression, TGF- $\beta$  also induces the expression of a number of protein components of the ECM, such as FN, as well as proteins that inhibit ECM degradation, such as PAI-1 (28). Some transformed cells no longer respond to TGF- $\beta$ -mediated effects on the cell cycle but retain the effects on ECM proteins (48, 49). To examine this possibility, we determined the effects of TGF- $\beta$ 1 treatment on PAI-1 and FN synthesis by the SCC lines. TGF- $\beta$ 1 induced a 3–4-fold increase in the levels of both cell-associated and secreted PAI-1 (Fig. 2A) and stimulated the synthesis of FN (Fig. 2B) in nonneoplastic HaCat keratinocyte cultures. In contrast, PAI-1 levels remained unchanged in A253, FaDu, and SqCC/Y1 cells. Moreover, although SqCC/Y1 cells produced relatively low but detectable

amounts of FN, synthesis was not increased in response to TGF- $\beta$ 1 treatment. The effects of TGF- $\beta$ 1 on FN synthesis in A253 and FaDu cells could not be ascertained, because neither cell line produced detectable amounts of this ECM protein (Fig. 2B). Although many malignant cell lines fail to secrete FN, a complete absence of FN expression by tumor cells is decidedly uncommon (50). In any event, these experiments indicate that the SCC lines fail to exhibit any of the typical cellular responses to TGF- $\beta$ .

To prove that the T $\beta$ R-II gene mutations were solely responsible for the TGF- $\beta$ -resistant phenotype of A253 and SqCC/Y1 cells and to determine more precisely how they affected receptor function, we introduced the two mutations into a full-length mouse wild-type T $\beta$ R-II cDNA. We used a mammalian expression vector in which wild-type and mutant T $\beta$ R-II cDNAs were placed under the transcriptional control of a CMV promoter. We studied the effects of the two mutations on receptor function in a series of transient transfection assays using T47D breast carcinoma cells as target. This is an ideal cell line to use for this type of experiment, because transfection of wild-type T $\beta$ R-II restores all responses to TGF- $\beta$  (45). Two separate reporter gene constructs, pCAL2 and p3TP-Lux, were used to assess the dual types of responses to TGF- $\beta$ . The repression of cyclin A promoter activity has been shown to correlate extremely well with the ability of cells (particularly keratinocytes) to respond to TGF- $\beta$ -mediated cell cycle arrest, and activation of the PAI-1 promoter reflects TGF- $\beta$ -induced PAI-1 production (43, 51). As shown in Fig. 3A, the expression of wild-type T $\beta$ R-II in T $\beta$ R-II-deficient T47D cells resulted in an approximately 50% reduction in cyclin A promoter activity compared to that of cells transfected with an inert control vector. In contrast, cyclin A promoter activity was not repressed in cells transfected with either the E526Q or the R537P receptor mutants (Fig. 3A). Repression of pCAL2 was independent of the addition of exogenous TGF- $\beta$ , presumably because, at high levels of expression, the T $\beta$ R-II and T $\beta$ R-I receptors tend to spontaneously dimerize and activate the signaling pathway (52–55). Alternatively, it is possible that bioactive TGF- $\beta$ 2 produced by T47D cells was sufficient to stimulate their TGF- $\beta$  receptors in an autocrine manner (56). Although attempts to block endogenous TGF- $\beta$ 2 activity by using a neutralizing anti-TGF- $\beta$ 2 antibody (R & D Systems) did not affect the results of the transfection experiments (data not shown), endogenous TGF- $\beta$ 2 may bind and activate the receptors intracellularly, as has

Fig. 2. Effect of TGF- $\beta$  on PAI-1 production and FN synthesis by keratinocytes and SCC cell lines. A, cells were grown to confluence in 6-well tissue culture dishes and treated with TGF- $\beta$ 1 at the indicated concentrations for 18 h. PAI-1 concentrations in culture supernatants and cell extracts were then analyzed separately using an ELISA. ■, HaCat; □, A253; ▤, FaDu; ▨, SqCC/Y1. The means  $\pm$  SE of four independent experiments are shown. B, pre-confluent cultures of HaCat keratinocytes or SCC cell lines were exposed to TGF- $\beta$ 1 (100 pM) for 18 h and to [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine during the last 4 h, followed by a one-step purification of FN from equal amounts of protein in the conditioned media or cell extracts using gelatin-Sepharose. Radiolabeled FN was resolved by 6% (w/v) SDS-PAGE and visualized by autoradiography as a single band with an apparent  $M_r$  of 220,000. FN was detectable in culture media and cell extracts of HaCat and SqCC/Y1 but not in FaDu or A253 cells.



