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GRANT NUMBER DAMD17-96-1-6024

TITLE: Transforming Growth Factor-ß Receptors in Human Breast Cancer

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REPORT DATE: May 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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DTIC QUALITY INSPECTED 1

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 1998	Annual (1 May 97 - 30 Apr 98)

4. TITLE AND SUBTITLE
Transforming Growth Factor-B Receptors in Humans

5. FUNDING NUMBERS
DAMD17-96-1-6024

6. AUTHOR(S)
Reiss, Michael, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University School of Medicine
New Haven, Connecticut 06520-8032

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

19980722 078

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This project addresses the question whether and, if so, how molecular lesions of the genes involved in the TGFß signaling pathway contribute to the origin and/or progression of breast cancer. We have cloned and determined the intron-exon structure of the TGFß type I receptor (TßR-I). This has allowed us to study this gene in a series of primary breast cancer specimens. Our most important finding is a much higher frequency of an exon 1 receptor variant among breast cancer cases than controls. Secondly, we have identified a serine to tyrosine mutation within the catalytic core of the TßR-I serine-threonine kinase domain in 2 of 31 primary and 5 of 12 metastatic breast cancer specimens. Thirdly, we have developped transient transfection assays to determine how specific TßR mutations affect affect receptor function. Using these assays we have shown that two previously identified TßR-II missense mutants are incapable of signaling because of the near complete loss of receptor kinase activity. Plans for the coming year include completing structural analyses of the TßR-I and -II genes in primary and metastatic breast cancer and defining the functional properties of the newly discovered TßR-I variant and mutant.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
46

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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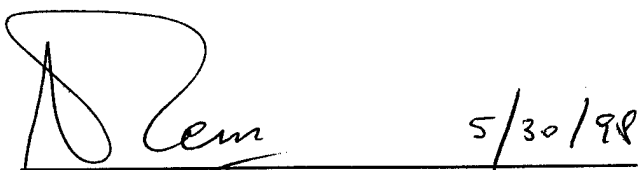

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INTRODUCTION

Transforming Growth Factor-ß (TGFß) 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 TGFß-mediated growth inhibition, while the TGFß they secrete apparently serves to enhance invasion into surrounding structures and perhaps their metastatic potential. The effects of TGFß on cell cycle progression are transduced by two cell surface receptors, TGFß type I (TßR-I) and -II receptors (TßR-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 TGFß-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 TGFß 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 TGFß 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 TGFß 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 TGFß, we wish to test the hypothesis that resistance of hormone-receptor positive cancers to tamoxifen is the result of inactivation of TGFß pathway genes.

BODY

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

Task 1. Screening for mutations in TGFß receptor genes in breast cancer

a. Identification of genetic alterations of TGFß-receptor genes in invasive breast cancer specimens. - Months 1-24

b. Identification of genetic alterations of TGFß-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 TGFß-receptor mutations

Cloning of TGFß-receptor mutants into mammalian expression vectors and transfection into TGFß-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 TßR genes in breast cancer

Test the hypothesis that genetic alterations of TGFß-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 TGFß receptor genes in breast cancer

Our initial studies of genes involved in TGFß signaling focused on the TßR-II gene. Using a chemical mismatch cleavage (CCM) assay, we were the first to identify missense mutations within the TßR-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 TßR genes also occur in primary tumors (particularly breast cancers) *in vivo*? and, if so (2) How do mutations in the TßR genes affect receptor function?

Before we could embark on a large study of the TßR 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 TßR-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 TßR-II and -I genes.

Genomic organization of TßR-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 TßR-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 TßR-I gene sequence. We determined the location of the intron-exon boundaries within the TßR-I gene by designing a series of oligonucleotides that matched sequences located at approximately 300 bp intervals along the TßR-I cDNA. These primers were then used for direct sequencing of purified BACH-559 DNA. We found that the TßR-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 TßR-I gene is approximately 31 kb in length. These results were recently published (1).

We have also resolved the genomic structure of the TßR-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 TßR-I and -II genes in this series. Preliminary results are presented here:

Primary carcinoma material is microdissected from 5 µm 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 µm microdissected paraffin-embedded tumor section using

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

Genotyping of TGFß 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 TßR-II, and each of the 9 exons (9 fragments) of TßR-I (**Table 1**). Primers used to amplify TßR-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 TßR-I gene were amplified using flanking intronic primer pairs (**Table 1**).

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

Sense primer	Exon	Antisense primer
5'gaggcgaggtttgctggggtgaggca-3'	Exon 1	5'-catgtttgagaaagagcaggagcgag-3'
5'-ctacacaatctttctctttttcc-3'	Exon 2	5'-gtttttcttgtagtatctagg-3'
5'-gtttatttcactcgaggcc-3'	Exon 3	5'-ggagaaacaattatgttac-3'
5'-gattgtgttgagtactattta-3'	Exon 4	5'-ggaaaagcaaatgttacagac-3'
5'-gcccaaccgaaatgttaattc-3'	Exon 5	5'-ggtagaactgcttatagaat-3'
5'-gcagtcatgtttaatttttgattc-3'	Exon 6	5'-gaacgcgtattaaatatagttg-3'
5'-tgtctgaaaggaggttcatcc-3'	Exon 7	5'-gaacaacttctgctcatgacg-3'
5'-gccttgcattagctgaataaat-3'	Exon 8	5'-gcttactaagcagaagcag-3'
5'-ggaaaatggtgcatgcatta-3'	Exon 9	5'-gagttcaggcaaagctgtag-3'

PCR primers used for the amplification of individual exons of the TßR-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 TßR-I coding region, respectively. All other primers are complementary to intronic sequences flanking each the exons of TßR-I.

The TßR 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 [γ-^{32}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.

The presence of any sequence alteration is always confirmed by repeated PCR-SSCP and sequencing using an independent aliquot of tumor-derived genomic DNA as starting material. Whether any mutations were somatic in nature or present in the germline is determined by analyzing genomic DNA isolated from normal tissue of the same patient.

Genotypic analysis of the TßR-I gene in breast cancer: At this point, we have completed a partial analysis of the TßR-I gene in this initial series of breast cancers. Our most important finding is a variant allele of the TßR-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)$_3$ TßR-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 TßR-I signal peptide (**Figure 2A, B**) (5). Comparative hydrophobicity plots of wild type and the del (GGC)$_3$ TßR-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.

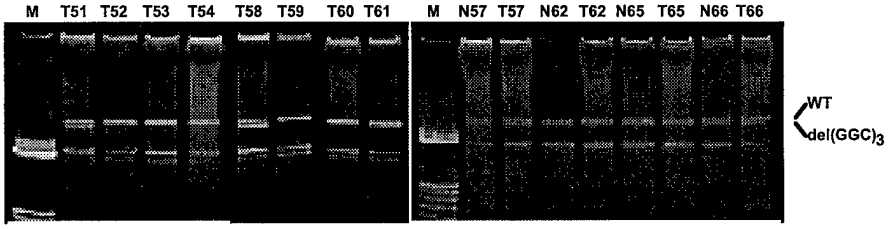


Figure 1

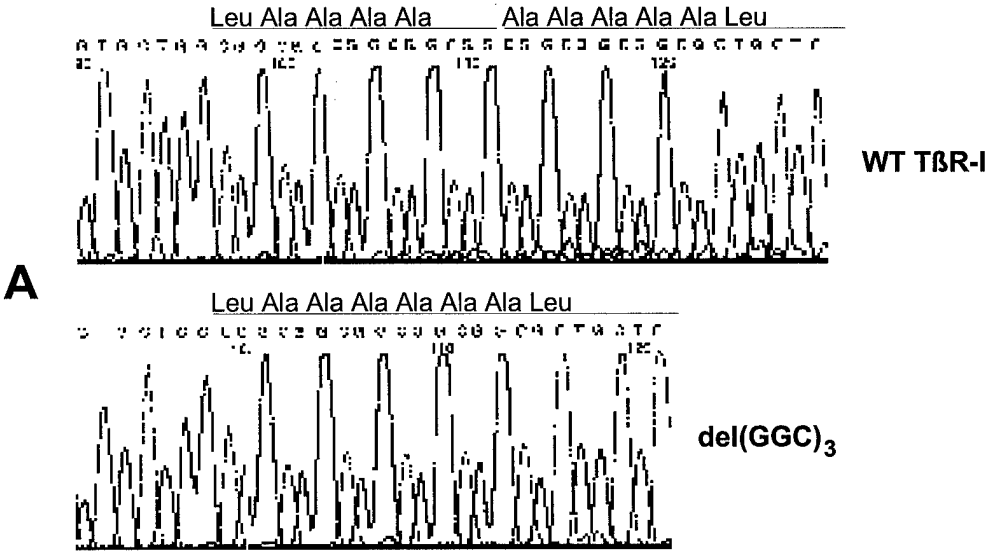
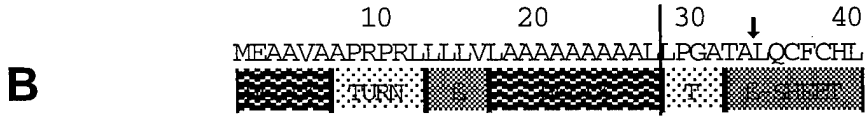


Figure 2



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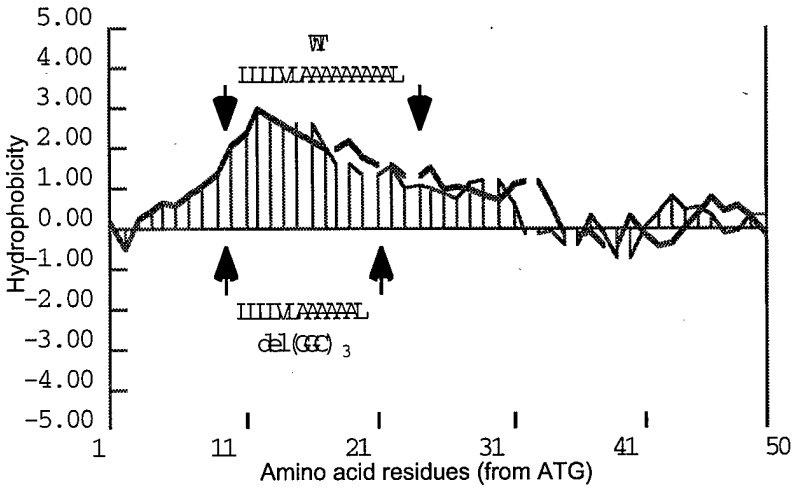
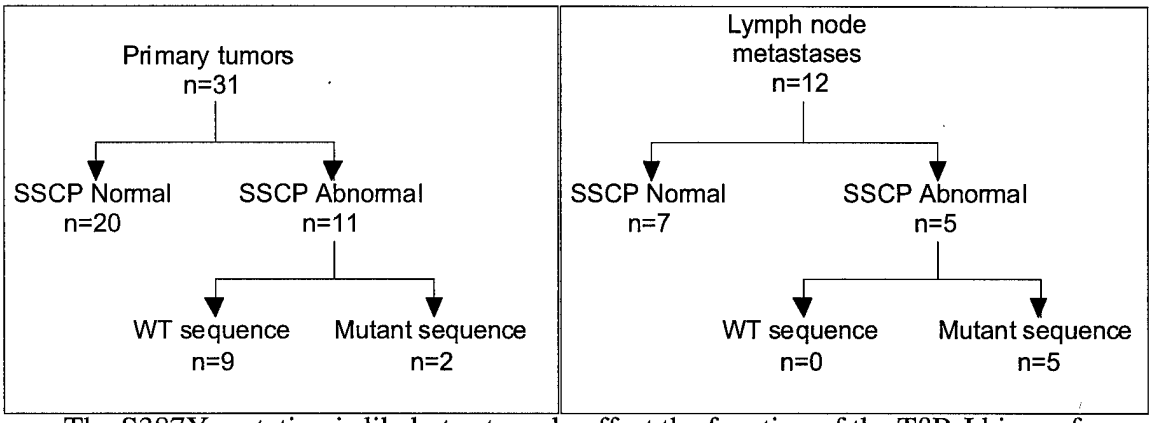


In order to determine whether there might be an association between the carrier state of the del(GGC)$_3$ TßR-I variant and the development of breast cancer, we determined the frequency of the del(GGC)$_3$ allele in a cohort of germline DNA samples from 43 independently and randomly selected individuals. Only one of these individuals was heterozygous for the del(GGC)$_3$ variant of TßR-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 del(GGC)$_3$ variant of TßR-I confers an increased cancer risk, presumably by decreasing the sensitivity of normal breast epithelial cells to TGFß.

We intend to confirm the validity of these results in a prospective case control study in collboration with Dr. Tongzhang Zheng (Dept. of Epidemiology & Public Health) at Yale. A positive outcome of such a would have several important implications for women's health. Particularly, the development of screening tests for carriers of the del(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 del(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 del(GGC)$_3$ variant of TßR-I and the presence or absence of lymph node metastases (Fisher's Exact test p value: 0.5804). Thus, the presence of the del (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 TßR-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 TßR-I Gene Exon 7 by SSCP and DNA Sequencing in BC Specimens



The S387Y mutation is likely to strongly affect the function of the TßR-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 TGFß 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 TßR-I kinase by introducing two ß-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. TßR-I-homologous Serine-Threonine Kinases-Catalytic Cores

Protein Kinase	Ligand	Catalytic core	Substrate
• TßR-I • BMP2/4R • BMPR-IA • BMPR-IB • Phosphorylase B kinase γ - muscle • Phosphorylase B kinase γ - liver	TGFß BMP2/4	GTKRYMAPEVLDDSINMKHFESFKRADIYA	Smad2; Smad3 Smad1 Phosphorylase
• TßR-II	TGFß	----------E -R----------D --	TßR-I
• ActR-IB • TSR-I	Activin Activin/TGFß	----------E -T----------D --	Smad2; Smad3
• TSR-IB	Activin/TGFß	----------E -Q----------D --	Unknown
• ActR-II • ActR-IIB	Activin	----------E -A----------D --	ActR-I
• RAC-α Ser/Thr kinase, AKT1 • RAC-ß Ser/Thr kinase, AKT2	Unknown	----------E -N----------D --	Unknown

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

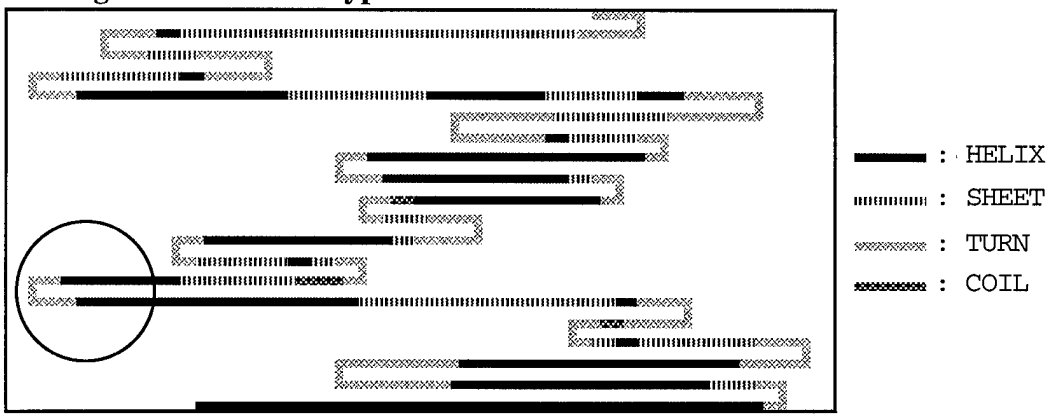
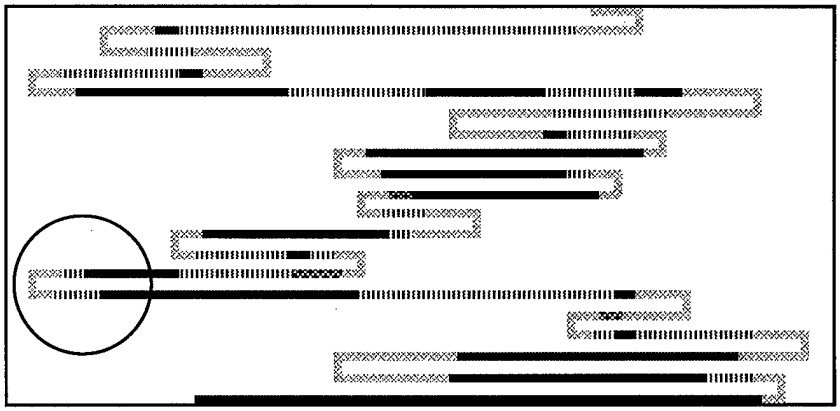


Figure 4B. S387Y Mutant TßR-I Serine-Threonine Kinase Domain



In summary, we have identified two particular structural alterations of the TßR-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 TßR-I and -II genes in primary and metastatic breast cancers.

Task 2. Determination of the functional consequences of TGFß-receptor mutations

As indicated above, we were the first to identify missense mutants (R537P and E526Q) of the TßR-II gene in the TGFß-resistant human head-&-neck SCC cell lines A253 and SqCC/Y1. Because we anticipate that we will encounter mutations in the TßR-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 TßR-II gene mutations were solely responsible for the TGFß-resistant phenotype and to determine more precisely how they affected receptor function, we introduced the two mutations into a full-length mouse wild type TßR-II cDNA. We used a mammalian expression vector in which wild type and mutant TßR-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 TßR-II restores all responses to TGFß (6). Two separate luciferase reporter gene constructs, pCAL2 and 3TP-Lux, were used to assess the dual types of responses to TGFß: Repression of the cyclin A promoter activity (pCAL2) has been shown to correlate extremely well with the ability of epithelial

cells to respond to TGFß-mediated cell cycle arrest, and activation of the PAI-1 promoter (3TP-Lux) reflects TGFß-induced PAI-1 production (7, 8). As shown in **Figure 5A**, expression of wild type TßR-II in TßR-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 TGFß, presumably because, at high levels of expression, the TßR-II and -I receptors can spontaneously dimerize and activate the signaling pathway (9-12). Alternatively, it is possible that bioactive TGFß2 produced by T47D cells is sufficient to stimulate their TGFß receptors in an autocrine manner (13). Although attempts to block endogenous TGFß2 activity by using a neutralizing anti-TGFß2 antibody (R & D Systems) did not affect the results of the transfection experiments (data not shown), endogenous TGFß2 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 TßR-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 TGFß-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 TGFß signaling with respect to both cell cycle regulation and extracellular matrix protein induction.

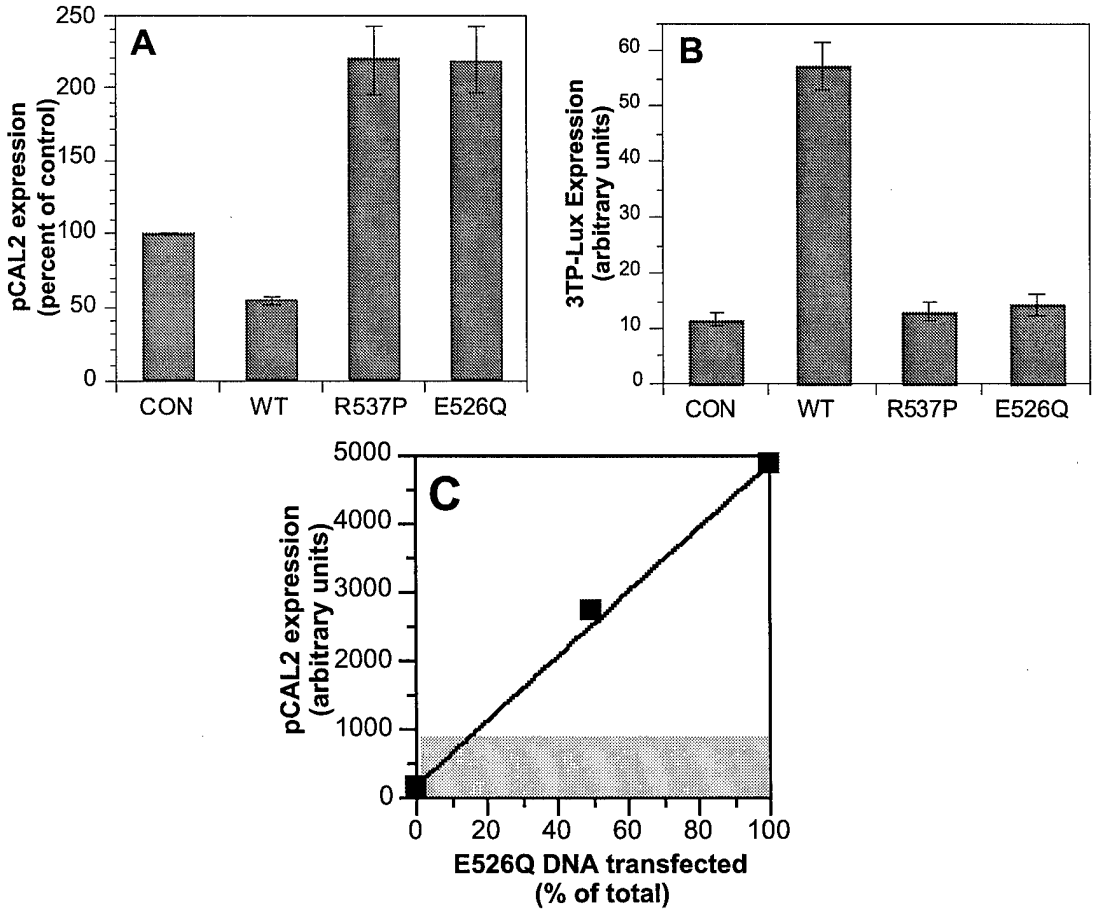


Figure 5. Functional analysis of TßR-II mutants

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 TßR-II receptor proteins are particularly potent dominant-negatives that completely abrogate all of the responses to TGFß when transfected into normal endothelial and bronchial cells (19-22).

Dominant-negative mutants may interfere with TGFß signaling by competing with wild type TßR-II for heterodimerization with TßR-I molecules or by sequestering co-expressed wild type TßR-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 TßR-II, even though the protein is not detectable by ligand binding assays, TßR-II-specific mRNA is undetectable by Northern blot analysis, and the cells are unresponsive to TGFß (6). In order to test whether the TßR-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 TßR-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 TßR-II mutants suggests the possibility that the proliferation of tumor cells that express such mutants might be stimulated by TGFß. 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 TGFß (23, 24). This switch in responsiveness to TGFß has been attributed to the use of an alternative TGFß 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 TßR-II mutants.

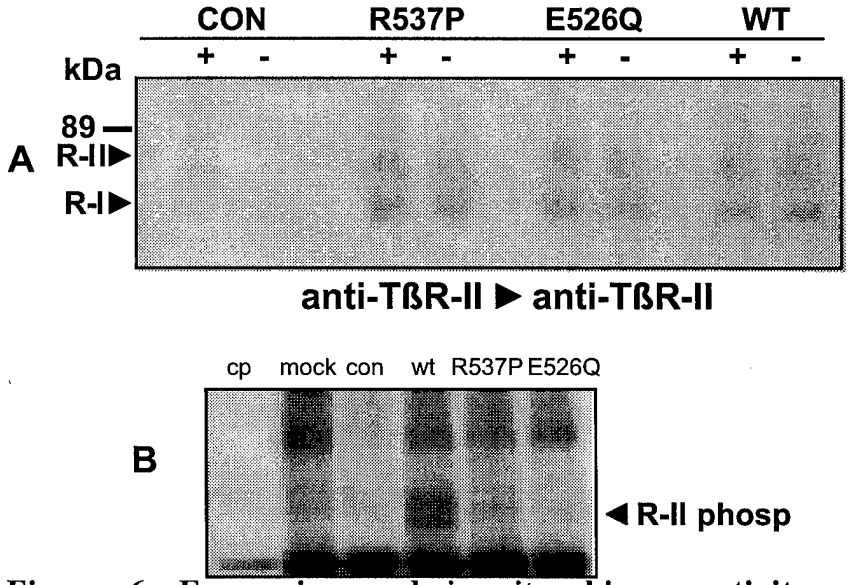


Figure 6. Expression and *in vitro* kinase activity of TßR-II mutants

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 TßR-IIs in transfected cells, parallel dishes of [^{35}S]-methionine and -cysteine labeled cells were subjected to double immunoprecipitation with anti-TßR-II antiserum (**Figure 6A**). Discreet bands of approximately equal intensity at the level of 65 kDa corresponding to the TßR-II receptor were detected in extracts from both wild type- and mutant TßR-II-transfected cells. Moreover, a second band of approximately 55 kDa representing co-immunoprecipitated TßR-I, was also seen in all three cases. No receptor protein was detected in control transfected cells. These results indicate that the two TßR-II mutations did not affect receptor protein expression nor the

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

Interestingly, this receptor phenotype is clearly distinct from that described for a closely related TßR-II mutant in which a proline residue at position 525 is replaced by a leucine (18). This P525L TßR-II mutant receptor is capable of binding ligand and of forming a complex with TßR-I but fails to transphosphorylate TßR-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 TßR-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 TßR-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 TßR-II was easily detectable in cells transfected with wild type TßR-II, while such phosphoproteins were barely detectable in cells transfected with either of the two TßR-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 TßR-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 TßR-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 TßR-II exodomains (31). Such truncated receptors have been shown to inhibit TGFß 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 TGFß-resistance by inhibiting cell surface expression of TGFß 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 TßR-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 TGFß, 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 TßR-II gene mutants. Both missense mutations in the TßR-II gene that we had previously identified in human head-&-neck cancer cells largely abolish the capability of the TGFß receptor system to transduce TGFß's effects on cell cycle progression as well as induction of ECM-associated proteins. In addition, our study indicates that these TßR-II mutants inhibit TßR-I function in a dominant-negative fashion.

CONCLUSIONS

1. The intron-exon organization of both the TßR-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 TßR-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 TßR-II gene mutants. These assays have allowed us to assess the effects of individual mutations on the ability of TGFß to inhibit cell cycle progression and to induce extracellular matrix-related target genes.

PUBLICATIONS RESULTING FROM THIS WORK

Reiss M, Barcellos-Hoff MH: The role of Transforming Growth Factor-ß 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-ß type I receptor gene. Genomics 46:278-283, 1997

De, M., Yan, W., De Jonge, R. R., Garrigue-Antar, L., Vellucci, V. F., and Reiss, M. Functional characterization of transforming growth factor-ß type II receptor mutants in human cancer, Cancer Res. 58:1986-92, 1998.

REFERENCES

1. Vellucci, V. F. and Reiss, M. Cloning and genomic organization of the human Transforming Growth Factor-ß type I receptor gene, Genomics. *46:* 278-283, 1997.

2. Lu, S., Zhang, W., Akiyama, Y., Nomizu, T., and Yuasa, Y. Genomic structure of the transforming growth factor ß type II receptor gene and its mutations in hereditary nonpolyposis colorectal cancers, Cancer Res. *56:* 4595-4598, 1996.

3. Chen, T., Dhingra, K., Sahin, A., Sneige, N., Hortobagyi, G., and Aldaz, C. M. Technical approach for the study of the genetic evolution of breast cancer from paraffin-embedded tissue sections, Breast Cancer Res Treat. *39:* 177-85, 1996.

4. Hongyo, T., Buzard, G. S., Calvert, R. J., and Weghorst, C. M. 'Cold SSCP': a simple, rapid and non-radioactive method for optimized single-strand conformation polymorphism analyses, Nucleic Acids Res. *21:* 3637-42, 1993.

5. Franzén, P., Ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C.-H., and Miyazono, K. Cloning of a TGFß type I receptor that forms a heterodimeric complex with the TGFß type II receptor, Cell. *75:* 681-692, 1993.

6. Kalkhoven, E., Roelen, B. A. J., de Winter, J. P., Mummery, C. L., van den Eijnden-van Raaij, A. J. M., van der Saag, P. T., and van der Burg, B. Resistance to transforming growth factor ß and activin due to reduced receptor expression in human breast tumor cell lines, Cell Growth Diff. *6:* 1151-1156, 1995.

7. Satterwhite, D. J., Aakre, M. E., Gorska, A. E., and Moses, H. L. Inhibition of cell growth by TGFß1 is associated with inhibition of B-*myb* and *cyclin A* in both BALB/MK and Mv1Lu cells, Cell Growth Diff. *5:* 789-799, 1994.

8. Feng, X.-H., Filvaroff, E. H., and Derynck, R. Transforming Growth Factor-ß (TGFß)-induced down-regulation of cyclin A expression requires a functional TGFß receptor complex, J. Biol. Chem. *270:* 24237-24245, 1995.

9. Ventura, F., Doody, J., Liu, F., Wrana, J. L., and Massagué, J. Reconstitution and transphosphorylation of TGF-beta receptor complexes, EMBO J. *13:* 5581-5589, 1994.

10. Chen, F. and Weinberg, R. A. Biochemical evidence for the autophosphorylation and transphosphorylation of transforming growth factor ß receptor kinases, Proc. Natl. Acad. Sci. USA. *92:* 1565-1569, 1995.

11. Vivien, D., Attisano, L., Wrana, J. L., and Massague, J. Signaling activity of homologous and heterologous transforming growth factor-beta receptor kinase complexes, J. Biol. Chem. *270:* 7134-7141, 1995.

12. Feng, X. H. and Derynck, R. Ligand-independent activation of transforming growth factor (TGF) beta signaling pathways by heteromeric cytoplasmic domains of TGF-beta receptors, J Biol Chem. *271:* 13123-9, 1996.

13. Kalkhoven, E., Kwakkenbos-Isbrucker, L., Mummery, C. L., deLaat, S. W., van den Eijnden-van Raaij, A. J. M., van der Saag, P. T., and van der Burg, B. The role of TGF-ß production in growth inhibition of breast tumor cells by progestins, Int. J. Cancer. *61:* 80-86, 1995.

14. Rahimi, N., Tremblay, E., McAdam, L., Park, M., Schwall, R., and Elliott, B. Identification of a hepatocyte growth factor autocrine loop in a murine mammary carcinoma, Cell Growth Differ. *7:* 263-270, 1996.

15. Chen, R.-H., Ebner, R., and Derynck, R. Inactivation of the type II receptor reveals two receptor pathways for the diverse TGFß activities, Science. *260:* 1335-1338, 1993.

16. Wrana, J. L., Attisano, L., Cárcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.-F., and Massagué, J. TGFß signals through a heteromeric protein kinase receptor complex, Cell. *71:* 1003-1014, 1992.

17. Wieser, R., Attisano, L., Wrana, J. L., and Massagué, J. Signaling activity of Transforming Growth Factor ß type II receptors lacking specific domains in the cytoplasmic region, Mol. Cell. Biol. *13:* 7239-7247, 1993.

18. Carcamo, J., Zentella, A., and Massagué, J. Disruption of transforming growth factor ß signaling by a mutation that prevents transphosphorylation within the receptor complex, Mol. Cell. Biol. *15:* 1573-1581, 1995.

19. Brand, T. and Schneider, M. D. Inactive type II and type I receptors for TGFß are dominant inhibitors of TGFß-dependent transcription, J. Biol. Chem. *270:* 8274-8284, 1995.

20. Choi, M. E. and Ballerman, B. J. Inhibition of capillary morphogenesis and associated apoptosis by dominant negative mutant transforming growth factor-ß receptors, J. Biol. Chem. *270:* 21144-21150, 1995.

21. Yamamoto, H., Ueno, H., Ooshima, A., and Takeshita, A. Adenovirus-mediated transfer of a truncated transforming growth factor-ß (TGFß) type II receptor completely and specifically abolishes diverse signaling by TGFß in vascular wall cells in primary culture, J. Biol. Chem. *271:* 16253-16259, 1996.

22. Zhao, J., Bu, D., Lee, M., Slavkin, H. C., Hall, F. L., and Warburton, D. Abrogation of transforming growth factor-beta type II receptor stimulates embryonic mouse lung branching morphogenesis in culture, Dev Biol. *180:* 242-57, 1996.

23. Fan, D., Chakrabarty, S., Seid, C., Bell, C. W., Schackert, H., Morikawa, K., and Fidler, I. J. Clonal stimulation of human colon carcinomas and human renal carcinomas mediated by TGFß1, Cancer Commun. *1:* 117-125, 1989.

24. Hsu, S., Huang, F., Hafez, M., Winawer, S., and Friedman, E. Colon carcinoma cells switch their response to transforming growth factor ß1 with tumor progression, Cell Growth Diff. *5:* 267-275, 1994.

25. Yan, Z. F., Winawer, S., and Friedman, E. Two different signal transduction pathways can be activated by transforming growth factor beta 1 in epithelial cells, J Biol Chem. *269:* 13231-13237, 1994.

26. Taylor, S. S., Knighton, D. R., Zheng, J., Ten Eyck, L. F., and Sowadski, J. M. Structural framework for the protein kinase family, Annu. Rev. Cell Biol. *8:* 429-462, 1992.

27. Zhang, F., Strand, A., Robbins, D., Cobb, M. H., and Goldsmith, E. J. Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution, Nature. *367:* 704-711, 1994.

28. Lu, S.-L., Akiyama, Y., Nagasaki, H., Saitoh, K., and Yuasa, Y. Mutations of the transforming growth factor-ß type II receptor gene and genomic instability in hereditary nonpolyposis colorectal cancer, Biochem. Biophys. Res. Comm. *216:* 452-457, 1995.

29. Takenoshita, S., Tani, M., Nagashima, M., Hagiwara, K., Bennett, W. P., Yokota, J., and Harris, C. C. Mutation analysis of coding sequences of the entire transforming growth factor beta type II receptor gene in sporadic human colon cancer using genomic DNA and intron primers, Oncogene. *14:* 1255-1258, 1997.

30. Garrigue-Antar, L., Munoz-Antonia, T., Antonia, S. J., Gesmonde, J., Vellucci, V. F., and Reiss, M. Missense mutations of the transforming growth factor beta type II receptor in human head and neck squamous carcinoma cells, Cancer Res. *55:* 3982-3987, 1995.

31. Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., Brattain, M., and Willson, J. K. V. Inactivation of the type II TGFß receptor in colon cancer cells with microsatellite instability, Science. *268:* 1336-1338, 1995.

32. Lin, H. Y., Moustakas, A., Knaus, P., Wells, R. G., Henis, Y. I., and Lodisch, H. F. The soluble exoplasmic domain of the type II transforming growth factor (TGFß) receptor, J. Biol. Chem. *270:* 2747-2754, 1995.

33. Wang, J., Sun, L., Myeroff, L., Wang, X., Gentry, L. E., Yang, J., Liang, J., Zborowska, E., Markowitz, S., Willson, J. K. V., and Brattain, M. G. Demonstration that mutation of the type II transforming growth factor ß receptor inactivates its tumor suppressor activity in replication error-positive colon carcinoma cells, J. Biol. Chem. *270:* 22044-22049, 1995.

34. Knaus, P. I., Lindemann, D., DeCoteau, J. F., Perlman, R., Yankelev, H., Hille, M., Kadin, M. E., and Lodish, H. F. A dominant inhibitory mutant of the type II transforming growth factor beta receptor in the malignant progression of a cutaneous T-cell lymphoma, Mol Cell Biol. *16:* 3480-3489, 1996.