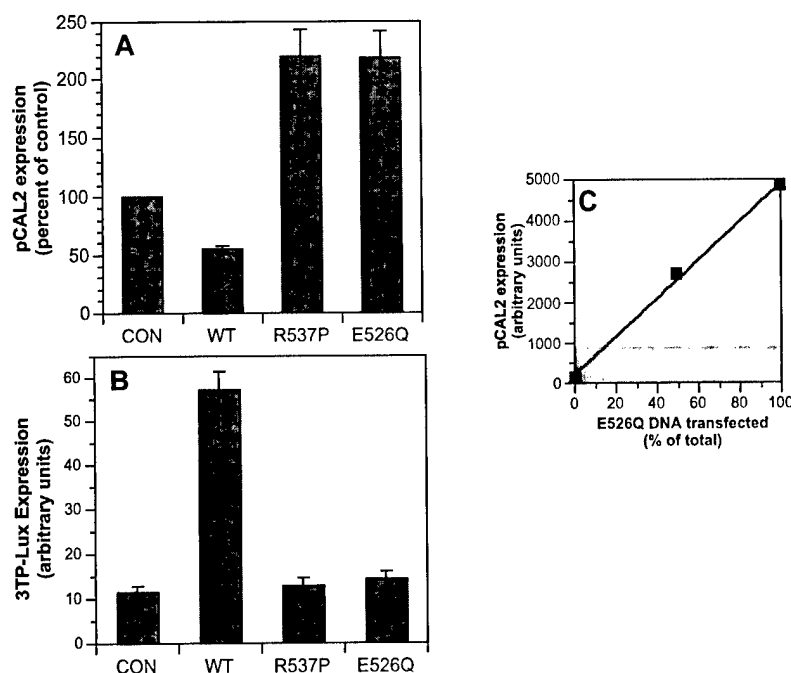


Fig. 3. Effects of transfected wild-type and mutant T $\beta$ R-II receptor on TGF- $\beta$ -regulated gene expression. T47D cells were cotransfected with plasmids expressing either wild-type (WT) or mutant (E526Q or R537P) T $\beta$ R-II receptor and pCAL2 (A) or p3TP-Lux (B) in conjunction with pRL-CMV, and luciferase activities in cell extracts were measured 48 h later, as described in "Materials and Methods." Results were normalized for Renilla luciferase activity to correct for differences in transfection efficiency between experiments. A, cyclin A promoter activity (pCAL2) was inhibited by 46% in cells transfected with the wild-type T $\beta$ R-II, whereas cells transfected with the E526Q or R537P mutants expressed approximately twice the amount of luciferase activity detected in control vector-transfected control cells. The means  $\pm$  SE from four independent experiments are shown. B, in cells transfected with wild-type T $\beta$ R-II, PAI-1 promoter (3TP-Lux) activity was increased 6-fold in response to TGF- $\beta$ 1 treatment, whereas no increase over control levels was noted in cells transfected with either of the two T $\beta$ R-II mutants. C, to determine whether the E526Q mutant was dominant negative, T47D cells were transfected with 1  $\mu$ g of wild-type T $\beta$ R-II, 1  $\mu$ g of E526Q, or with 0.5  $\mu$ g of each plasmid combined. The gray bar represents the pCAL2 activity in cells transfected with the control vector. The linear relationship between the amount of wild-type:E526Q DNA ratio and pCAL2 activity indicates that the activation of the cyclin A promoter in cells transfected with the T $\beta$ R-II mutant cannot be ascribed to a dominant-negative effect.

been noted for platelet-derived growth factor and hepatocyte growth factor (57).

In cells transfected with wild-type T $\beta$ R-II, the activity of the PAI-1 promoter was increased approximately 6-fold over that of the controls, whereas no increase was observed in cells transfected with either receptor mutant (Fig. 3B). Thus, both mutations abolish TGF- $\beta$ -dependent cell cycle arrest as well its effects on ECM-associated proteins, indicating that the amino acid residues at positions 526 and 537 are critical for normal TGF- $\beta$  signaling with respect to both cell cycle regulation and ECM protein induction.

Interestingly, both the E526Q and R537P mutants caused a 2-fold increase in cyclin A promoter activity over the control levels (Fig. 3A). This observation raised the possibility that both mutants have a strong dominant-negative effect. Besides several other missense mutants within the juxtamembrane and kinase domains (46, 58–60), COOH-terminal-truncated T $\beta$ R-II proteins are particularly potent dominant-negatives that completely abrogate all of the responses to TGF- $\beta$  when transfected into normal endothelial and bronchial cells (61–64).

Dominant-negative mutants may interfere with TGF- $\beta$  signaling by competing with wild-type T $\beta$ R-II for heterodimerization with T $\beta$ R-I molecules or by sequestering coexpressed wild-type T $\beta$ R-II molecules in homodimeric complexes. However, if this interpretation is correct, it would imply that T47D cells do, in fact, express a low level of wild-type T $\beta$ R-II, although the protein is not detectable by ligand binding assays, T $\beta$ R-II-specific mRNA is undetectable by Northern blot analysis, and the cells are unresponsive to TGF- $\beta$  (45). To test whether the T $\beta$ R-II mutants were dominant negative, we cotransfected them into T47D cells together with the wild-type construct. As shown in Fig. 3C, the pCAL2 activity we measured in cells transfected with a 1:1 ratio of E526Q mutant and wild-type T $\beta$ R-II cDNA

was exactly what one would expect if the mutant cDNA had no repressive activity of its own and did not affect repression of pCAL2 by the wild-type receptor. Similar results were obtained with the R537P mutant (data not shown). Thus, based on these results, it is very unlikely that the induction of pCAL2 activity in cells expressing mutant receptors is due to a dominant-negative effect.

The increase in cyclin A promoter activity induced by the two T $\beta$ R-II mutants suggests the possibility that the proliferation of tumor cells that express such mutants might be stimulated by TGF- $\beta$ . Such a seemingly paradoxical response to this growth inhibitor has been reported in a number of different tumor systems. For example, highly metastatic human colorectal carcinoma sublines are frequently stimulated by TGF- $\beta$  (65, 66). This switch in responsiveness to TGF- $\beta$  has been attributed to the use of an alternative TGF- $\beta$  signaling pathway in which two particular myelin basic protein kinases that are normally inhibited become activated (67). It is conceivable that the activation of this alternative signaling pathway results in the induction of cyclin A expression and, consequently, in the stimulation of cell proliferation. These questions will best be addressed by generating T47D cell lines that are stably transfected with the T $\beta$ R-II mutants.

To rule out that the observed differences in reporter gene activity were simply due to variations in the levels of expression of wild-type and mutant T $\beta$ R-IIs in transfected cells, parallel dishes of [ $^{35}$ S]methionine- and [ $^{35}$ S]cysteine-labeled cells were subjected to double IP with anti-T $\beta$ R-II antiserum (Fig. 4A). Discrete bands of approximately equal intensity at the level of 65 kDa corresponding to the T $\beta$ R-II receptor were detected in extracts from both wild-type- and mutant T $\beta$ R-II-transfected cells. Moreover, a second band of approximately 55 kDa, representing coimmunoprecipitated T $\beta$ R-I, was also seen in all three cases. No receptor protein was detected in control-transfected cells. These results indicate that the two T $\beta$ R-II mutations

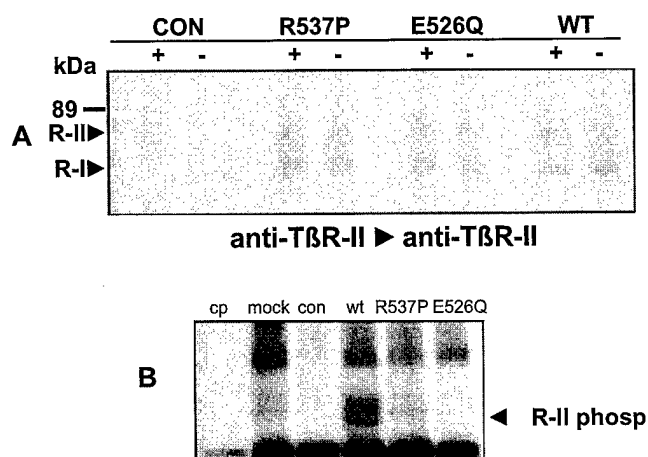


Fig. 4. Expression and *in vitro* kinase activity of transfected wild-type and mutant T $\beta$ R-II receptor in T47D cells. A, T47D cells were transfected with plasmids expressing either wild-type (WT) or mutant (E526Q or R537P) T $\beta$ R-II cDNAs, radiolabeled using methionine and [ $^{35}$ S]cysteine, and subjected to double IP with anti-T $\beta$ R-II antiserum as described in "Materials and Methods" (A). Discrete bands of approximately equal intensity at the level of 65 kDa corresponding to the T $\beta$ R-II receptor were detected in extracts from wild-type and mutant T $\beta$ R-II-transfected cells. Moreover, a second band of approximately 55 kDa, representing coimmunoprecipitated T $\beta$ R-I, was also present in all three cases. No receptor protein was detected in cells transfected with the control plasmid (CON). B, T $\beta$ R receptor complexes were harvested from transfected T47D cells by IP using  $\alpha$ R-II antibody and incubated in the presence of [ $\gamma$ - $^{32}$ P]ATP. After the *in vitro* kinase reaction, extracts were boiled for 5 min in the presence of 1% SDS, reprecipitated with  $\alpha$ R-II, and resolved by 7.5% (w/v) SDS-PAGE. A phosphorylated species of ~65 kDa corresponding to T $\beta$ R-II was easily detectable in the cells transfected with wild-type T $\beta$ R-II (wt), whereas these phosphoproteins were barely detectable in mutant T $\beta$ R-II-transfected cells (R537P and E526Q). Phosphoproteins were not detected in control transfectants (con), in wild-type-transfected cells immunoprecipitated in the presence of excess control peptide (cp), nor in mock-transfected cells (mock). These experiments were performed three different times with consistently similar results.

did not affect receptor protein expression or the ability of the receptor mutants to form heteromeric complexes with endogenous T $\beta$ R-I receptors.

Interestingly, this receptor phenotype is clearly distinct from that described for a closely related T $\beta$ R-II mutant in which a proline residue at position 525 is replaced by a leucine (60). This P525L T $\beta$ R-II mutant receptor is capable of binding ligand and of forming a complex with T $\beta$ R-I but fails to transphosphorylate T $\beta$ R-I (60). However, P525L differs from the E526Q and R537P mutants in that its kinase activity does not seem to be impaired, as demonstrated by normal levels of T $\beta$ R-II autophosphorylation (60). In addition, the P525L mutant is clearly dominant negative, whereas our two mutants are not (60). Thus, mutations that affect two neighboring amino acids (525 and 526) display very different phenotypes, thereby underscoring the functional importance of the COOH-terminal portion of the kinase domain. This is also underscored by the fact that the arginine residue at position 528 is highly conserved among all protein kinases and forms an ion pair with an equally conserved glutamic acid residue in subdomain VIII that is essential for kinase function (68, 69). Thus, it is likely that mutations involving codon 528 identified in gastric carcinomas (31, 32, 36) also severely disrupt T $\beta$ R-II receptor function.