APPENDICES

Reprints

Breast Cancer Research and Treatment **45**: 81–95, 1997.
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Hypothesis

Transforming growth factor-β in breast cancer: A working hypothesis

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Key words: breast cancer, TGFβ, activation, resistance, receptors, tamoxifen, chemoprevention

Summary

Transforming Growth Factor-β (TGFβ) 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 TGFβ-mediated growth arrest, and TGFβ can act as an anti-tumor promoter. In contrast, advanced breast cancers are mostly refractory to TGFβ-mediated growth inhibition and produce large amounts of TGFβ, 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 TGFβ during progression is the consequence of the activation of the latent TGFβ that is produced and deposited into the tumor microenvironment, thereby driving the clonal expansion of TGFβ-resistant tumor cells. While tumor cells themselves may activate TGFβ, recent observations suggest that environmental tumor promoters or carcinogens, such as ionizing radiation, can cause stromal fibroblasts to activate TGFβ by epigenetic mechanisms. As the biological effects of the anti-estrogen tamoxifen may well be mediated by TGFβ, 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-β (TGFβ), 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, TGFβ 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 TGFβ1 targeted to the mammary epithelium inhibits the normal development of the ductal and lobular epithelium in a dose-dependent manner [6,

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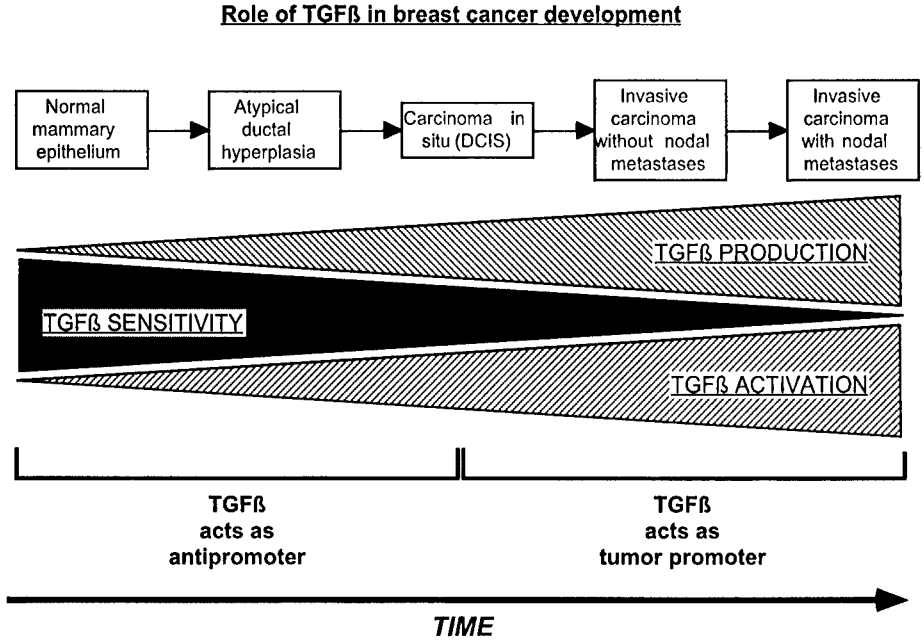

Figure 1. Proposed role of TGFβ in mammary carcinogenesis. Human breast cancers at successive stages of tumor progression appear to be associated with increasing production of TGFβ, suggesting that this growth factor is providing a selective advantage. In parallel, one sees the preferential outgrowth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

7]. In the adult mammary gland, TGFβ appears to control the massive cell death and restructuring that takes place during post-lactational involution [8]. Thus, TGFβ 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 TGFβ has also been implicated in mammary carcinogenesis. One view that has been proposed is that TGFβ 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 TGFβ which somehow promotes tumor progression, while the tumor cells themselves are refractory to TGFβ-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 TGFβ 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 TGFβ by the tumor cells, the activation of latent TGFβ within the microenvironment, and the clonal expansion of tumor cells that are resistant to TGFβ on the basis of inactivation of genes encoding TGFβ receptors or perhaps other elements of the signaling pathway. This working hypothesis reconciles the two seemingly contrary views of TGFβ's role in breast cancer and has important consequences for chemoprevention as well as therapy of the disease.

Production of TGFβ by breast cancer cells increases during neoplastic progression

The TGFβs (TGFβ1–3) comprise a family of highly conserved dimeric 25 kDa polypeptides that are ubiquitously expressed in normal mammalian tis-

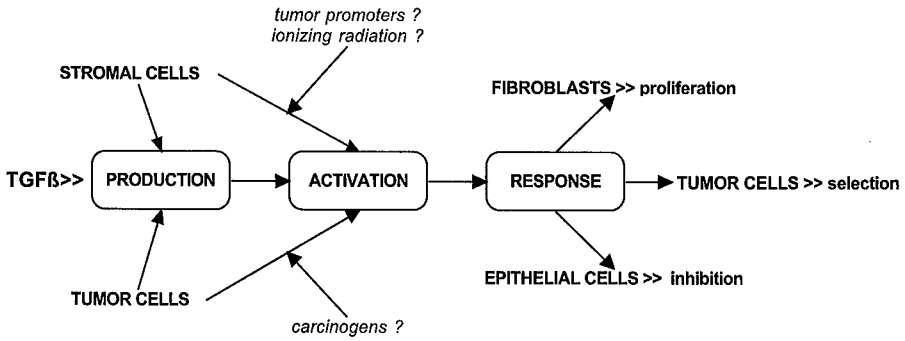

Figure 2. Putative relationship between TGFβ production, activation, and biological effects in invasive breast cancer. Either the epithelial tumor cells themselves or surrounding stromal fibroblasts can be the source of tumor associated TGFβ. Similarly, activation of latent TGFβ can be caused by genetic 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 TGFβ 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 TGFβ-resistant epithelial tumor cell clones.

sues [11]. TGFβs exert two major biological effects on epithelial cells. First, picomolar amounts of TGFβ are able to arrest human mammary epithelial cells (HME) at the G_1/S boundary, resulting in complete inhibition of DNA replication and clonal growth [2, 3]. Secondly, TGFβ 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 TGFβ was first suggested by tissue culture models. For example, 184 HME cells and oncogene-transformed sublines are exceedingly sensitive to TGFβ-mediated growth arrest, and secrete barely detectable amounts of TGFβ [3]. In contrast, most cell lines derived from invasive human breast carcinomas secrete much larger amounts of TGFβ, mostly TGFβ2 [3, 14, 15]. In primary human breast cancers, TGFβ 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 TGFβ 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 TGFβ, 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 TGFβ 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 TGFβ 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 TGFβ might promote tumor progression. One possibility is that TGFβ 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 TGFβ *in vitro* or had been transfected with a TGFβ1 expression vector were significantly more tumorigenic when they were injected into nude mice than control cells [20, 21]. Another possibility is that tumor-derived TGFβ1 acts as an immune suppressor, and allows the tumor cells to escape from immune surveillance [22–24].

Table 1. Effects of TGFβ 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_{50} (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)	v-*mos*	ADG	0.7	63%	100	[3]
Man	A1N4H HMEC (weakly tumorigenic)	v-Ha-*ras*	ADG	1.1	78.3%	≥ 10	[3]
Man	A1N4MH HMEC (weakly tumorigenic)	v-*mos* + v-Ha-*ras*	ADG	1.5	82.7%	100	[3]
Man	A1N4TH HMEC (highly tumorigenic)	SV40 (large T) + v-Ha-*ras*	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]
			ADG	NA	86.5%	100	
Man	MCF-7	Adenocarcinoma (ER positive)	ADG	> 100	10%	100	[14]
			TI	> 1000	No inhibition	1000	
			AIG	> 100	No inhibition	100	
Man	MCF-7L	Adenocarcinoma (ER positive)	ADG	> 100	10%	100	[14]
			TI	> 1000	No inhibition	1000	
			AIG	> 100	No inhibition	100	
Man	T47D	Adenocarcinoma (ER positive)	ADG	> 100	No inhibition	100	[14]
			TI	> 1000	No inhibition	1000	
Man	ZR75-1	Adenocarcinoma (ER positive)	ADG	> 100	20%	100	[14]
			TI	> 1000	20%	1000	
			AIG	> 100	20%	100	
Man	MDA 330	Adenocarcinoma (ER negative)	ADG	> 100	10%	100	[14]
			TI	> 1000	40%	1000	
			AIG	> 100	45%	100	
Man	BT20	Adenocarcinoma (ER negative)	ADG	> 100	30%	100	[14]
			TI	> 1000	10%	1000	
			AIG	2	80%	100	
Man	MDA-MB-231	Adenocarcinoma (ER negative)	ADG	100	50%	100	[14]
			TI	100	60%	1000	
			AIG	0.5	100%	100	

Activation of TGFβ in the tumor microenvironment

As indicated above, the malignant progression of breast cancer appears to be associated with the increased production and secretion of TGFβ by the tumor cells themselves. However, the active form of TGFβ1 is derived from a 390-amino acid precursor which is processed into a homodimer of the mature 112 amino acid carboxy-terminal TGFβ1 peptide in a non-covalent association with a dimer of the processed N-terminal pro-segment, called the latency-associated peptide (LAP). This latent TGFβ complex is secreted but unable to bind to TGFβ receptors unless the biologically active mature TGFβ is dissociated from the LAP [25]. Since TGFβ receptors are apparently ubiquitously expressed [26] and latent TGFβ is abundant in all tissues [11], release from the latent complex is the key control of TGFβ's biological activity. This so-called activation is considered the critical event that regulates TGFβ function *in vivo* [27]. TGFβ 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, TGFβ 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 TGFβ 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_{50} (pM)	Maximal inhibition	Concentration (pM)	Ref.
Man	HS578T	Adenocarcinoma (ER negative)	ADG TI	100 5	50% 85%	100 1000	[14]
Man	MCF-7	Adenocarcinoma (estrogen dependent)	TI	200	58%	400	[49]
Man	MCF-7	Adenocarcinoma (estrogen independent)	TI	400–1000	65%	1000	[49]
Man	MDA-MB-231	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 TGFβ between non-neoplastic HMEC and human breast cancer cells *in vitro* as well as *in vivo*. The average IC_{50} of TGFβ 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 TGFβ-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.

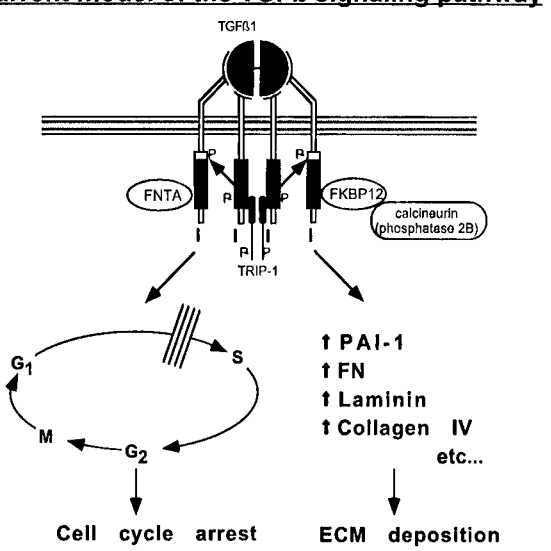

Figure 3. Current model of TGFβ signaling pathway. TGFβ1 binds to TβR-II homodimers followed by the recruitment of one (or more) TβR-I receptor molecules into a stable ternary complex with TβR-II, which results in the phosphorylation of the GS domain of TβR-I by the TβR-II kinase. The α-subunit of the *ras* farnesyltransferase (FNTA) and FKBP-12 are associated with TβR-I. FKBP-12 functions as a bridge molecule between TβR-I and the Ca^{2+}/calmodulin-dependent protein phosphatase 2B (calcineurin), while FNTA is involved in activation of the *ras* pathway. The function of the TβR-II-associated protein TRIP-I is unknown. TGFβ induces the expression of cyclin-dependent kinase inhibitors, such as $p15^{INK4B}$ and $p21^{WAF-1}$, whereas it inhibits expression of cyclins A and E, as well as cdk4, presumably leading to cell cycle arrest in mid- to late G_1. In addition, TGFβ 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 TGFβ that they produce. *In vitro*, a significant fraction of the TGFβ 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 TGFβ *in vivo* is not clear because none of the published immunohistochemical studies employed antibodies that specifically recognize the activated form of the TGFβ. 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 TGFβ, the increased levels of uPA in tumors may cause TGFβ 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 TGFβ activation [34]. Alternatively, environmental agents may cause TGFβ 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 TGFβ. Using antibodies that discriminate between the active and latent forms of TGFβ [41], we found that latent TGFβ was abundant in the normal mammary gland, but that active TGFβ was restricted to epithelial structures, where it was faintly detectable. However, at 1 hr after radiation exposure, latent TGFβ immunoreactivity was strikingly diminished in the adipose stroma, while active TGFβ 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 TGFβ 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, TGFβ 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 TGFβ. For example, phorbol ester treatment of carcinogen-initiated skin rapidly induces TGFβ mRNA and protein immunoreactivity [42, 43] that most likely reflects the production of active TGFβ, since the antibody used in these studies reacts specifically with active TGFβ [41, 44]. Similarly, phenobarbital induces active TGFβ immunoreactivity in normal liver tissue, resulting in the selective clonal expansion of transformed hepatocytes that express decreased levels of TGFβ 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 TGFβ activation causes a selective expansion of cells in which mutations confer resistance to TGFβ, as is observed, for example, in diethylnitrosamine-induced liver tumors [46]. Conversely, one would predict that transformation in which TGFβ activation does not occur would not be associated with TGFβ-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 TGFβ), thereby promoting the selective outgrowth of mutant cells that display a particular phenotype (in this case, TGFβ-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 TGFβ on breast carcinoma cells

In vitro, mammary epithelial cell lines range from being exquisitely sensitive to being completely refractory to TGFβ-mediated growth inhibition (Table 1). For instance, spontaneously immortalized HMEC (A1N4 cells) are nearly as sensitive to TGFβ 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 TGFβ 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 TGFβ, depending, to some extent, on whether cells are grown in monolayer or in soft-agar. By and large, TGFβs 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 TGFβ 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 TGFβ in two studies [47, 48], but not in a third [14]. Another possibility is that TGFβ-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 TGFβ-mediated growth inhibition, whereas estrogen-independent sublines are refractory to TGFβ [49]. In any event, even if modest differences in TGFβ sensitivity exist among breast carcinoma cell lines, the average IC_{50} of TGFβ 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 TGFβ1 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 TGFα, 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 TGFβ did not result in any suppression of tumor growth *in vivo* [51]. In aggregate, these studies provide compelling evidence that overexpression of TGFβ1 can markedly suppress *de novo* mammary tumor development, but that this effect is lost once invasive carcinomas have arisen.

Mechanisms of escape from TGFβ control

In order to understand the mechanisms that cause tumor cells to become refractory to TGFβ-mediated cell cycle arrest, it is necessary to examine the molecular components of the TGFβ signaling pathway. The biological effects of TGFβ1 are transduced by two interacting and interdependent receptor subtypes, type I (TβR-I) and type II (TβR-I) (Figure 3) [52, 53]. Both are highly conserved transmembrane serine-threonine receptor kinases [54–56]. Binding of TGFβ1 to TβR-II homodimers is followed by the recruitment of TβR-I receptor molecules into a stable heterotetrameric complex with TβR-II (Figure 3) [57]. This triggers the phosphorylation of the juxtamembrane (GS) domain of TβR-I by the TβR-II kinase [58, 59]. The activated TβR-I kinase presumably phosphorylates downstream elements in the signaling cascade [59].

A number of proteins which physically interact with TβR-I [60] or with the TβR-II receptor [61], may be required for signaling. For example, FKBP-12 functions as a bridge molecule between TβR-I and the Ca^{2+}/calmodulin-dependent protein phosphatase 2B (calcineurin) [60, 62]. Moreover, the α-subunit of the *ras* farnesyltransferase (FNTA), which is also associated with TβR-I, is phosphorylated and released upon TGFβ stimulation, indicating that activation of *ras* and the mitogen-activated protein kinase cascade may play a role in mediating the cellular responses to TGFβ [63–66]. Further downstream, TGFβ induces the expression of the cyclin-dependent kinase inhibitors $p15^{INK4B}$ and $p21^{WAF-1}$ [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 TGFβ. We and others [71, 72] have shown that cells that express mutant forms of the p53 tumor suppressor protein lose sensitivity to TGFβ. Secondly, Okamoto et al. [73] noted TGFβ-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 TGFβ, 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 TGFβ responsiveness is inactivation of either one of the two TβR receptors [58, 77]. There appears to be a direct quantitative relationship between the level of TβR-II expression in tumor cell lines and TGFβ-responsiveness. For example, somatic cell fusion of two different, TGFβ-refractory, TβR-II-negative carcinoma cell lines gave rise to hybrids that re-expressed TβR-II protein and regained sensitivity to TGFβ [78]. Several TGFβ-resistant human breast cancer cell lines fail to express TβR-II mRNA transcripts [52, 79]. Moreover, transfection with a TβR-II expression vector results in the restoration of TGFβ-responsiveness *in vitro* and, most importantly, in suppression of tumorigenicity *in vivo* [79, 80]. Besides TβR-II, genetic defects of the TβR-I receptor can also result in TGFβ resistance: Kim et al. [81] recently identified a human prostatic carcinoma cell line that was refractory to TGFβ because of loss of TβR-I expression as a consequence of gene rearrangement. The relationship between loss of TβR expression and TGFβ resistance appears to hold up *in vivo* as well. For example, Kadin et al. [82] demonstrated that the loss of TβR-II transcripts parallels the progressive loss of TGFβ responsiveness in cells obtained from serial biopsies of a single patient with progressive Ki-1$^+$ cutaneous T-cell lymphoma. Similarly, malignant CD4+ lymphocytes obtained from patients with Sézary syndrome are also refractory to TGFβ and fail to express TβR-II cell surface receptors [83].