In our previous study (29), *in vitro* kinase assays using immunoprecipitated receptors from A253 and SqCC/Y1 cells had suggested that the E526Q and R537P mutants differed with respect to their kinase activity. Specifically, the level of autophosphorylated T $\beta$ R-II protein was significantly reduced in immunoprecipitates from SqCC/Y1 cells, whereas it was dramatically increased in immunoprecipitates from A253 cells, suggesting that the R537P mutant serine-threonine kinase might be constitutively activated (29). To determine whether these differences could be directly attributed to the two

missense mutations, we assessed the *in vitro* kinase activity of the two cloned mutants in immunoprecipitates from transiently transfected T47D cells (29). As shown in Fig. 4B, a phosphorylated protein of ~65 kDa corresponding to T $\beta$ R-II was easily detectable in cells transfected with wild-type T $\beta$ R-II, whereas such phosphoproteins were barely detectable in cells transfected with either of the two T $\beta$ R-II mutants. Thus, both the E526Q and the R537P mutations have a dramatic negative impact on the receptor kinase activity. The finding that the E526Q mutation results in a dramatic reduction in the kinase activity of T $\beta$ R-II (Fig. 4) is in accord with our previous observation that immunoprecipitated receptor preparations from SqCC/Y1 cells were largely devoid of *in vitro* kinase activity (29). However, the loss of kinase activity of the R537P mutant (Fig. 4) contrasts with the previous observation that TGF- $\beta$  receptor complexes immunoprecipitated from A253 cells displayed an increased level of auto- and transphosphorylation (29). One possible explanation lies in an amino acid sequence difference between the human and mouse T $\beta$ R-II at position 416. In the human T $\beta$ R-II sequence, the serine residue at position 416 becomes autophosphorylated by an intermolecular mechanism after receptor homodimerization (70). This phosphorylation step seems to regulate signal transduction, because it inhibits the transphosphorylation of T $\beta$ R-I and the transduction of the growth-inhibitory signal (70). In contrast to the human sequence, the mouse T $\beta$ R-II sequence lacks a potential phosphorylation site at position 416. Thus, if the high level of T $\beta$ R-II autophosphorylation observed in A253 extracts is due to hyperphosphorylation on serine 416, this may account for the differences in autophosphorylation patterns of T $\beta$ R-II observed between A253 cells (which express a human mutant R537P receptor) and T47D cells transfected with mutant R537P mouse T $\beta$ R-II cDNA. We are currently investigating this possibility.

Mutations of the T $\beta$ R-II gene associated with human neoplasms seem to cluster in two particular hot spots. Mutations in the 5' half of the gene result in the synthesis of truncated and presumably soluble T $\beta$ R-II exodomains (see Ref. 71). Such truncated receptors have been shown to inhibit TGF- $\beta$  signaling in a dominant-negative manner (72, 73). The second group of mutations seems to be centered on the COOH-terminal portion of the serine-threonine kinase domain, particularly subdomains X and XI (29–32, 36). Knaus *et al.* (30) demonstrated recently that a D404G mutation found in a case of cutaneous T-cell lymphoma caused TGF- $\beta$  resistance by inhibiting the cell surface expression of TGF- $\beta$  receptors in a dominant-negative manner. However, up to this point, the impact of these various COOH-terminal missense mutations on the receptor kinase activity or the signaling function of the receptor protein itself had not been addressed. The current study demonstrates that two different mutations within the COOH terminus of the T $\beta$ R-II receptor that we had identified in human SCC cells abolish the signaling function of the receptor, because they cause a nearly complete loss of serine-threonine kinase activity. These results indicate that these mutant receptors are responsible for the resistance of A253 and SqCC/Y1 cells to TGF- $\beta$  and provide strong evidence for a role of these mutants in the pathogenesis of the head and neck tumors they were found in.

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# Cloning and Genomic Organization of the Human Transforming Growth Factor- $\beta$ Type I Receptor Gene

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**Transforming growth factor- $\beta$  (TGF $\beta$ ) regulates cell cycle progression by a unique signaling mechanism that involves its binding to the type II (T $\beta$ R-II) TGF $\beta$  receptor and activation of type I (T $\beta$ R-I). Both are transmembrane serine-threonine receptor kinases. As various types of human tumor cells are often refractory to TGF $\beta$ -mediated cell cycle arrest, it is likely that the T $\beta$ R-I receptor is inactivated in many of these cases. We determined the intron-exon organization of the *TGFBR1* gene. We report here that this gene is approximately 31 kb in length and consists of nine exons. The organization of the segment of the *TGFBR1* gene that encodes the C-terminal portion of the serine-threonine kinase domain appears to be highly conserved between members of the R-I gene family. This information should facilitate and expedite the structural analysis of *TGFBR1* in human tumors and possibly other disease states.** © 1997 Academic Press

**Key Words:** transforming; growth factor beta; receptor; intron; exon; activin.

## INTRODUCTION

Transforming growth factor- $\beta$  (TGF $\beta$ ) types 1, 2, and 3 belong to a large superfamily of secreted polypeptides that also includes activin, inhibin, and the bone morphogenetic proteins (BMPs) (Massagué *et al.*, 1994). The primary role of these proteins in adult mammalian organisms is to regulate cell proliferation and differentiation in a wide variety of tissues (Roberts and Sporn, 1993). In addition, the members of the TGF $\beta$  superfamily play a fundamental role in the development of the body plan during early embryogenesis in organisms ranging from *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Xenopus laevis* to mammals (Massagué, 1996).

The intracellular signals from all members of the TGF $\beta$  superfamily of proteins appear to be transduced

by a mechanism that is unique to this class of signaling peptides and involves ligand-specific pairs of transmembrane serine-threonine kinase receptors (Massagué, 1996). In most cases, free ligand binds primarily to the type II receptor (R-II) (Wrana *et al.*, 1992, 1994). Interestingly, R-II receptor kinase activity is detectable independent of ligand binding (Wrana *et al.*, 1992, 1994). Once ligand is bound to its R-II receptor, the corresponding member of the type I receptor (R-I) family is recruited into a ternary complex, typically in a heterotetrameric configuration with two R-II molecules and a single ligand dimer (Anders and Leof, 1996; Chen and Derynck, 1994; Luo and Lodish, 1996). The formation of this ternary complex causes the R-II kinase to phosphorylate specific serine residues within the highly conserved GS domain located immediately upstream of the serine-threonine kinase domain of the R-I receptor (Wieser *et al.*, 1993). This phosphorylation step activates the R-I kinase, which is the necessary first step in transducing the signal downstream. Thus, the R-I molecule is the primary signal transducer (Massagué, 1996). This idea is supported by the observation that a constitutively activated T $\beta$ R-I molecule is capable of signaling independently of the presence of either ligand or R-II receptor (Carcamo *et al.*, 1994; Wieser *et al.*, 1995). Postreceptor events in TGF $\beta$  signaling are primarily mediated by members of the *D. melanogaster mothers-against-dpp* (MAD) superfamily of proteins (Derynck and Zhang, 1996). In response to TGF $\beta$  binding, two of these proteins, Smad2 and Smad3, become transiently associated with the activated T $\beta$ R-I receptor and are phosphorylated, presumably by the T $\beta$ R-I kinase (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996). Following their activation, Smad 2 and -3 accumulate in the nucleus in heteromeric complexes with a third member of the MAD superfamily, Smad4 (Lagna *et al.*, 1996; Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996). The Smad4 carboxy-terminal domain has been shown to be a potent activator of transcription when fused to a Gal4 DNA binding domain, but it is not yet clear whether native Smad4 binds DNA directly or functions as a transcriptional coactivator *in vivo* (Chen *et al.*, 1996; Lagna *et al.*, 1996; Liu *et al.*, 1996). Target genes for TGF $\beta$ -dependent transcriptional activation include

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*INK4B* and *WAF1*, which encode the cyclin-dependent kinase inhibitors p15 and p21, respectively. These proteins play a critical role in inducing cell cycle arrest (Datto *et al.*, 1995; Hannon and Beach, 1994).

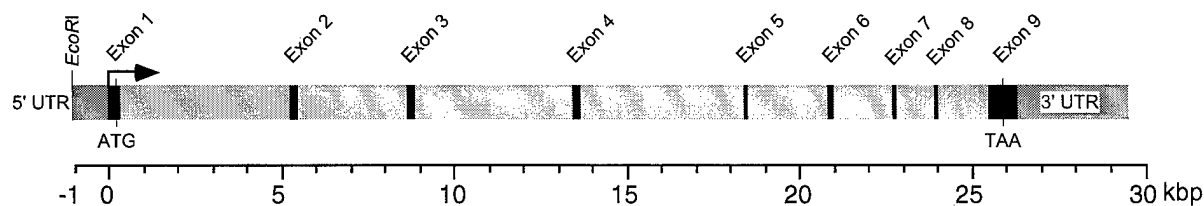
Human malignant neoplastic cells are frequently refractory to TGF $\beta$ -mediated cell cycle arrest (Fynan and Reiss, 1993). In principle, resistance may be caused by functional inactivation of any of the essential signaling intermediates. Thus far, structural alterations including homozygous deletions, mutations, and truncations of the *TGFBR2*, *SMAD2*, and *SMAD4* genes have been found in human tumors (De Jonge *et al.*, 1997; Eppert *et al.*, 1996; Garrigue-Antar *et al.*, 1995; Hahn *et al.*, 1996; Markowitz *et al.*, 1995; Park *et al.*, 1994). In addition, loss of *TGFBR2* mRNA expression occurs frequently in human esophageal and small cell lung carcinomas (De Jonge *et al.*, 1997; Garrigue-Antar *et al.*, 1996).

Up to this point, only limited evidence for inactivation of the primary transducing R-I receptor in human cancer has been presented. Loss of *TGFBR1* mRNA expression due to gene rearrangement has been noted in a single prostatic carcinoma cell line (Kim *et al.*, 1996). Moreover, pancreatic carcinoma cell lines often express markedly reduced levels of *TGFBR1* mRNA (Baldwin *et al.*, 1996). The main stumbling block in conducting a comprehensive analysis of the role of the *TGFBR1* gene in cancer has been the lack of information regarding its genomic organization. To facilitate studies of structural alterations of the *TGFBR1* gene in human neoplasms, we determined the intron-exon organization of the gene and methods for analyzing individual exons. We report here that the *TGFBR1* gene is approximately 31 kb and consists of nine exons. The organization of the segment of the gene that encodes the C-terminal portion of the serine-threonine

## A

| Exon | 3' Splice acceptor | 5' End of exon          | Exon size (bp) | 3' End of exon | 5' Splice donor | Estimated intron size (bp) |
|------|--------------------|-------------------------|----------------|----------------|-----------------|----------------------------|
| 1    |                    | TGCCTGGCGG <sup>1</sup> | 329            | GGGGCGACGG     | gtgagcggcg      | 4,900                      |
| 2    | cttttccag          | CGTTACAGTG              | 246            | CCAACACTG      | gtaagtgtga      | 3,200                      |
| 3    | ccttttccag         | TAAAGTCATC              | 231            | TCTGGCTCAG     | gtaacataat      | 4,550                      |
| 4    | ttacctttag         | GTTTACCATT              | 231            | GACAATAAAG     | gtctgtaaca      | 4,700                      |
| 5    | tgttttacag         | ACAATGGTAC              | 168            | GGTACCCAAG     | gtaattctat      | 2,300                      |
| 6    | gattctttag         | GAAAGCCAGC              | 157            | GAACAAAAAG     | gtatactttt      | 1,700                      |
| 7    | ttttttttag         | GTACATGGCC              | 125            | TCCATTGGTG     | gtaaattgct      | 1,100                      |
| 8    | ctgatacag          | GAATTCATGA              | 131            | GAGCTGTGAA     | gtgagtattt      | 1,400                      |
| 9    | tttctttag          | GCCTTGAGAG              | ≥ 845          |                |                 |                            |