The importance of loss of TβR-II expression in

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

Besides loss of TβR-II expression, we recently identified several mutants of the TβR-II receptor gene in human squamous head-&-neck carcinoma cell lines that account for their resistance to TGFβ1-mediated cell cycle arrest [87]. Mutational inactivation of the TβR-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 TβR-I gene have not yet been described, it is likely that they occur in human cancers as well.

At this point, structural information about TGFβ genes in primary human breast cancers is extremely limited. Ke et al. [92] were unable to detect any TβR-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 TβR-II missense mutations which are likely to affect receptor kinase function (L. Garrigue-Antar and M. Reiss, unpublished observations). Thus, genetic alterations of the TβR-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 TGFβ. While, in the early stages of breast cancer development, mammary epithelial cells are sensitive to growth inhibition by TGFβ, TGFβ-resistance and the accompanying higher levels of TGFβ 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 TGFβ 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 TGFβ may be associated with the risk of enhancing growth or invasiveness of tumors that have arisen in a microenvironment that contains activated TGFβ. In these cases, targeting TGFβ 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 TGFβ. Treatment of human ER-positive breast cancer cell lines with tamoxifen *in vitro* induces the production and secretion of TGFβ 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 TGFβ, and that this was dependent on the expression of functional ER. Paradoxically, ER-negative breast cancer cell lines appear to be more sensitive to TGFβ-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 TGFβ-mediated growth arrest because they do not express TGFβ 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 TGFβ 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 TGFβ isoforms *in vivo*: Thus, several studies have demonstrated elevations of plasma levels of TGFβ2 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 TGFβ isoforms in breast cancer specimens. Although some of these studies suggested that TGFβ1 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 TGFβ isoform induced in breast cancer-associated stromal fibroblasts following tamoxifen treatment is TGFβ2 [99, 100]. Although none of these studies formally prove that TGFβ mediates the effects of tamoxifen on breast tumor growth, they strongly suggest a relationship between the induction of TGFβ2 in tumor stroma with elevations of TGFβ2 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 TGFβ-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 TGFβ-mediated cell cycle arrest. This may also explain why primary breast cancers that are clinically resistant to tamoxifen express the highest levels of TGFβ1 [106]. Moreover, tumors that express low levels of uPA are more likely to responsed to tamoxifen than those that express high uPA, in which TGFβ is presumably activated [107]. Consequently, uncovering the molecular basis of TGFβ 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 TGFβ 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 TGFβ activation and TGFβ-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 TGFβ 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 TGFβ 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 TGFβ1 by mammary carcinoma cells [115]. Expression of M6P/IGF-IIR facilitates the activation of TGFβ, thereby restoring the autocrine growth inhibitory loop and preventing the outgrowth of transformed cell clones.

Conversely, in the treatment of advanced TGFβ-resistant breast cancers, tamoxifen may be ineffective and perhaps may even promote tumor growth. Similarly, resistance of tumor cells to TGFβ may negate some of the cytotoxic effects of radiation treatment, and radiation-dependent activation of TGFβ 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 TGFβ. In fact, radiation-induced TGFβ may even be detrimental in the case of a TGFβ-sensitive breast tumor, as TGFβ-dependent cell cycle arrest may allow for repair of DNA damage. Two different approaches to prevent inflammation-associated fibrosis by inactivating TGFβ have been successfully tested in experimental models [28, 93]. These include the use of neutralizing antibodies to TGFβ [117] and the administration of the TGFβ-binding protein, decorin [118]. Based on the idea that tumor-derived TGFβ 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 TGFβ2 mRNA by enhancing immune rejection [24]. Similar approaches might be developed for the treatment of advanced breast cancers that are associated with TGFβ activation.

In summary, the detection of activated TGFβ in breast cancer stroma and/or the identification of molecular lesions in the TGFβ signaling system may provide new clinical tools to distinguish tumors in which augmentation of TGFβ 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 TGFβ 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].

Acknowledgements

Supported, in part, by U.S. Dept. of Defense Breast Cancer Research Program Grants DAMD 17-96-1-6024 (to MR) and DAMD 17-96-1-6716 (to MHB-H), as well as U.S. Public Health Service award CA 51841 (to MHB-H) from the National Cancer Institute.

References

1. Cordon-Cardo C: Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. Am J Pathol 147: 545–560, 1995
2. Hosobuchi M, Stampfer MR: Effects of transforming growth factor β on growth of human mammary epithelial cells in culture. *In Vitro* Cell Develop Biol 25: 705–713, 1989
3. Valverius EM, Walker-Jones D, Bates SE, Stampfer MR, Clark R, McCormick F, Dickson RB, Lippman ME: Production of and responsiveness to transforming growth factor-β in normal and oncogene-transformed human mammary epithelial cells. Cancer Res 49: 6269–6274, 1989
4. Robinson SD, Silberstein GB, Roberts AB, Flanders KC, Daniel CW: Regulated expression and growth inhibitory effects of transforming growth factor-beta isoforms in mouse mammary gland development. Development 113: 867–878, 1991
5. Silberstein GB, Flanders KC, Roberts AB, Daniel CW: Regulation of mammary morphogenesis: evidence for extracellular matrix-mediated inhibition of ductal budding by transforming growth factor-beta 1. Dev Biol 152: 354–362, 1992
6. Pierce DFJ, Johnson MD, Matsui Y, Robinson SD, Gold LI, Purchio AF, Daniel CW, Hogan BLM, Moses H: Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mouse expressing active TGFβ1. Genes & Dev 7: 2308–2317, 1993
7. Jhappan C, Geiser AG, Kordon EC, Bagheri D, Hennighausen AB, Roberts AB, Smith GH, Merlino G: Targeting expression of a transforming growth factor β1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. EMBO J 12: 1835–1845, 1993
8. Strange R, Li F, Saurer S, Burkhardt A, Friis RR: Apoptotic cell death and tissue remodelling during mouse mammary gland involution. Development 115: 49–58, 1992
9. Wakefield LM, Letterio JJ, Geiser AG, Flanders KC, O'Shaughnessy J, Roberts AB, Sporn MB: Transforming growth factor-beta's in mammary tumorigenesis: promoters or antipromoters? Prog Clin Biol Res 391: 133–148, 1995
10. Fynan TM, Reiss M: Resistance to inhibition of cell growth by transforming growth factor-β and its role in oncogenesis. Crit Rev Oncogenesis 4: 493–540, 1993
11. Thompson NL, Flanders KC, Smith JM, Ellingsworth LR, Roberts AB, Sporn MB: Expression of transforming growth factor β1 in specific cells and tissues of adult and neonatal mice. J Cell Biol 108: 661–669, 1988
12. Li L, Hu J-S, Olson EN: Different members of the *jun* proto-oncogene family exhibit distinct patterns of expression

in response to type β transforming growth factor. J Biol Chem 265: 1556–1562, 1990
13. Stampfer MR, Yaswen P, Alhadeff M, Hosoda J: TGF beta induction of extracellular matrix associated proteins in normal and transformed human mammary epithelial cells in culture is independent of growth effects. J Cell Physiol 155: 210–221, 1993
14. Arteaga CL, Tandon AK, Von Hoff DD, Osborne CK: Transforming growth factor-β: potential autocrine growth inhibitor of estrogen receptor-negative human breast cancer cells. Cancer Res 48: 3898–3904, 1988
15. Kalkhoven E, Kwakkenbos-Isbrücker L, Mummery CL, De Laat SW, van den Eijnden-van Raaij AJM, van der Saag PT, van der Burg B: The role of TGFβ production in growth inhibition of breast-tumor cells by progestins. Int J Cancer 61: 80–86, 1995
16. Walker RA, Dearing SJ: Transforming growth factor beta 1 in ductal carcinoma *in situ* and invasive carcinomas of the breast. Eur J Cancer 28: 641–644, 1992
17. McCune BK, Mullin BR, Flanders KC, Jaffurs WJ, Mullen LT, Sporn MB: Localization of transforming growth factor-beta isotypes in lesions of the human breast [see comments]. Hum Pathol 23: 13–20, 1992
18. Dalal BI, Keown PA, Greenberg AH: Immunocytochemical localization of secreted transforming growth factor-beta 1 to the advancing edges of primary tumors and to lymph node metastases of human mammary carcinoma. Am J Pathol 143: 381–389, 1993
19. Gorsch SM, Memoli VA, Stukel TA, Gold LI, Arrick BA: Immunohistochemical staining for transforming growth factor β1 associates with disease progression in human breast cancer. Cancer Res 52: 6949–6952, 1992
20. Welch DR, Fabra A, Nakajima M: Transforming growth factor β stimulates mammary adenocarcinoma cell invasion and metastatic potential. Proc Natl Acad Sci USA 87: 7678–7682, 1990
21. Arrick BA, Lopez AR, Elfman F, Ebner R, Damsky CH, Derynck R: Altered metabolic and adhesive properties and increased tumorigenesis associated with increased expression of transforming growth factor beta 1. J Cell Biol 118: 715–726, 1992
22. Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT: Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. J Clin Invest 92: 2569–2576, 1993
23. Arteaga CL, Carty-Dugger T, Moses HL, Hurd SD, Pietenpol JA: Transforming growth factor beta 1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice. Cell Growth Differ 4: 193–201, 1993
24. Levin NA, Brzoska P, Gupta N, Minna JD, Gray JW, Christman MF: Identification of frequent novel genetic alterations in small cell lung carcinoma. Cancer Res 54: 5086–5091, 1994
25. Miyazono K, Hellman U, Wernstedt C, Heldin C-H: Latent high molecular weight complex of transforming growth factor-β from human platelets: a high molecular weight complex containing precursor sequences. J Biol Chem 263: 6407–6415, 1988
26. Massagué J: Receptors for the TGFβ family. Cell 69: 1067–1070, 1992
27. Miyazono K, Heldin C-H: Latent forms of TGFβ: molecular structure and mechanisms of activation. In: Bock GR, Marsh J (eds) Clinical Applications of TGFβ. Wiley, Chichester, 1991, pp 81–92
28. Border WA, Noble NA: Transforming growth factor β in tissue fibrosis. N Engl J Med 331: 1286–1292, 1994
29. Flaumenhaft R, Rifkin DB: The extracellular regulation of growth factor action. Mol Cell Biol 3: 1057–1065, 1992
30. Foekens JA, Schmitt M, van Putten WL, Peters HA, Bontenbal M, Janicke F, Klijn JG: Prognostic value of urokinase-type plasminogen activator in 671 primary breast cancer patients. Cancer Res 52: 6101–6105, 1992
31. Janicke F, Schmitt M, Pache L, Ulm K, Harbeck N, Hofler H, Graeff H: Urokinase (uPA) and its inhibitor PAI-1 are strong and independent prognostic factors in node-negative breast cancer. Breast Cancer Res Treat 24: 195–208, 1993
32. Grondahl-Hansen J, Christensen IJ, Rosenquist C, Brunner N, Mouridsen HT, Dano K, Blichert-Toft M: High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. Cancer Res 53: 2513–2521, 1993
33. Odekon LE, Blasi F, Rifkin DB: Requirement for receptor-bound urokinase in plasmin-dependent cellular conversion of latent TGF-beta to TGF-beta. J Cell Physiol 158: 398–407, 1994
34. Nielsen BS, Sehested M, Timshel S, Pyke C, Dano K: Messenger RNA for urokinase plasminogen activator is expressed in myofibroblasts adjacent to cancer cells in human breast cancer. Lab Invest 74: 168–177, 1996
35. Barcellos-Hoff MH: Radiation-induced transforming growth factor β and subsequent extracellular matrix reorganization in murine mammary gland. Cancer Res 53: 3880–3886, 1993
36. Mackie EJ, Halfter W, Liverani D: Induction of tenascin in healing wounds. J Cell Biol 107: 2757–2767, 1988
37. Laurent TC, Fraser RE: Hyaluronan. FASEB J 6: 2397–2404, 1992
38. Chiquet-Ehrismann R, Mackie EJ, Pearson CA, Sakakura T: Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. Cell 47: 131–139, 1986
39. Mackie EJ, Chiquet-Ehrismann R, Pearson CA, Inaguma Y, Taya K, Kawarada Y, Sakakura T: Tenascin is a stromal marker for epithelial malignancy in the mammary gland. Proc Natl Acad Sci USA 84: 4621–4625, 1987
40. Howeedy AA, Virtanen I, Laitinen L, Gould NS, Koukoulis GK, Gould VE: Differential distribution of tenascin in

the normal, hyperplastic, and neoplastic breast. Lab Invest 63: 798–806, 1990
41. Barcellos-Hoff MHR, Derynck R, Tsang L-S, Weatherbee JA: Transforming growth factor-β activation in irradiated murine mammary gland. J Clin Invest 93: 892–899, 1994
42. Fowlis DJ, Flanders KC, Duffie E, Balmain A, Akhurst RJ: Discordant localisation of TGFβ RNA and protein during chemical carcinogenesis in mouse skin. Cell Growth Differ 3: 81–91, 1992
43. Esherick JS, DiCunto F, Flanders KC, Missero C, Dotto GP: Transforming growth factorβ1 induction is associated with transforming growth factorβ2 and β3 down-modulation in 12-O-tetradecanoylphorbol-13-acetate-induced skin hyperplasia. Cancer Res 53: 5507–5512, 1993
44. Barcellos-Hoff MH, Ehrhart EJ, Kalia M, Jirtle R, Flanders K, Tsang ML-S: Immunohistochemical detection of active TGFβ *in situ* using engineered tissue. Am J Pathol 147: 1228–1237, 1995
45. Jirtle RL, Hankinc GR, Reisenbichler H, Boyer IJ: Regulation of mannose-6-phosphate/insulin-like growth factor-II receptors and transforming growth factor beta during liver tumor promotion with phenobarbital. Carinogenesis 15: 1473–1478, 1994
46. Mansbach JM, Mills JJ, Boyer IJ, De Souza AT, Hankins GR, Jirtle RL: Phenobarbital selectively promotes initiated cells with reduced TGF beta receptor levels. Carcinogenesis 17: 171–174, 1996
47. Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R, Dickson RB: Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48: 417–428, 1987
48. Zugmaier G, Ennis BW, Deschauer B, Katz D, Knabbe C, Wilding G, Daly P, Lippman ME, Dickson RB: Transforming growth factor type β1 and β2 are equipotent growth inhibitors of human breast cancer cell lines. J Cell Physiol 141: 353–361, 1989
49. Herman ME, Katzenellenbogen BS: Alterations in transforming growth factor-alpha and -beta production and cell responsiveness during the progression of MCF-7 human breast cancer cells to estrogen-autonomous growth. Cancer Res 54: 5867–5874, 1994
50. Pierce DF Jr, Gorska AE, Chytil A, Meise KS, Page DL, Coffey RJ Jr, Moses HL: Mammary tumor suppression by transforming growth factor beta 1 transgene expression. Proc Natl Acad Sci USA 92: 4254–4258, 1995
51. Zugmaier G, Paik S, Wilding G, Knabbe C, Bano M, Lupu R, Deschauer B, Simpson S, Dickson RB, Lippman M: Transforming growth factor β1 induces cachexia and systemic fibrosis without an antitumor effect in nude mice. Cancer Res 51: 3590–3594, 1991
52. Lin HY, Wang X-F, Ng-Eaton E, Weinberg RA, Lodish HF: Expression cloning of the TGFβ type II receptor, a functional transmembrane serine/threonine kinase. Cell 68: 775–785, 1992
53. Franzén P, Ten Dijke P, Ichijo H, Yamashita H, Schulz P, Heldin C-H, Miyazono K: Cloning of a TGFβ type I receptor that forms a heterodimeric complex with the TGFβ type II receptor. Cell 75: 681–692, 1993
54. Lin HY, Moustakas A: TGF-beta receptors: structure and function. Cell Mol Biol 40: 337–349, 1994
55. ten Dijke P, Ichijo H, Franzen P, Schulz P, Saras J, Toyoshima H, Heldin C-H, Miyazono K: Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. Oncogene 8: 2879–2887, 1993
56. Attisano L, Wrana JL, Lopez-Casillas F, Massagué J: TGF beta receptors and actions. Biochim Biophys Acta 1222: 71–80, 1994
57. Chen R-H, Derynck R: Homomeric interactions between type II transforming growth factor-β receptors. J Biol Chem 269: 22868–22874, 1994
58. Wrana JL, Attisano L, Cárcamo J, Zentella A, Doody J, Laiho M, Wang X-F, Massagué J: TGFβ signals through a heteromeric protein kinase receptor complex. Cell 71: 1003–1014, 1992
59. Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J: Mechanism of activation of the TGFβ receptor. Nature 370: 341–347, 1994
60. Wang T, Donahoe P, Zervos AS: Specific interaction of type I receptors of the TGFβ family with the immunophilin FKBP-12. Science 265: 674–676, 1994
61. Chen R-H, Miettinen PJ, Maruoka EM, Choy L, Derynck R: A WD-domain protein that is associated with and phosphorylated by the type II TGF-β receptor. Nature 377: 548–552, 1995
62. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159, 1987
63. Yan ZF, Winawer S, Friedman E: Two different signal transduction pathways can be activated by transforming growth factor beta 1 in epithelial cells. J Biol Chem 269: 13231–13237, 1994
64. Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K: Identification of a member of the MAPKKK family as a potential mediator of TGFβ signal transduction. Science 270: 2008–2011, 1995
65. Fischer JR, Darjes H, Lahm H, Schindel M, Drings P, Krammer PH: Constitutive secretion of bioactive transforming growth factor beta 1 by small lung cancer cell lines. Eur J Cancer 30A: 2125–2129, 1994
66. Nørgaard P, Damstrup L, Rygaard K, Spang-Thomsen M, Skovgaard Poulsen H: Growth suppression by transforming growth factor beta 1 of human-cell lung cancer cell lines is associated with expression of the type II receptor. Br J Cancer 69: 802–808, 1994
67. Hannon GJ, Beach D: $p15^{INK4B}$ is a potential effector of TGFβ-induced cell cycle arrest. Nature 371: 257–261, 1994
68. Li J-M, Nichols MA, Chandrasekharan S, Xiong Y, Wang X-F: Transforming growth factor β activates the promoter

of cyclin-dependent kinase inhibitor $p15^{INK4B}$ through an Sp1 consensus site. J Biol Chem 270: 26750–26753, 1995