## B



<sup>1</sup> Transcription start sequence (Bloom, *et al.*, 1996)

**FIG. 1.** Intron-exon organization of the human *TGFBR1* gene. (A) Nucleotide sequences at the 5' and 3' ends of each exon as well as intronic flanking sequences are provided. All splice sites follow the ag-gt rule for splice-donor and -acceptor sequences. (B) Scaled representation of the *TGFBR1* gene. Indicated sizes of individual introns are based on information derived from Southern blot analysis, direct sequencing of BACH-559 DNA, and/or PCR amplification of individual introns using flanking oligonucleotide primer sets (data not shown). Exon 9 extends at least to the 3' end of the published *ALK-5* sequence (Franzén *et al.*, 1993). No sequence information is available for the shaded portion of the 3' untranslated region (3'UTR), which is at least 3.2 kb in length (Franzén *et al.*, 1993). 5'UTR, 5' untranslated region.

[illegible]

**B**

|        |     |            |             |            |            |            |     |
|--------|-----|------------|-------------|------------|------------|------------|-----|
|        |     | 10         | 20          | 30         | 40         | 50         |     |
| TGFB1  | 1   | ISEGTTLKDL | YDMMTSGSG   | SGLPLLVQRT | IARTIVLQES | IGKGRFGEVW | 50  |
| ACVR1B | 1   | LSKDKTLQDL | VYDLSTSGSG  | SGLPLFVQRT | VARTIVLQEI | IGKGRFGEVW | 50  |
| BMP1A  | 1   | IPVGESLKDL | IDQSQSSGSG  | SGLPLLVQRT | IAKQIQMVQR | VKGGRYGEVW | 50  |
| BMP1B  | 1   | IPPGESLRDL | IEQSQSSGSG  | SGLPLLVQRT | IAKQIQMVQR | IGKGRYGEVW | 50  |
| ACVRL1 | 1   | EQGDTMLGDL | LDSDCTTSGS  | SGLPLFVQRT | VARQVALVEC | VKGGRYGEVW | 50  |
|        |     | 60         | 70          | 80         | 90         | 100        |     |
| TGFB1  | 51  | RGKWRGEEVA | VKIFSSREER  | SWFREAEIYQ | TVLMRHENIL | GFIADNKN   | 100 |
| ACVR1B | 51  | RGRWRGGDVA | VKIFSSREER  | SWFREAEIYQ | TVLMRHENIL | GFIADNKN   | 100 |
| BMP1A  | 51  | MGKWRGEKVA | VKVFFTTEEA  | SWFRETEIYQ | TVLMRHENIL | GFIADIKGT  | 100 |
| BMP1B  | 51  | MGKWRGEKVA | VKVFFTTEEA  | SWFRETEIYQ | TVLMRHENIL | GFIADIKGT  | 100 |
| ACVRL1 | 51  | RGLWHGESVA | VKIFSSRDEQ  | SWFRETEIYN | TVLLRHENIL | GFIASDMTSR | 100 |
|        |     | 110        | 120         | 130        | 140        | 150        |     |
| TGFB1  | 101 | GTWTQLWLV  | DYHEHGSFDF  | YLNRYTVTVE | GMIKLALSTA | SGLAHLHMEI | 150 |
| ACVR1B | 101 | GTWTQLWLV  | DYHEHGSFDF  | YLNRYTVTIE | GMIKLALSAA | SGLAHLHMEI | 150 |
| BMP1A  | 101 | GSWTQLYLIT | DYHENGSLYD  | FLKCATLDTR | ALFKLAYSAA | CGLCHLHTEI | 150 |
| BMP1B  | 101 | GSWTQLYLIT | DYHENGSLYD  | FLKSTFLDAK | SMLKLAYSSV | SGLCHLHTEI | 150 |
| ACVRL1 | 101 | NSSTQLWLIT | DYHEHGSFDF  | FLQRQTLEPH | LALRLAVSAA | CGLAHLHMEI | 150 |
|        |     | 160        | 170         | 180        | 190        | 200        |     |
| TGFB1  | 151 | VGTQGKPAIA | HRDLKSKNII  | VKKNGTCCIA | DLGLAVRHDS | ATDTIDIAPN | 200 |
| ACVR1B | 151 | VGTQGKPGIA | HRDLKSKNII  | VKKNGMCAIA | DLGLAVRHDA | VTDTIDIAPN | 200 |
| BMP1A  | 151 | YGTQGKPAIA | HRDLKSKNII  | IKKNGSCCIA | DLGLAVKFNS | DTNEVDVPLN | 200 |
| BMP1B  | 151 | ESTQGKPAIA | HRDLKSKNII  | VKKNGTCCIA | DLGLAVKFIS | DTNEVDIPPN | 200 |
| ACVRL1 | 151 | FGTQGKPAIA | HRDFKSRNVL  | VKSNIQCCIA | DLGLAVMHSQ | GSDYLDIGNN | 200 |
|        |     | 210        | 220         | 230        | 240        | 250        |     |
| TGFB1  | 201 | HRVGTKRYMA | PEVLDDSIINM | KHFESFKRAD | IYAMGLVFW  | IARRCSIGGI | 250 |
| ACVR1B | 201 | QRVGTKRYMA | PEVLDEITINM | KHFDSFKCAD | IYALGLVYWE | IARRCNSGGV | 250 |
| BMP1A  | 201 | TRVGTKRYMA | PEVLDESINM  | NHFQPYIMAD | IYSFGLIWE  | MARRCITGGI | 250 |
| BMP1B  | 201 | TRVGTKRYMP | PEVLDESINM  | NHFQSYIMAD | MYSFGLIWE  | VARRCNSGGI | 250 |
| ACVRL1 | 201 | PRVGTKRYMA | PEVLDEQIRT  | DCFESYKWT  | IWAFGLVLWE | IARRTIVNGI | 250 |
|        |     | 260        | 270         | 280        | 290        | 300        |     |
| TGFB1  | 251 | HEDYQLPYD  | LVPSPDSVEE  | MRKVVCQKL  | RPNIPNRWQS | CEALRVMAKI | 300 |
| ACVR1B | 251 | HEEYQLPYD  | LVPSPDSIEE  | MRKVVCQKL  | RPNIPNRWQS | YEALRVMGKM | 300 |
| BMP1A  | 251 | VEEYQLPYN  | MVPSDPSYED  | MREIVCVKRL | RPIVSNRWNS | DECLRAVLKL | 300 |
| BMP1B  | 251 | VEEYQLPYD  | LVPSPDSYED  | MREIVCIKKL | RPSFNRWSS  | DECLROMGKL | 300 |
| ACVRL1 | 251 | VEDYRPPFYD | VVPNDPSFED  | MKKVVCVDQ  | TPTIPNRLAA | DPVLSGLAQM | 300 |
|        |     | 310        | 320         | 330        | 340        | 350        |     |
| TGFB1  | 301 | MRECWYANGA | ARLTALRIKK  | TLSQLSQEG  | IKM.....   | 350        |     |
| ACVR1B | 301 | MRECWYANGA | ARLTALRIKK  | TLSQLSVOED | VKI.....   | 350        |     |
| BMP1A  | 301 | MSECWAHNP  | SRLTALRIKK  | TLAKMVESQD | VKI.....   | 350        |     |
| BMP1B  | 301 | MTECWAHNP  | SRLTALRVKK  | TLAKMSESQD | IKL.....   | 350        |     |
| ACVRL1 | 301 | MRECWYPNPS | ARLTALRIKK  | TLQKISNSPE | KPKVIQ.... | 350        |     |

**FIG. 2.** TGF $\beta$  superfamily protein type I receptor genes. (A) Comparison of the locations of introns ( $\blacktriangledown$ ) within the *TGFB1* gene with those reported for the human *ACVR1B* (*ALK-4*, *SKR2*) ( $\square$ ) and *ACVRL1* (*ALK-1*, *TSR1*) ( $\triangle$ ) genes. The segments of these genes that encode the serine-threonine kinase domains are bracketed. Stop codons are boxed. (B) Amino acid sequences of human type I receptors for TGF $\beta$ , activin, or bone morphogenetic proteins are highly conserved (55–75% sequence homology) with respect to the region that encodes the GS- (overlined) and serine-threonine kinase domains (brackets). *TGFB1* (*ALK-5*, Ten Dijke *et al.*, 1993); *ACVR1B* (Carcamo *et al.*, 1994) (*ALK-4*, Ten Dijke *et al.*, 1993; *SKR2*, Xu *et al.*, 1994); *BMP1A* (*ALK-3*, Ten Dijke *et al.*, 1993); *BMP1B* (*ALK-6*, Ten Dijke *et al.*, 1994a,b); *ACVRL1* (*ALK-1*, Ten Dijke *et al.*, 1993; *TSR-1*, Attisano *et al.*, 1993).

kinase domain is highly conserved between members of the R-I gene family, while the 5' ends of these genes show considerable divergence. This information should facilitate and expedite the structural analysis of *TGFB1* in human tumors and possibly other disease states.

## MATERIALS AND METHODS

**Isolation and purification of bacterial artificial chromosome (BAC) DNA.** Bacterial stocks were plated on LB-agar supplemented with 12.5  $\mu$ g/ml chloramphenicol. A 30-ml culture of LB with chloramphenicol was seeded from a single colony and grown overnight at 37°C to confluency. Cells were pelleted and gently resuspended in 1 ml GTE buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0). Bacteria were then lysed by sequential treatment with lysozyme (50 mg/ml), 0.2 M NaOH/1% (w/v) SDS, and 3 M potassium

acetate (pH 4.8), each for 5 min on ice. Following clarification of the lysate by centrifugation at 10,000g for 10 min, the supernatant was carefully removed and incubated in the presence of 50  $\mu$ g/ml RNase A and 10 units/ml RNase One (Promega) for 1 h at 37°C, followed by phenol/chloroform extraction and isopropyl alcohol precipitation of the DNA from the aqueous phase. Typically, between 5 and 10  $\mu$ g of BAC DNA can be harvested from a 30-ml culture.