69. Geng Y, Weinberg RA: Transforming growth factor β effects on expression of G1 cyclins and cyclin-dependent protein kinases. Proc Natl Acad Sci USA 90: 10315–10319, 1993
70. Ewen ME, Sluss HK, Whitehouse LL, Livingston DM: TGFβ inhibition of cdk4 synthesis is linked to cell cycle arrest. Cell 74: 1009–1020, 1993
71. Gerwein BI, Spillare E, Forrestier K, Lehman TA, Kispert J, Welsh JA, Pfeifer AMA, Lechner JF, Baker SJ, Vogelstein B, Harris CC: Mutant p53 can induce tumorigenic conversion of human bronchial epithelial cells and reduce their responsiveness to a negative growth factor, transforming growth factor β1. Proc Natl Acad Sci USA 89: 2759–2763, 1992
72. Reiss M, Vellucci VF, Zhou Z-L: Loss of sensitivity to the anti-proliferative effect of transforming growth factor-β1 associated with the expression of mutant p53 tumor suppressor gene in murine keratinocytes. Cancer Res 53: 899–904, 1993
73. Okamoto A, Jiang W, Kim SJ, Spillare EA, Stoner G, Weinstein IB, Harris CC: Overexpression of human cyclin D1 reduces the transforming growth factor beta (TGF-beta) type II receptor and growth inhibition by TGF-beta 1 in an immortalized human esophageal epithelial cell line. Proc Natl Acad Sci USA 91: 11570–11580, 1994
74. Buckley MF, Sweeney KJ, Hamilton JA, Sini RL, Manning DL, Nicholson RI, deFazio A, Watts CK, Musgrove EA, Sutherland RL: Expression and amplification of cyclin genes in human breast cancer. Oncogene 8: 2127–2133, 1993
75. Zukerberg LR, Yang WI, Gadd M, Thor AD, Koerner FC, Schmidt EV, Arnold A: Cyclin D1 (PRAD1) protein expression in breast cancer: approximately one-third of infiltrating mammary carcinomas show overexpression of the cyclin D1 oncogene. Mod Pathol 8: 560–567, 1995
76. Weinstat-Saslow D, Merino MJ, Manrow RE, Lawrence JA, Bluth RF, Wittenbel KD, Simpson JF, Page DL, Steeg PS: Overexpression of cyclin D mRNA distinguishes invasive and *in situ* breast carcinomas from non-malignant lesions. Nature Med 1: 1257–1260, 1995
77. Laiho M, Weis FMB, Boyd FT, Ignotz RA, Massagué J: Responsiveness to transforming growth factor-β (TGFβ) restored by complementation between cells defective in TGFβ receptors I and II. J Biol Chem 266: 9108–9112, 1991
78. Geiser AG, Burmester JK, Webbink R, Roberts AB, Sporn MB: Inhibition of growth by transforming growth factor-β following fusion of two nonresponsive human carcinoma cell lines. J Biol Chem 267: 2588–2593, 1992
79. Kalkhoven E, Roelen BAJ, de Winter JP, Mummery CL, van den Eijnden-van Raaij AJM, van der Saag PT, van der Burg B: Resistance to transforming growth factor β and activin due to reduced receptor expression in human breast tumor cell lines. Cell Growth Diff 6: 1151–1156, 1995
80. Sun L, Wu G, Willson JK, Zborowska E, Yang J, Rajkarunanayake I, Wang J, Gentry LE, Wang XF, Brattain MG: Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. J Biol Chem 269: 26449–26455, 1994
81. Kim IY, Ahn H-J, Zelner DJ, Shaw JW, Sensibar JA, Kim J-H, Kato M, Lee C: Genetic change in transforming growth factor-β (TGF-β) receptor type I gene correlates with insensitivity to TGFβ1 in human prostate cancer cells. Cancer Res 56: 44–48, 1996
82. Kadin ME, Cavaille-Coll MW, Gertz R, Massagué J, Cheifetz S, George D: Loss of receptors for transforming growth factor β in human T-cell malignancies. Proc Natl Acad Sci USA 91: 6002–6006, 1994
83. Capocasale RJ, Lamb RJ, Vonderheid EC, Fox FE, Rook AH, Nowell PC, Moore JS: Reduced surface expression of transforming growth factor β receptor type II in mitogen-activated T cells from Sézary patients. Proc Natl Acad Sci USA 92: 5501–5505, 1995
84. Jakowlew SB, Mathias A, Chung P, Moody TW: Expression of transforming growth factor beta ligand and receptor messenger RNAs in lung cancer cell lines. Cell Growth Differ 6: 465–476, 1995
85. Damstrup L, Rygaard K, Spang-Thomsen M, Skovgaard Poulsen H: Expression of transforming growth factor beta (TGF beta) receptors and expression of TGF beta 1, TGF beta 2 and TGF beta 3 in human small cell lung cancer cell lines. Br J Cancer 67: 1015–1021, 1993
86. Friess H, Yamanaka Y, Büchler M, Beger HG, Kobrin MS, Baldwin RL, Korc M: Enhanced expression of the type II transforming growth factor β receptor in human pancreatic cancer cells without alteration of type II receptor expression. Cancer Res 53: 2704–2707, 1993
87. Garrigue-Antar L, Munoz-Antonia T, Antonia SJ, Gesmonde J, Vellucci VF, Reiss M: Missense mutations of the transforming growth factor beta type II receptor in human head and neck squamous carcinoma cells. Cancer Res 55: 3982–3987, 1995
88. Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, Fan RS, Zborowska E, Kinzler KW, Vogelstein B, Brattain M, Willson JKV: Inactivation of the type II TGFβ receptor in colon cancer cells with microsatellite instability. Science 268: 1336–1338, 1995
89. Myeroff LL, Parsons R, Kim S-J, Hedrick L, Cho KR, Orth K, Mathis M, Kinzler KW, Lutterbaugh J, Park K, Bang Y-J, Lee HY, Park J-G, Lynch HT, Roberts AB, Vogelstein B, Markowitz SD: A transforming growth factor β type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. Cancer Res 55: 5545–5547, 1995
90. Parsons R, Myeroff LL, Liu B, Willson JKV, Markowitz SD, Kinzler KW, Vogelstein B: Microsatellite instability and mutations of the transforming growth factor β type II receptor gene in colorectal cancer. Cancer Res 55: 5548–5550, 1995
91. Pavelic K, Antonic M, Pavelic L, Pavelic J, Pavelic Z, Spaventi S: Human lung cancers growing on extracellular matrix: expression of oncogenes and growth factors. Anticancer Res 12: 2191–2196, 1992

92. Ke Y, Stoner GD, Demetrick DJ, Bennett WP: Mutation analysis of the transforming growth factor beta type II receptor in sporadic human cancers of the pancreas, liver and breast. Proc Am Assoc Cancer Res 37: 116, 1996
93. Roth JA, Mukhopadhyay T, Tainksky MA, Fang K, Casson AG, Schneider PM: Molecular approaches to prevention and therapy of aerodigestive tract cancers. Monogr Natl Cancer Inst 15–21, 1992
94. Ji H, Stout LE, Zhang Q, Zhang R, Leung HT, Leung BS: Absence of transforming growth factor-beta responsiveness in the tamoxifen growth-inhibited human breast cancer cell line CAMA-1. J Cell Biochem 54: 332–342, 1994
95. Kopp A, Jonat W, Schmahl M, Knabbe C: Transforming growth factor beta 2 (TGF-beta 2) levels in plasma of patients with metastatic breast cancer treated with tamoxifen. Cancer Res 55: 4512–4515, 1995
96. Knabbe C, Kopp A, Hilgers W, Lang D, Muller V, Zugmaier G, Jonat W: Regulation and role of TGF beta production in breast cancer. Ann NY Acad Sci 784: 263–276, 1996
97. Butta A, MacLennan K, Flanders KC, Sacks NPM, Smith I, McKinna A, Dowsett M, Wakefield LM, Sporn MB, Baum M, Colletta AA: Induction of transforming growth factor β1 in human breast cancer *in vivo* following tamoxifen treatment. Cancer Res 52: 4261–4264, 1992
98. Murray PA, Gomm J, Ricketts D, Powles T, Coombes RC: The effect of endocrine therapy on the levels of oestrogen and progesterone receptor and transforming growth factor-beta 1 in metastatic human breast cancer: an immunocytochemical study. Eur J Cancer 30A: 1218–1222, 1994
99. MacCallum J, Keen JC, Bartlett JM, Thompson AM, Dixon JM, Miller WR: Changes in expression of transforming growth factor beta mRNA isoform in patients undergoing tamoxifen therapy. Br J Cancer 74: 474–478, 1996
100. Benson JR, Wakefield LM, Baum M, Colletta AA: Synthesis and secretion of transforming growth factor beta isoforms by primary cultures of human breast tumour fibroblasts *in vitro* and their modulation by tamoxifen. Br J Cancer 74: 352–358, 1996
101. Group EBCTC: Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. Lancet 339: 1–15, 71–85, 1992
102. McGuire WL, Vollmer EP, Carbone PP: Estrogen Receptors in Human Breast Cancer. Raven Press, New York, 1975
103. Johnston SRD, Saccani-Jotti G, Smith IE, Salter J, Newby J, Coppen M, Ebbs SR, Dowsett M: Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. Cancer Res 55: 3331–3338, 1995
104. Karnik PS, Kulkarni S, Liu XP, Budd GT, Bukowski RM: Estrogen receptor mutations in tamoxifen-resistant breast cancer. Cancer Res 54: 349–353, 1994
105. Osborne CK, Fuqua SA: Mechanisms of tamoxifen resistance. Breast Cancer Res Treat 32: 49–55, 1994
106. Thompson AM, Kerr DJ, Steel CM: Transforming growth factor beta 1 is implicated in the failure of tamoxifen therapy in human breast cancer. Br J Cancer 63: 609–614, 1991
107. Foekens JA, Look MP, Peters HA, van Putten WL, Portengen H, Klijn JG: Urokinase-type plasminogen activator and its inhibitor PAI-1: predictors of poor response to tamoxifen therapy in recurrent breast cancer. J Natl Cancer Inst 87: 751–756, 1995
108. Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, Samid D, Duan DS, Gnarra JR, Linehan WM, Baylin SB: Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. Proc Natl Acad Sci USA 91: 9700–9704, 1994
109. Fisher B, Costantino J, Redmond C, Fisher E, Margolese R, Dimitrov N, Wolmark N, Wickerham DL, Deutsch M, Ore L, Mamounas E, Poller W, Kavanah M: Lumpectomy compared with lumpectomy and radiation therapy for the treatment of intraductal breast cancer. N Engl J Med 328: 1581–1586, 1993
110. Dickens TA, Colletta AA: The pharmacological manipulation of members of the transforming growth factor beta family in the chemoprevention of breast cancer. Bioassays 15: 71–74, 1993
111. Bush TL, Helzlsouer KJ: Tamoxifen for the primary prevention of breast cancer: a review and critique of the concept and trial. Epidemiol Rev 15: 233–243, 1993
112. Powles TJ, Jones AL, Ashley SE, O'Brien ME, Tidy VA, Treleavan J, Cosgrove D, Nash AG, Sacks N, Baum M, et al.: The Royal Marsden Hospital pilot tamoxifen chemoprevention trial. Breast Cancer Res Treat 31: 73–82, 1994
113. Kojima S, Rifkin DB: Mechanism of retinoid-induced activation of latent transforming growth factor-beta in bovine endothelial cells. J Cell Physiol 155: 323–332, 1993
114. Costa A, Formelli F, Chiesa F, Decensi A, De Palo G, Veronesi U: Prospects of chemoprevention of human cancers with the synthetic retinoid fenretinide. Cancer Res 54: 1994
115. Jirtle RL, Haag JD, Ariazi EA, Gould MN: Increased mannose-6-phosphate/insulin-like growth factor II receptor and transforming growth factor-β1 levels during monoterpene-induced regression of mammary tumors. Cancer Res 53: 3849–3852, 1993
116. Nørgaard P, Spang-Thomsen M, Poulsen HS: Expression and autoregulation of transforming growth factor β receptor mRNA in small-cell lung cancer cell lines. Br J Cancer 73: 1037–1043, 1996
117. Kashii T, Mizushima Y, Monno S, Nakagawa K, Kobayashi M: Gene analysis of K-, H-ras, p53, and retinoblastoma susceptibility genes in human lung cancer cell lines by the polymerase chain reaction/single-strand conformation polymorphism method. J Cancer Res Clin Oncol 120: 143–148, 1994
118. Niklinski J, Furman M: Clinical tumour markers in lung cancer. Eur J Cancer Prev 4: 129–138, 1995

[CANCER RESEARCH 58, 1986–1992, May 1, 1998]

Functional Characterization of Transforming Growth Factor β Type II Receptor Mutants in Human Cancer[1]

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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 β type II receptor (TβR-II) serine-threonine kinase in two human squamous carcinoma cell lines. These cell lines are resistant to transforming growth factor β-mediated inhibition of growth. Moreover, treatment with transforming growth factor β 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 TβR-II cDNAs were expressed in TβR-II-deficient T47D cells. Cyclin A promoter activity was reduced by 50% in cells expressing wild-type TβR-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 TβR-II.

INTRODUCTION

TGF-β[4] 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-β 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-β is to enable the cellular microenvironment to regulate epithelial cell proliferation by exerting G_1-S-phase cell cycle checkpoint control. This is illustrated by the fact that, *in vitro*, TGF-β 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-β-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-β-mediated growth control is caused by inactivation of the TGF-β signaling pathway and represents an important step in the malignant transformation of epithelial cells (8, 9).

The TGF-β signal is transduced by a unique mechanism that involves a pair of transmembrane serine-threonine kinase receptors (10). Free ligand binds primarily to the TβR-II. Two TβR-I molecules are then recruited into a ternary complex with two TβR-II molecules and a single ligand dimer. The formation of this ternary complex allows the TβR-II kinase to phosphorylate specific serine residues located within the highly conserved so-called GS domain immediately upstream of the TβR-I serine-threonine kinase domain. This activation of the TβR-I kinase is the necessary first step in further transducing the signal downstream (10). Postreceptor events in TGF-β signaling are mediated by homologues of the *Drosophila* mother-against-dpp protein (11, 12). In response to TGF-β binding, two of these proteins, Smad2 and Smad3, become transiently associated with the activated TβR-I receptor and are phosphorylated, presumably by the TβR-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_2-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-β1 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-β 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 *TβR-I, TβR-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-β signaling pathway contributes to malignant progression.

Recently, we identified missense mutations at codons 526 and 537 of the *TβR-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 TβR-II have been noted in cutaneous T-cell lymphoma cells (30). The clonal selection of tumor cells bearing these TβR-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 *TβR-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 *TβR-II* gene completely abolishes the transduction of all cellular responses to TGF-β by dramatically reducing receptor serine-threonine kinase activity.

Received 12/3/97; accepted 3/4/98.

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

[1] Supported in part by USPHS Award CA41556 from the National Cancer Institute and United States Department of Defense Breast Cancer Research Program Grant DAMD17-96-1-6024.

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[4] The abbreviations used are: TGF-β, transforming growth factor β; SCC, squamous cell carcinoma; TβR-II, type II TGF-β receptor; TβR-I, type I TGF-β receptor; ECM, extracellular matrix; PAI-1, plasminogen activator inhibitor type 1; TβS-T, Tris-buffered saline with Triton-X100; FN, fibronectin; CMV, cytomegalovirus; IP, immunoprecipitation; αR-II, anti-TβR-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^{++} 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-β1 (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-β1 (100 pM). During the final 4 h of an 18-h incubation, cells were exposed to 150 μCi/ml [^{35}S]methionine and [^{35}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 μl of conditioned medium or cell lysate were incubated in the presence of 100 μl of gelatin-Sepharose (Pharmacia Biotech) and 3 μl 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 β-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 TβR-II cDNA under the transcriptional control of a CMV promoter (43). It was legitimate to use the mouse TβR-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 *Hin*dIII/*Xba*I digestion, the newly generated 470-nucleotide *Hin*dIII/*Xba*I fragment was ligated into the p122 backbone that had been prepared by partial *Hin*dIII/*Xba*I digestion. The presence of the specific desired mutation was confirmed by restriction digestion and DNA sequencing.

Reporter Gene Assays. The signaling function of mutant αR-IIs was assessed in transient transfection assays into T47D breast carcinoma cells. This cell line is convenient, because it is refractory to TGF-β and fails to express detectable levels of TβR-II mRNA or protein, and all responses to TGF-β can be restored by reexpressing wild-type TβR-II (45). Two separate reporter gene constructs were used to assess the different types of responses to TGF-β: (*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-β 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 × 10^5 cells/well in 6-well cluster dishes and allowed to adhere overnight at 37°C. Before transfection, 10 μl of Lipofectin (Life Technologies, Inc.) were diluted into 700 μl of Opti-MEM (Life Technologies, Inc.) and incubated at 20°C for 45 min. Lipofectin was then combined with plasmid DNA (1 μg TβR-II plasmid DNA and 2 μg reporter plasmid/300 μl 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-β1. 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 μg) 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 μl) was mixed with 100 μl 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 μCi/ml [^{35}S]methionine and [^{35}S]cysteine (50 Ci/mmol; DuPont New England Nuclear) for 4 h, cell lysates were prepared from TGF-β1-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 μg/ml leupeptin]. Lysate containing 1 mg of total protein was incubated with 1 μg of polyclonal rabbit antibody directed against the TβR-II receptor (αR-II, R220) (Santa Cruz Biotechnology) for 2 h at 4°C on a rotating shaker, followed by the addition of 40 μl 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 αR-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× 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 αR-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 μg of αR-II antibody for 2 h at 4°C on a rotating shaker, followed by the addition of 40 μl 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 μl of kinase buffer [20 mM HEPES (pH 7.7), 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA (pH 8), 0.05% (v/v) Triton X-100, 0.5 mM DTT, 20 mM β-glycerol phosphate, 100 μM sodium orthovanadate, 4 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride] containing 20 μM [γ-^{32}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 αR-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 αR-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 TβR-II gene (29). We had shown previously that TGF-β 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-β is illustrated in Fig. 1; whereas proliferation of nonneoplastic HaCat keratinocytes was inhibited by approximately 75% at concentrations of TGF-β1 of ≥25 pM, the anchorage-dependent growth of A253 and SqCC/Y1 cells was not affected by TGF-β1,

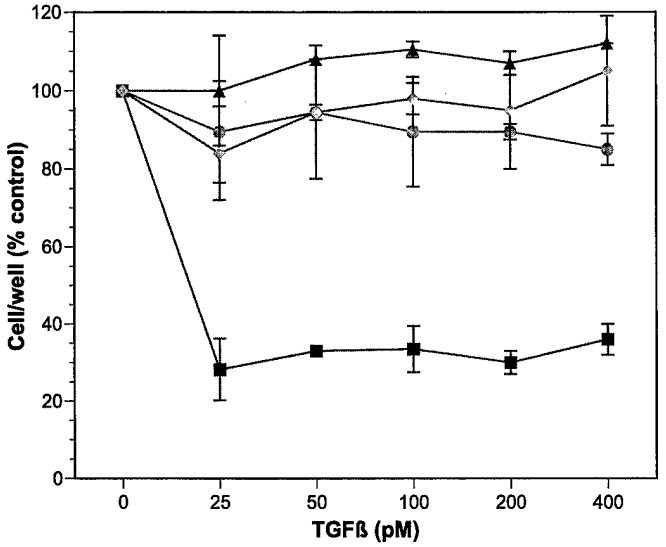

Fig. 1. Effect of TGF-β1 on cell proliferation of keratinocytes and SCC cell lines *in vitro*. Cells (10^4 cells/well) were plated in 24-well cluster dishes, followed by the addition of TGF-β1 24 h later at the indicated concentrations. Medium and TGF-β1 were replaced after 3 days, and cell numbers in 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 ± 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-β action, Smad4 (47), was similarly unaffected by treatment with TGF-β1.

Besides inhibiting cell cycle progression, TGF-β 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-β-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-β1 treatment on PAI-1 and FN synthesis by the SCC lines. TGF-β1 induced a 3–4-fold increase in the levels of both cell-associated and secreted PAI-1 (Fig. 2*A*) and stimulated the synthesis of FN (Fig. 2*B*) 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-β1 treatment. The effects of TGF-β1 on FN synthesis in A253 and FaDu cells could not be ascertained, because neither cell line produced detectable amounts of this ECM protein (Fig. 2*B*). 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-β.

To prove that the TβR-II gene mutations were solely responsible for the TGF-β-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 TβR-II cDNA. We used a mammalian expression vector in which wild-type and mutant TβR-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 TβR-II restores all responses to TGF-β (45). Two separate reporter gene constructs, pCAL2 and p3TP-Lux, were used to assess the dual types of responses to TGF-β. 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-β-mediated cell cycle arrest, and activation of the PAI-1 promoter reflects TGF-β-induced PAI-1 production (43, 51). As shown in Fig. 3*A*, the expression of wild-type TβR-II in TβR-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. 3*A*). Repression of pCAL2 was independent of the addition of exogenous TGF-β, presumably because, at high levels of expression, the TβR-II and TβR-I receptors tend to spontaneously dimerize and activate the signaling pathway (52–55). Alternatively, it is possible that bioactive TGF-β2 produced by T47D cells was sufficient to stimulate their TGF-β receptors in an autocrine manner (56). Although attempts to block endogenous TGF-β2 activity by using a neutralizing anti-TGF-β2 antibody (R & D Systems) did not affect the results of the transfection experiments (data not shown), endogenous TGF-β2 may bind and activate the receptors intracellularly, as has

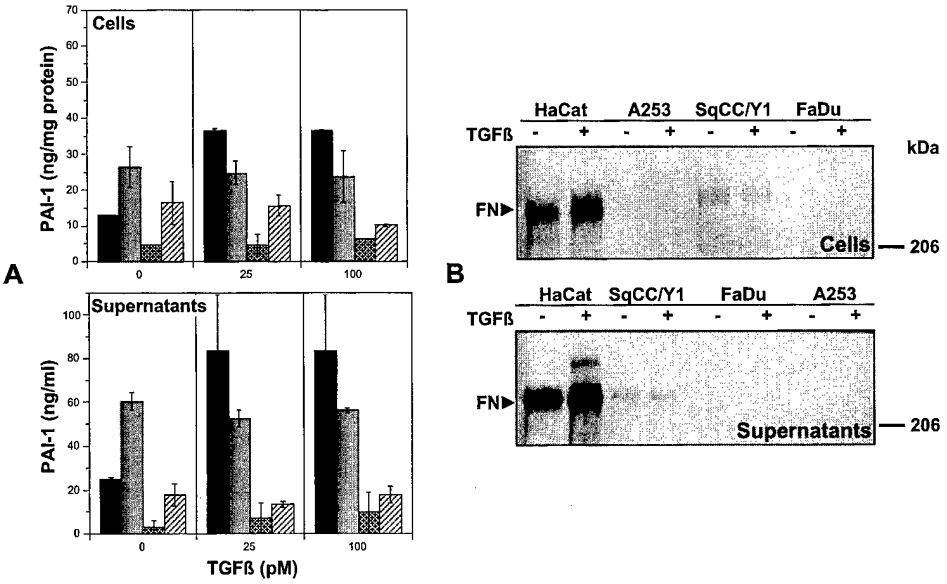

Fig. 2. Effect of TGF-β on PAI-1 production and FN synthesis by keratinocytes and SCC cell lines. *A*, cells were grown to confluence in 6-well tissue culture dishes and treated with TGF-β1 at the indicated concentrations for 18 h. PAI-1 concentrations in culture supernatants and cell extracts were then analyzed separately using an ELISA. ■, HaCat; ▨, A253; ▩, FaDu; ▨, SqCC/Y1. The means ± SE of four independent experiments are shown. *B*, preconfluent cultures of HaCat keratinocytes or SCC cell lines were exposed to TGF-β1 (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.

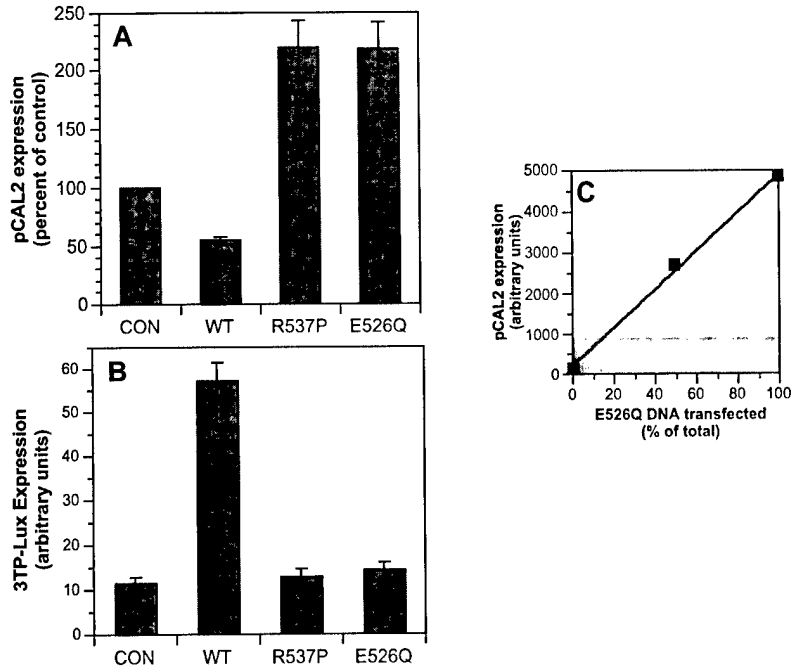

Fig. 3. Effects of transfected wild-type and mutant TβR-II receptor on TGF-β-regulated gene expression. T47D cells were cotransfected with plasmids expressing either wild-type (*WT*) or mutant (*E526Q* or *R537P*) TβR-II receptor and pCAL2 (*A*) or p3TP-Lux (*B*) in conjunction with pRL-CMV, and luciferase 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 TβR-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 ± SE from four independent experiments are shown. *B*, in cells transfected with wild-type TβR-II, PAI-1 promoter (*3TP-Lux*) activity was increased 6-fold in response to TGF-β1 treatment, whereas no increase over control levels was noted in cells transfected with either of the two TβR-II mutants. *C*, to determine whether the E526Q mutant was dominant negative, T47D cells were transfected with 1 μg of wild-type TβR-II, 1 μg of E526Q, or with 0.5 μg 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 TβR-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 TβR-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. 3*B*). Thus, both mutations abolish TGF-β-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-β 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. 3*A*). 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 TβR-II proteins are particularly potent dominant-negatives that completely abrogate all of the responses to TGF-β when transfected into normal endothelial and bronchial cells (61–64).

Dominant-negative mutants may interfere with TGF-β signaling by competing with wild-type TβR-II for heterodimerization with TβR-I molecules or by sequestering coexpressed wild-type TβR-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 TβR-II, although the protein is not detectable by ligand binding assays, TβR-II-specific mRNA is undetectable by Northern blot analysis, and the cells are unresponsive to TGF-β (45). To test whether the TβR-II mutants were dominant negative, we cotransfected them into T47D cells together with the wild-type construct. As shown in Fig. 3*C*, the pCAL2 activity we measured in cells transfected with a 1:1 ratio of E526Q mutant and wild-type TβR-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 TβR-II mutants suggests the possibility that the proliferation of tumor cells that express such mutants might be stimulated by TGF-β. 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-β (65, 66). This switch in responsiveness to TGF-β has been attributed to the use of an alternative TGF-β 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 TβR-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 TβR-IIs in transfected cells, parallel dishes of [^{35}S]methionine- and [^{35}S]cysteine-labeled cells were subjected to double IP with anti-TβR-II antiserum (Fig. 4*A*). Discrete bands of approximately equal intensity at the level of 65 kDa corresponding to the TβR-II receptor were detected in extracts from both wild-type- and mutant TβR-II-transfected cells. Moreover, a second band of approximately 55 kDa, representing coimmunoprecipitated TβR-I, was also seen in all three cases. No receptor protein was detected in control-transfected cells. These results indicate that the two TβR-II mutations

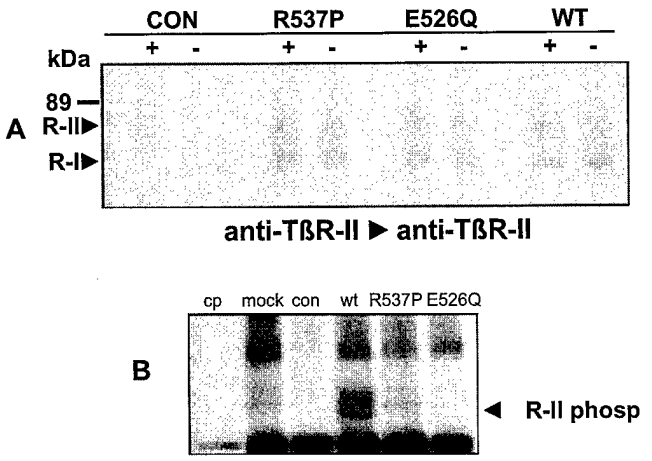

Fig. 4. Expression and *in vitro* kinase activity of transfected wild-type and mutant TβR-II receptor in T47D cells. *A*, T47D cells were transfected with plasmids expressing either wild-type (*WT*) or mutant (*E526Q* or *R537P*) TβR-II cDNAs, radiolabeled using methionine and [^{35}S]cysteine, and subjected to double IP with anti-TβR-II antiserum as described in "Materials and Methods" (*A*). Discrete bands of approximately equal intensity at the level of 65 kDa corresponding to the TβR-II receptor were detected in extracts from wild-type and mutant TβR-II-transfected cells. Moreover, a second band of approximately 55 kDa, representing coimmunoprecipitated TβR-I, was also present in all three cases. No receptor protein was detected in cells transfected with the control plasmid (*CON*). *B*, TβR receptor complexes were harvested from transfected T47D cells by IP using αR-II antibody and incubated in the presence of [γ-^{32}P]ATP. After the *in vitro* kinase reaction, extracts were boiled for 5 min in the presence of 1% SDS, reprecipitated with αR-II, and resolved by 7.5% (w/v) SDS-PAGE. A phosphorylated species of ~65 kDa corresponding to TβR-II was easily detectable in the cells transfected with wild-type TβR-II (*wt*), whereas these phosphoproteins were barely detectable in mutant TβR-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 TβR-I receptors.

Interestingly, this receptor phenotype is clearly distinct from that described for a closely related TβR-II mutant in which a proline residue at position 525 is replaced by a leucine (60). This P525L TβR-II mutant receptor is capable of binding ligand and of forming a complex with TβR-I but fails to transphosphorylate TβR-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 TβR-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 TβR-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 TβR-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. 4*B*, a phosphorylated protein of ~65 kDa corresponding to TβR-II was easily detectable in cells transfected with wild-type TβR-II, whereas such phosphoproteins were barely detectable in cells transfected with either of the two TβR-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 TβR-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-β 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 TβR-II at position 416. In the human TβR-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 TβR-I and the transduction of the growth-inhibitory signal (70). In contrast to the human sequence, the mouse TβR-II sequence lacks a potential phosphorylation site at position 416. Thus, if the high level of TβR-II autophosphorylation observed in A253 extracts is due to hyperphosphorylation on serine 416, this may account for the differences in autophosphorylation patterns of TβR-II observed between A253 cells (which express a human mutant R537P receptor) and T47D cells transfected with mutant R537P mouse TβR-II cDNA. We are currently investigating this possibility.

Mutations of the TβR-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 TβR-II exodomains (see Ref. 71). Such truncated receptors have been shown to inhibit TGF-β 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-β resistance by inhibiting the cell surface expression of TGF-β 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 TβR-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-β and provide strong evidence for a role of these mutants in the pathogenesis of the head and neck tumors they were found in.

ACKNOWLEDGMENTS

Plasmids p122 and pCAL2 were generous gifts from Dr. R. Derynck. The p3TP-Lux was a gift from Dr. M. Centrella.

REFERENCES

1. Thompson, A. M., Kerr, D. J., and Steel, C. M. Transforming growth factor β1 is implicated in the failure of tamoxifen therapy in human breast cancer. Br. J. Cancer, *63:* 609–614, 1991.