**Determination of the *TGFB1* gene exon-intron organization.** The genomic organization of the *TGFB1* gene was determined by direct sequencing of purified BAC DNA or of PCR-amplified BAC DNA fragments. Sequencing was carried out using the SequiTherm EXCEL DNA Sequencing kit (Epicentre Technologies, Madison, WI). For each sequencing reaction, 2.5–5  $\mu$ g of ethanol-precipitated BAC DNA was resuspended in 5  $\mu$ l water. Sequencing primers (27 nucleotides in length) were end-labeled with [ $\gamma$ - $^{32}$ P]ATP (DuPont/NEN; 6000 Ci/mmol) using T4 polynucleotide kinase. BAC DNA was then subjected to manual cycle sequencing using 1.5  $\mu$ l SequiTherm EXCEL DNA polymerase per reaction and the following cycling parameters: 1 cycle of 5 min at 95°C, followed by 35 cycles of 1 min at 95°C

and 1 min at 68°C. Termination dyes were then added, and the reaction products were heated to 70°C for 5 min, cooled on ice, and resolved on an 8% sequencing gel. Gels were then fixed, dried, and exposed to Kodak X-Omat AR5 film. The locations of putative intron-exon boundaries and their flanking intronic sequences were determined by direct sequencing using oligonucleotide primers that matched *TGFBR1* exon sequences at sites evenly distributed throughout the coding region (Franzén *et al.*, 1993). The positions of boundaries were confirmed by sequencing in the reverse direction from sites in neighboring exons.

## RESULTS AND DISCUSSION

To obtain genomic clones that contained the *TGFBR1* gene, a human genomic library cloned into BACs (Shizuya *et al.*, 1992) was screened using a full-length *TGFBR1* cDNA (*ALK-5*; Franzén *et al.*, 1993) at Genome Systems, Inc. (St. Louis, MO). We obtained three individual BAC clones (BACH-527, -559, -564) that hybridized with the *ALK-5* probe in two sequential screens. To confirm that the BAC clones contained *TGFBR1*-specific genomic sequences and to generate a preliminary restriction map, purified BAC DNA was subjected to Southern blot analysis using three different DNA fragments derived from the 5' end, an internal portion, and the 3' untranslated region of the *TGFBR1* cDNA, respectively. Two of the three clones (BACH-559 and -564) hybridized strongly with all three *ALK-5* probes, even under stringent conditions, indicating that both of these BAC clones encompassed the complete coding sequence as well as the 3' untranslated portion of the *TGFBR1* gene (data not shown). Using two nested sets of oligonucleotide primers specific for *TGFBR1* cDNA sequences and reaction conditions optimized for long-range PCR, we were able to amplify DNA fragments from BACH-559, indicating that this clone contains the full-length human *TGFBR1* gene.

To determine the location of the intron-exon boundaries within the *TGFBR1* gene, we designed a series of oligonucleotides that matched sequences located at approximately 300-bp intervals along the *ALK-5* (*TGFBR1*) cDNA, which were then used for direct sequencing of purified BACH-559 DNA. The position of each intron-exon boundary was confirmed by sequencing in the reverse orientation based on the sequence of the second exon flanking the same intron. As shown in Fig. 1, the *TGFBR1* gene consists of nine exons, with sizes ranging from 125 to more than 845 bp. Exon 1 contains the ATG start codon and extends at least as far upstream as the transcription start site (Bloom *et al.*, 1996). Most of the extracellular domain of the receptor is encoded by the first two exons. Exon 3 encodes the transmembrane domain, the juxtamembrane region, and the GS-domain. The serine-threonine kinase domain is encoded by exons 4 through 9. Exon 9 encodes the C-terminus of the protein and extends at least as far as the 3' end of the published *ALK-5* sequence (Franzén *et al.*, 1993). Each of the exon-intron junctions follow the GT-AG rule for splice-donor and -acceptor sites. Based on the Southern blot analysis,

direct sequencing of BACH-559 DNA, and PCR amplification of individual introns using flanking exonic oligonucleotide primer sets (data not shown), we were able to calculate the molecular sizes of the individual introns (Fig. 1). We estimate that the human *TGFBR1* gene is approximately 31 kb in length.

The genomic organization of the segment of the human *SKR2* gene (which appears to be identical to *ACVR1B* and *ALK-4*) that encodes the serine-threonine kinase domain has been reported previously (Xu *et al.*, 1994). In addition, the intron-exon structure of the human *ACVRL1* gene (identical to *ALK-1* and *TSR1*) has recently been deposited with GenBank (Accession Nos. U77707-U77713). The intron-exon junctions within the 3' end of the *SKR2/ACVR1B* gene can be precisely aligned with those we identified between exons 5 and 9 of the human *TGFBR1* gene (Fig. 2A). In addition, exons 8 and 9 of *TGFBR1* are precisely aligned with exons 9 and 10 of the *ACVRL1* gene (Fig. 2A). Moreover, there is a high degree of amino acid sequence homology between *TGFBR1* (*ALK-5*) and other members of the human R-I receptor family with respect to the juxtamembrane and serine-threonine kinase domains (Fig. 2B). Thus, it is likely that these genes were derived from a common ancestral gene that encoded the terminal portion of the receptor serine-threonine kinase domain and that the divergence in their evolution affected mainly the segment that encodes the extracellular portion of the receptor proteins. Our findings should greatly facilitate and expedite the structural analysis of the *TGFBR1* gene in human tumors and possibly other disease states. In this regard, it is of interest that germline mutations of the *ACVRL1* and endoglin (an accessory TGF $\beta$  receptor) genes have recently been found to be associated with the two principal hereditary hemorrhagic teleangiectasia syndromes (Johnson *et al.*, 1996; McAllister *et al.*, 1995).

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