2. Rifkin, D. B., Kojima, S., Abe, M., and Harpel, J. G. TGF-β. Structure, function, and formation. Thromb. Haemostasis, *70:* 177–179, 1993.
3. Flaumenhaft, R., and Rifkin, D. B. The extracellular regulation of growth factor action. Mol. Cell. Biol., *3:* 1057–1065, 1992.
4. Border, W. A., and Noble, N. A. Transforming growth factor β in tissue fibrosis. N. Engl. J. Med., *331:* 1286–1292, 1994.
5. Reiss, M., and Dibble, C. L. Reinitiation of DNA synthesis in quiescent mouse keratinocytes: regulation by polypeptide hormones, cholera toxin, dexamethasone, and retinoic acid. In Vitro (Rockville), *24:* 537–544, 1988.
6. Ichijo, H., Momose, F., and Miyazono, K. Biological effects and binding properties of transforming growth factor-β on human oral squamous carcinoma cells. Exp. Cell Res., *187:* 263–269, 1990.
7. Reiss, M., and Stash, E. B. High frequency of resistance of human squamous carcinoma cells to the anti-proliferative action of transforming growth factor-β. Cancer Commun., *2:* 363–369, 1990.
8. Fynan, T. M., and Reiss, M. Resistance to inhibition of cell growth by transforming growth factor-β and its role in oncogenesis. Crit. Rev. Oncog., *4:* 493–540, 1993.
9. Reiss, M. Transforming growth factor-β and cancer. A love-hate relationship? Oncol. Res., in press, 1998.
10. Massagué, J. TGF-β signaling: receptors, transducers, and MAD proteins. Cell, *85:* 947–950, 1996.
11. Derynck, R., and Zhang, Y. Intracellular signaling: the Mad way to do it. Curr. Biol., *6:* 1226–1229, 1996.
12. Macias-Silva, M., Abdollah, S., Hoodless, P. A., Pirone, R., Attisano, L., and Wrana, J. L. MADR2 is a substrate of the TGF-β receptor and its phosphorylation is required for nuclear accumulation and signaling. Cell, *87:* 1215–1224, 1996.
13. Chen, Y., Lebrun, J. J., and Vale, W. Regulation of transforming growth factor β- and activin-induced transcription by mammalian Mad proteins. Proc. Natl. Acad. Sci. USA, *93:* 12992–12997, 1996.
14. Lagna, G., Hata, A., Hemmati-Brivanlou, A., and Massagué, J. Partnership between DPC4 and SMAD proteins in TGF-β signaling pathways. Nature (Lond.), *383:* 832–836, 1996.
15. Chen, X., Rubock, M. J., and Whitman, M. A transcriptional partner for MAD proteins in TGFβ signaling. Nature (Lond.), *383:* 691–696, 1996.
16. de Winter, J. P., Roelen, B. A., ten Dijke, P., van der Burg, B., and van den Eijnden-van Raaij, A. J. DPC4 (SMAD4) mediates transforming growth factor-β1 (TGF-β1) induced growth inhibition and transcriptional response in breast tumour cells. Oncogene, *14:* 1891–1899, 1997.
17. Hata, A., Lo, R. S., Wotton, D., Lagna, G., and Massagué, J. Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. Nature (Lond.), *388:* 82–87, 1997.
18. Shi, Y., Hata, A., Lo, R. S., Massagué, J., and Pavletich, N. P. A structural basis for mutational inactivation of the tumour suppressor Smad4. Nature (Lond.), *388:* 87–93, 1997.
19. Kim, J., Johnson, K., Chen, H. J., Carroll, S., and Laughon, A. *Drosophila* Mad binds to DNA and directly mediates activation of *vestigial* by decapentaplegic. Nature (Lond.), *388:* 304–308, 1997.
20. Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G., and Whitman, M. Smad4 and FAST-1 in the assembly of activin-responsive factor. Nature (Lond.), *389:* 85–89, 1997.
21. Polyak, K., Kato, J., Solomon, M. J., Sherr, C. J., Massagué, J., Roberts, J. M., and Koff, A. $p27^{kip1}$, a cyclin-cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. Genes Dev., *8:* 9–22, 1994.
22. Hannon, G. J., and Beach, D. $p15^{INK4B}$ is a potential effector of TGF-β-induced cell cycle arrest. Nature (Lond.), *371:* 257–261, 1994.
23. Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y., and Wang, X. F. Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc. Natl. Acad. Sci. USA, *92:* 5545–5549, 1995.
24. Reynisdottir, I., Polyak, K., Iavarone, A., and Massagué, J. Kip/Cip and Ink4 cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-β. Genes Dev., *9:* 1831–1845, 1995.
25. Ewen, M. E., Sluss, H. K., Whitehouse, L. L., and Livingston, D. M. TGFβ inhibition of cdk4 synthesis is linked to cell cycle arrest. Cell, *74:* 1009–1020, 1993.
26. Geng, Y., and Weinberg, R. A. Transforming growth factor-β effects on expression of G_1 cyclins and cyclin-dependent protein kinases. Proc. Natl. Acad. Sci. USA, *90:* 10315–10319, 1993.
27. Iavarone, A., and Massagué, J. Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-β in cells lacking the CDK inhibitor p15. Nature (Lond.), *387:* 417–422, 1997.
28. Roberts, A. B., Heine, U. I., Flanders, K. C., and Sporn, M. B. Transforming growth factor-β. Major role in regulation of extracellular matrix. Ann. N. Y. Acad. Sci., *580:* 225–232, 1990.
29. Garrigue-Antar, L., Munoz-Antonia, T., Antonia, S. J., Gesmonde, J., Vellucci, V. F., and Reiss, M. Missense mutations of the transforming growth factor β type II receptor in human head and neck squamous carcinoma cells. Cancer Res., *55:* 3982–3987, 1995.
30. Knaus, P. I., Lindemann, D., DeCoteau, J. F., Perlman, R., Yankelev, H., Hille, M., Kadin, M. E., and Lodish, H. F. A dominant inhibitory mutant of the type II transforming growth factor β receptor in the malignant progression of a cutaneous T-cell lymphoma. Mol. Cell. Biol., *16:* 3480–3489, 1996.
31. Lu, S., Zhang, W., Akiyama, Y., Nomizu, T., and Yuasa, Y. Genomic structure of the transforming growth factor β type II receptor gene and its mutations in hereditary nonpolyposis colorectal cancers. Cancer Res., *56:* 4595–4598, 1996.
32. Takenoshita, S., Tani, M., Nagashima, M., Hagiwara, K., Bennett, W. P., Yokota, J., and Harris, C. C. Mutation analysis of coding sequences of the entire transforming growth factor β type II receptor gene in sporadic human colon cancer using genomic DNA and intron primers. Oncogene, *14:* 1255–1258, 1997.
33. Eppert, K., Scherer, S. W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L-C., Bapat, B., Gallinger, S., Andrulis, I., Thomsen, G. H., Wrana, J. L., and Attisano, L. MADR2 maps to 18q21 and encodes a TGF-β MAD-related protein that is functionally mutated in colorectal carcinoma, Cell, *86:* 543–552, 1996.
34. Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science (Washington DC), *271:* 350–353, 1996.
35. Arai, T., Akiyama, Y., Okabe, S., Ando, M., Endo, M., and Yuasa, Y. Genomic structure of the human Smad3 gene and its infrequent alterations in colorectal cancers. Cancer Lett., in press, 1998.
36. Lu, S-L., Akiyama, Y., Nagasaki, H., Saitoh, K., and Yuasa, Y. Mutations of the transforming growth factor-β type II receptor gene and genomic instability in hereditary nonpolyposis colorectal cancer. Biochem. Biophys. Res. Commun., *216:* 452–457, 1995.
37. Hanks, S. K., and Quinn, A. M. Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Methods Enzymol., *200:* 38–81, 1991.
38. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.
39. Reiss, M., Pitman, S. W., and Sartorelli, A. C. Modulation of the terminal differentiation of human squamous carcinoma cells *in vitro* by all-*trans*-retinoic acid. J. Natl. Cancer Inst., *74:* 1015–1023, 1985.
40. Rangan, S. R. A new human cell line (FaDu) from a hypopharyngeal carcinoma. Cancer (Phila.), *29:* 117–121, 1972.
41. Keydar, I., Chen, L., Karby, S., Weiss, F. R., Delarea, J., Radu, M., Chaitcik, S., and Brenner, H. J. Establishment and characterization of a cell line of human breast carcinoma origin. Eur. J. Cancer, *15:* 659–670, 1979.
42. Laiho, M., Weis, F. M. B., Boyd, F. T., Ignotz, R. A., and Massagué, J. Responsiveness to transforming growth factor-β (TGFβ) restored by complementation between cells defective in TGFβ receptors I and II. J. Biol. Chem., *266:* 9108–9112, 1991.
43. Feng, X-H., Filvaroff, E. H., and Derynck, R. Transforming growth factor-β (TGFβ)-induced down-regulation of cyclin A expression requires a functional TGFβ receptor complex. J. Biol. Chem., *270:* 24237–24245, 1995.
44. Lawler, S., Candia, A. F., Ebner, R., Shum, L., Lopez, A. R., Moses, H. L., Wright, C. V., and Derynck, R. The murine type II TGF-β receptor has a coincident embryonic expression and binding preference for TGF-β 1. Development (Camb.), *120:* 165–175, 1994.
45. Kalkhoven, E., Roelen, B. A. J., de Winter, J. P., Mummery, C. L., van den Eijnden-van Raaij, A. J. M., van der Saag, P. T., and van der Burg, B. Resistance to transforming growth factor β and activin due to reduced receptor expression in human breast tumor cell lines. Cell Growth Differ., *6:* 1151–1156, 1995.
46. Wrana, J. L., Attisano, L., Cárcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X-F., and Massagué, J. TGFβ signals through a heteromeric protein kinase receptor complex. Cell, *71:* 1003–1014, 1992.
47. Reiss, M., Santoro, V., De Jonge, R. R., and Vellucci, V. F. Transfer of chromosome 18 into human head and neck squamous carcinoma cells: evidence for tumor suppression by Smad4/DPC4. Cell Growth Differ., *8:* 407–415, 1997.
48. Geiser, A. G., Burmester, J. K., Webbink, R., Roberts, A. B., and Sporn, M. B. Inhibition of growth by transforming growth factor-β following fusion of two nonresponsive human carcinoma cell lines. J. Biol. Chem., *267:* 2588–2593, 1992.
49. Sankar, S., Mahooti-Brooks, N., Bensen, L., McCarthy, T. L., Centrella, M., and Madri, J. A. Modulation of transforming growth factor β receptor levels on microvascular endothelial cells during *in vitro* angiogenesis. J. Clin. Invest., *97:* 1436–1446, 1996.
50. Ruoslahti, E. Fibronectin and its $\alpha_5\beta_1$ integrin receptor in malignancy. Invasion Metastasis, *14:* 87–97, 1994.
51. Satterwhite, D. J., Aakre, M. E., Gorska, A. E., and Moses, H. L. Inhibition of cell growth by TGFβ1 is associated with inhibition of B-*myb* and *cyclin A* in both BALB/MK and Mv1Lu cells. Cell Growth Differ., *5:* 789–799, 1994.
52. Ventura, F., Doody, J., Liu, F., Wrana, J. L., and Massagué, J. Reconstitution and transphosphorylation of TGF-β receptor complexes. EMBO J., *13:* 5581–5589, 1994.
53. Chen, F., and Weinberg, R. A. Biochemical evidence for the autophosphorylation and transphosphorylation of transforming growth factor β receptor kinases. Proc. Natl. Acad. Sci. USA, *92:* 1565–1569, 1995.
54. Vivien, D., Attisano, L., Wrana, J. L., and Massagué, J. Signaling activity of homologous and heterologous transforming growth factor-β receptor kinase complexes. J. Biol. Chem., *270:* 7134–7141, 1995.
55. Feng, X. H., and Derynck, R. Ligand-independent activation of transforming growth factor (TGF) β signaling pathways by heteromeric cytoplasmic domains of TGF-β receptors. J. Biol. Chem., *271:* 13123–13129, 1996.
56. Kalkhoven, E., Kwakkenbos-Isbrucker, L., Mummery, C. L., deLaat, S. W., van den Eijnden-van Raaij, A. J. M., van der Saag, P. T., and van der Burg, B. The role of TGF-β production in growth inhibition of breast tumor cells by progestins. Int. J. Cancer, *61:* 80–86, 1995.
57. Rahimi, N., Tremblay, E., McAdam, L., Park, M., Schwall, R., and Elliott, B. Identification of a hepatocyte growth factor autocrine loop in a murine mammary carcinoma. Cell Growth Differ., *7:* 263–270, 1996.
58. Chen, R-H., Ebner, R., and Derynck, R. Inactivation of the type II receptor reveals two receptor pathways for the diverse TGFβ activities. Science (Washington DC), *260:* 1335–1338, 1993.

59. Wieser, R., Attisano, L., Wrana, J. L., and Massagué, J. Signaling activity of transforming growth factor β type II receptors lacking specific domains in the cytoplasmic region. Mol. Cell. Biol., *13:* 7239–7247, 1993.
60. Carcamo, J., Zentella, A., and Massagué, J. Disruption of transforming growth factor β signaling by a mutation that prevents transphosphorylation within the receptor complex. Mol. Cell. Biol., *15:* 1573–1581, 1995.
61. Brand, T., and Schneider, M. D. Inactive type II and type I receptors for TGFβ are dominant inhibitors of TGFβ-dependent transcription. J. Biol. Chem., *270:* 8274–8284, 1995.
62. Choi, M. E., and Ballerman, B. J. Inhibition of capillary morphogenesis and associated apoptosis by dominant negative mutant transforming growth factor-β receptors. J. Biol. Chem., *270:* 21144–21150, 1995.
63. Yamamoto, H., Ueno, H., Ooshima, A., and Takeshita, A. Adenovirus-mediated transfer of a truncated transforming growth factor-β (TGFβ) type II receptor completely and specifically abolishes diverse signaling by TGFβ in vascular wall cells in primary culture. J. Biol. Chem., *271:* 16253–16259, 1996.
64. Zhao, J., Bu, D., Lee, M., Slavkin, H. C., Hall, F. L., and Warburton, D. Abrogation of transforming growth factor-β type II receptor stimulates embryonic mouse lung branching morphogenesis in culture. Dev. Biol., *180:* 242–257, 1996.
65. Fan, D., Chakrabarty, S., Seid, C., Bell, C. W., Schackert, H., Morikawa, K., and Fidler, I. J. Clonal stimulation of human colon carcinomas and human renal carcinomas mediated by TGFβ1. Cancer Commun., *1:* 117–125, 1989.
66. Hsu, S., Huang, F., Hafez, M., Winawer, S., and Friedman, E. Colon carcinoma cells switch their response to transforming growth factor β1 with tumor progression. Cell Growth Differ., *5:* 267–275, 1994.
67. Yan, Z. F., Winawer, S., and Friedman, E. Two different signal transduction pathways can be activated by transforming growth factor β1 in epithelial cells. J. Biol. Chem., *269:* 13231–13237, 1994.
68. Taylor, S. S., Knighton, D. R., Zheng, J., Ten Eyck, L. F., and Sowadski, J. M. Structural framework for the protein kinase family. Annu. Rev. Cell Biol., *8:* 429–462, 1992.
69. Zhang, F., Strand, A., Robbins, D., Cobb, M. H., and Goldsmith, E. J. Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution. Nature (Lond.), *367:* 704–711, 1994.
70. Luo, K., and Lodish, H. F. Positive and negative regulation of type II TGF-β receptor signal transduction by autophosphorylation on multiple serine residues. EMBO J., *16:* 1970–1981, 1997.
71. Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., Brattain, M., and Willson, J. K. V. Inactivation of the type II TGFβ receptor in colon cancer cells with microsatellite instability. Science (Washington DC), *268:* 1336–1338, 1995.
72. Lin, H. Y., Moustakas, A., Knaus, P., Wells, R. G., Henis, Y. I., and Lodish, H. F. The soluble exoplasmic domain of the type II transforming growth factor (TGFβ) receptor. J. Biol. Chem., *270:* 2747–2754, 1995.
73. Wang, J., Sun, L., Myeroff, L., Wang, X., Gentry, L. E., Yang, J., Liang, J., Zborowska, E., Markowitz, S., Willson, J. K. V., and Brattain, M. G. Demonstration that mutation of the type II transforming growth factor β receptor inactivates its tumor suppressor activity in replication error-positive colon carcinoma cells. J. Biol. Chem., *270:* 22044–22049, 1995.

GENOMICS 46, 278–283 (1997)
ARTICLE NO. GE975023

Cloning and Genomic Organization of the Human Transforming Growth Factor-β Type I Receptor Gene

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Received June 20, 1997; accepted September 10, 1997

Transforming growth factor-β (TGFβ) regulates cell cycle progression by a unique signaling mechanism that involves its binding to the type II (TβR-II) TGFβ receptor and activation of type I (TβR-I). Both are transmembrane serine-threonine receptor kinases. As various types of human tumor cells are often refractory to TGFβ-mediated cell cycle arrest, it is likely that the TβR-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-β (TGFβ) 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 TGFβ 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 TGFβ 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 TβR-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 TGFβ signaling are primarily mediated by members of the *D. melanogaster mothers-against-dpp* (MAD) superfamily of proteins (Derynck and Zhang, 1996). In response to TGFβ binding, two of these proteins, Smad2 and Smad3, become transiently associated with the activated TβR-I receptor and are phosphorylated, presumably by the TβR-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 TGFβ-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).

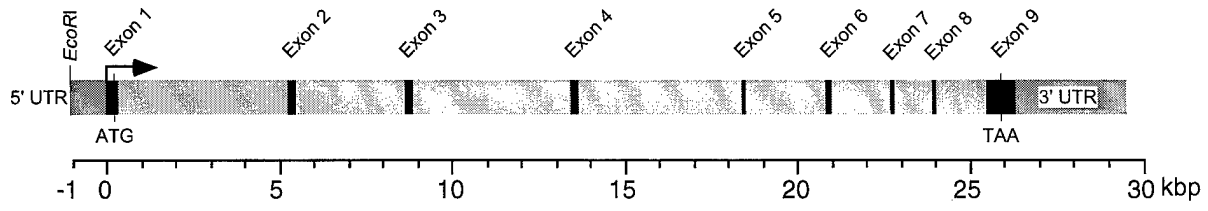
Human malignant neoplastic cells are frequently refractory to TGFβ-mediated cell cycle arrest (Fynan and Reiss, 1993). In principle, resistance may be caused by functional inactivation of any of the essential signaling intermediates. Thus far, structural alterations including homozygous deletions, mutations, 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

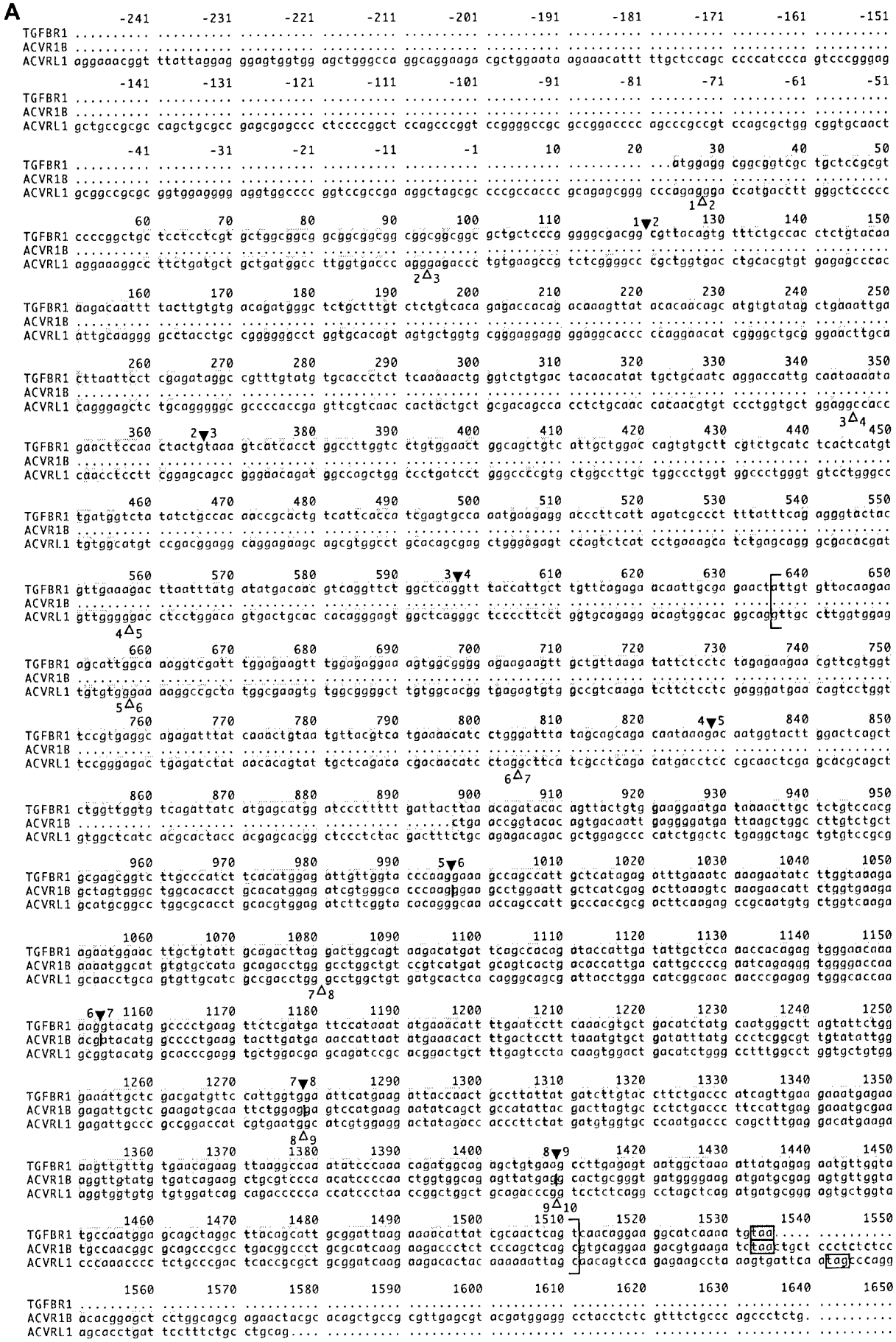
Exon	3′ Splice acceptor	5′ End of exon	Exon size (bp)	3′ End of exon	5′ Splice donor	Estimated intron size (bp)
1		TGCCTGGCGG[1]	329	GGGGCGACGG	gtgagcggcg	4,900
2	ctttttccag	CGTTACAGTG	246	CCAACTACTG	gtaagttgta	3,200
3	ccttttttcag	TAAAGTCATC	231	TCTGGCTCAG	gtaacataat	4,550
4	ttacctttag	GTTTACCATT	231	GACAATAAAG	gtctgtaaca	4,700
5	tgttttacag	ACAATGGTAC	168	GGTACCCAAG	gtaattctat	2,300
6	gattctttag	GAAAGCCAGC	157	GAACAAAAAG	gtatactttt	1,700
7	tttttttttag	GTACATGGCC	125	TCCATTGGTG	gtaaattgct	1,100
8	ctgatacag	GAATTCATGA	131	GAGCTGTGAA	gtgagtattt	1,400
9	tttcttgtag	GCCTTGAGAG	≥ 845			

B



[1] 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.


A
TGFBR1
ACVR1B
ACVRL1

B

```
                  10         20         30         40         50
TGFBR1     1 ISEGTTLKDL IYDMTTSGSG SGLPLLVQRT IARTIVLQES IGKGRFGEVW 50
ACVR1B     1 LSKDKTLQDL VYDLSTSGSG SGLPLFVQRT VARTIVLQEI IGKGRFGEVW 50
BMPR1A     1 IPVGESLKDL IDQSQSSGSG SGLPLLVQRT IAKQIQMVRQ VGKGRYGEVW 50
BMPR1B     1 IPPGESLRDL IEQSQSSGSG SGLPLLVQRT IAKQIQMVKQ IGKGRYGEAR 50
ACVRL1     1 EQGDTMLGDL LDSDCTTGSG SGLPFLVQRT VARQVALVEC VGKGRYGEVW 50

                  60         70         80         90         100
TGFBR1    51 RGKWRGEEVA VKIFSSREER SWFREAEIYQ TVMLRHENIL GFIAADNKDN 100
ACVR1B    51 RGRWRGGDVA VKIFSSREER SWFREAEIYQ TVMLRHENIL GFIAADNKDN 100
BMPR1A    51 MGKWRGEKVA VKVFFTTEEA SWFRETEIYQ TVLMRHENIL GFIAADIKGT 100
BMPR1B    51 MGKWRGEKVA VKVFFTTEEA SWFRETEIYQ TVLMRHENIL GFIAADIKGT 100
ACVRL1    51 RGLWHGESVA VKIFSSRDEQ SWFRETEIYN TVLLRHDNIL GFIASDMTSR 100

                  110        120        130        140        150
TGFBR1   101 GTWTQLWLVS DYHEHGSLFD YLNRYTVTVE GMIKLALSTA SGLAHLHMEI 150
ACVR1B   101 GTWTQLWLVS DYHEHGSLFD YLNRYTVTIE GMIKLALSAA SGLAHLHMEI 150
BMPR1A   101 GSWTQLYLIT DYHENGSLYD FLKCATLDTR ALLKLAYSAA CGLCHLHTEI 150
BMPR1B   101 GSWTQLYLIT DYHENGSLYD YLKSTTLDAK SMLKLAYSSV SGLCHLHTEI 150
ACVRL1   101 NSSTQLWLIT HYHEHGSLYD FLQRQTLEPH LALRLAVSAA CGLAHLHVEI 150

                  160        170        180        190        200
TGFBR1   151 VGTQGKPAIA HRDLKSKNIL VKKNGTCCIA DLGLAVRHDS ATDTIDIAPN 200
ACVR1B   151 VGTQGKPGIA HRDLKSKNIL VKKNGMCAIA DLGLAVRHDA VTDTIDIAPN 200
BMPR1A   151 YGTQGKPAIA HRDLKSKNIL IKKNGSCCIA DLGLAVKFNS DTNEVDVPLN 200
BMPR1B   151 FSTQGKPAIA HRDLKSKNIL VKKNGTCCIA DLGLAVKFIS DTNEVDIPPN 200
ACVRL1   151 FGTQGKPAIA HRDFKSRNVL VKSNLQCCIA DLGLAVMHSQ GSDYLDIGNN 200

                  210        220        230        240        250
TGFBR1   201 HRVGTKRYMA PEVLDDSINM KHFESFKRAD IYAMGLVFWE IARRCSIGGI 250
ACVR1B   201 QRVGTKRYMA PEVLDETINM KHFDSFKCAD IYALGLVYWE IARRCNSGGV 250
BMPR1A   201 TRVGTKRYMA PEVLDESLNK NHFQPYIMAD IYSFGLIIWE MARRCITGGI 250
BMPR1B   201 TRVGTKRYMP PEVLDESLNR NHFQSYIMAD MYSFGLILWE VARRCVSGGI 250
ACVRL1   201 PRVGTKRYMA PEVLDEQIRT DCFESYKWTD IWAFGLVLWE IARRTIVNGI 250

                  260        270        280        290        300
TGFBR1   251 HEDYQLPYYD LVPSDPSVEE MRKVVCEQKL RPNIPNRWQS CEALRVMAKI 300
ACVR1B   251 HEEYQLPYYD LVPSDPSIEE MRKVVCDQKL RPNIPNWWQS YEALRVMGKM 300
BMPR1A   251 VEEYQLPYYN MVPSDPSYED MREVVCVKRL RPIVSNRWNS DECLRAVLKL 300
BMPR1B   251 VEEYQLPYHD LVPSDPSYED MREIVCIKKL RPSFPNRWSS DECLRQMGKL 300
ACVRL1   251 VEDYRPPFYD VVPNDPSFED MKKVVCVDQQ TPTIPNRLAA DPVLSGLAQM 300

                  310        320        330        340        350
TGFBR1   301 MRECWYANGA ARLTALRIKK TLSQLSQQEG IKM..... ..........  350
ACVR1B   301 MRECWYANGA ARLTALRIKK TLSQLSVQED VKI..... ..........  350
BMPR1A   301 MSECWAHNPA SRLTALRIKK TLAKMVESQD VKI..... ..........  350
BMPR1B   301 MTECWAHNPA SRLTALRVKK TLAKMSESQD IKL..... ..........  350
ACVRL1   301 MRECWYPNPS ARLTALRIKK TLQKISNSPE KPKVIQ.... ..........  350
```

FIG. 2. TGFβ superfamily protein type I receptor genes. (**A**) Comparison of the locations of introns (▼) within the *TGFBR1* gene with those reported for the human *ACVR1B* (*ALK-4, SKR2*) (|) and *ACVRL1* (*ALK-1, TSR1*) (△) 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 TGFβ, 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).*TGFBR1* (*ALK-5,* Ten Dijke *et al.,* 1993); *ACVR1B* (Carcamo *et al.,* 1994) (*ALK-4,* Ten Dijke *et al.,* 1993; *SKR2,* Xu *et al.,* 1994); *BMPR1A* (*ALK-3,* Ten Dijke *et al.,* 1993); *BMPR1B* (*ALK-6,* Ten Dijke *et al.,* 1994a,b); *ACVRL1* (*ALK-1,* Ten Dijke *et al.,* 1993; *TSR-I,* 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 *TGFBR1* 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 μg/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 10,000*g* for 10 min, the supernatant was carefully removed and incubated in the presence of 50 μg/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 μg of BAC DNA can be harvested from a 30-ml culture.

Determination of the TGFBR1 gene exon–intron organization. The genomic organization of the *TGFBR1* 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 μg of ethanol-precipitated BAC DNA was resuspended in 5 μl water. Sequencing primers (27 nucleotides in length) were end-labeled with [γ-^{32}P]ATP (DuPont/NEN; 6000 Ci/mmol) using T4 polynucleotide kinase. BAC DNA was then subjected to manual cycle sequencing using 1.5 μl 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 Kodax 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 TGFβ 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).

ACKNOWLEDGMENTS

The authors express their gratitude to Dr. Michael Centrella (Dept. of Surgery, Yale Univ. School of Medicine) for the *ALK-5* cDNA probe and to Dr. Taiping Chen for many helpful discussions and suggestions. This work was supported, in part, by U.S. Public Health Service Grant CA41556 from the National Cancer Institute and U.S. Dept. of Defense Breast Cancer Research Program Grant DAMD17-96-1-6024.

REFERENCES

Anders, R. A., and Leof, E. B. (1996). Chimeric granulocyte/macrophage colony-stimulating factor transforming growth factor-beta (TGF-beta) receptors define a model system for investigating the role of homomeric and heteromeric receptors in TGF-beta signaling. *J. Biol. Chem.* **271:** 21758–21766.

Baldwin, R. L., Friess, H., Yokoyama, M., Lopez, M. E., Kobrin, M. S., Buchler, M. W., and Korc, M. (1996). Attenuated ALK5 receptor expression in human pancreatic cancer: Correlation with resistance to growth inhibition. *Int. J. Cancer* **67:** 283–288.

Bloom, B. B., Humphries, D. E., Kuang, P.-P., Fine, A., and Goldstein, R. H. (1996). Structure and expression of the promoter

for the R4/ALK5 human type I transforming growth factor-β receptor: Regulation by TGFβ. *Biochim. Biophys. Acta* **1312:** 243–248.

Carcamo, J., Weis, F. M., Ventura, F., Wieser, R., Wrana, J. L., Attisano, L., and Massague, J. (1994). Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor beta and activin. *Mol. Cell. Biol.* **14:** 3810–3821.

Chen, R.-H., and Derynck, R. (1994). Homomeric interactions between type II Transforming Growth Factor-β receptors. *J. Biol. Chem.* **269:** 22868–22874.

Chen, X., Rubock, M. J., and Whitman, M. (1996). A transcriptional partner for MAD proteins in TGFβ signaling. *Nature* **383:** 691–696.

Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y., and Wang, X. F. (1995). Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc. Natl. Acad. Sci. USA* **92:** 5545–5549.

De Jonge, R. R., Garrigue-Antar, L., Vellucci, V. F., and Reiss, M. (1997). Frequent inactivation of the transforming growth factor type II receptor in small cell lung carcinoma cells. *Oncol. Res.* **9:** 89–98.

Derynck, R., and Zhang, Y. (1996). Intracellular signalling: The Mad way to do it. *Curr. Biol.* **6:** 1226–1229.

Eppert, K., Scherer, S. W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L.-C., Bapat, B., Gallinger, S., Andrulis, I., Thomsen, G. H., Wrana, J. L., and Attisano, L. (1996). MADR2 maps to 18q21 and encodes a TGF-β MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* **86:** 543–552.

Franzén, P., Ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C.-H., and Miyazono, K. (1993). Cloning of a TGFβ type I receptor that forms a heterodimeric complex with the TGFβ type II receptor. *Cell* **75:** 681–692.

Fynan, T. M., and Reiss, M. (1993). Resistance to inhibition of cell growth by Transforming Growth Factor-β and its role in oncogenesis. *Crit. Rev. Oncogenesis* **4:** 493–540.

Garrigue-Antar, L., Munoz-Antonia, T., Antonia, S. J., Gesmonde, J., Vellucci, V. F., and Reiss, M. (1995). Missense mutations of the transforming growth factor beta type II receptor in human head and neck squamous carcinoma cells. *Cancer Res.* **55:** 3982–3987.

Garrigue-Antar, L., Souza, R. F., Vellucci, V. F., Meltzer, S. J., and Reiss, M. (1996). Loss of transforming growth factor-β type II receptor gene expression in human esophageal cancer. *Lab. Invest.* **75:** 263–272.

Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* **271:** 350–353. [See comments]

Hannon, G. J., and Beach, D. (1994). $p15^{INK4B}$ is a potential effector of TGF-β-induced cell cycle arrest. *Nature* **371:** 257–261.

Johnson, D. W., Berg, J. N., Baldwin, M. A., Gallione, C. J., Marondel, I., Yoon, S. J., Stenzel, T. T., Speer, M., Pericak-Vance, M. A., Diamond, A., Guttmacher, A. E., Jackson, C. E., Attisano, L., Kucherlapati, R., Porteous, M. E., and Marchuk, D. A. (1996). Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. *Nature. Genet.* **13:** 189–195.

Kim, I. Y., Ahn, H. J., Zelner, D. J., Shaw, J. W., Sensibar, J. A., Kim, J. H., Kato, M., and Lee, C. (1996). Genetic change in transforming growth factor beta (TGF-beta) receptor type I gene correlates with insensitivity to TGF-beta 1 in human prostate cancer cells. *Cancer Res.* **56:** 44–48.

Lagna, G., Hata, A., Hemmati-Brivanlou, A., and Massague, J. (1996). Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways. *Nature* **383:** 832–836.

Liu, F., Hata, A., Baker, J. C., Doody, J., Carcamo, J., Harland, R. M., and Massague, J. (1996). A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature* **381:** 620–622.

Luo, K., and Lodish, H. F. (1996). Signaling by chimeric erythropoietin-TGF-beta receptors: Homodimerization of the cytoplasmic domain of the type I TGF-beta receptor and heterodimerization with the type II receptor are both required for intracellular signal transduction. *EMBO J.* **15:** 4485–4496.

Macias-Silva, M., Abdollah, S., Hoodless, P. A., Pirone, R., Attisano, L., and Wrana, J. L. (1996). MADR2 is a substrate of the TGF-β receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* **87:** 1215–1224.

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., Brattain, M., and Willson, J. K. V. (1995). Inactivation of the type II TGFβ receptor in colon cancer cells with microsatellite instability. *Science* **268:** 1336–1338.

Massagué, J. (1996). TGF-β Signaling: Receptors, transducers, and MAD proteins. *Cell* **85:** 947–950.

Massagué, J., Attisano, L., and Wrana, J. L. (1994). The TGFβ family and its composite receptors. *Trends Cell Biol.* **4:** 172–178.

McAllister, K. A., Baldwin, M. A., Thukkani, A. K., Gallione, C. J., Berg, J. N., Porteous, M. E., Guttmacher, A. E., and Marchuk, D. A. (1995). Six novel mutations in the endoglin gene in hereditary hemorrhagic telangiectasia type 1 suggest a dominant-negative effect of receptor function. *Hum. Mol. Genet.* **4:** 1983–1985.

Park, K., Kim, S.-J., Bang, Y.-J., Park, J.-G., Kim, N. K., Roberts, A. B., and Sporn, M. B. (1994). Genetic changes in the transforming growth factor β (TGFβ) type II receptor gene in human gastric cancer cells: Correlation with sensitivity to TGFβ. *Proc. Natl. Acad. Sci. USA* **91:** 8772–8776.

Roberts, A. B., and Sporn, M. B. (1993). Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). *Growth Factors* **8:** 1–9.

Shizuya, H., Birren, B., Kim, U.-J., Mancino, V., Slepak, T., Tachiiri, Y., and Simon, M. (1992). Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc. Natl. Acad. Sci. USA* **89:** 8794–8797.

Wieser, R., Attisano, L., Wrana, J. L., and Massagué, J. (1993). Signaling activity of Transforming Growth Factor β type II receptors lacking specific domains in the cytoplasmic region. *Mol. Cell. Biol.* **13:** 7239–7247.

Wieser, R., Wrana, J. L., and Massague, J. (1995). GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. *EMBO J.* **14:** 2199–2208.

Wrana, J. L., Attisano, L., Cárcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.-F., and Massagué, J. (1992). TGFβ signals through a heteromeric protein kinase receptor complex. *Cell* **71:** 1003–1014.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massagué, J. (1994). Mechanism of activation of the TGFβ receptor. *Nature* **370:** 341–347.

Xu, J., Matsuzaki, K., McKeehan, K., Wang, F., Kan, M., and McKeehan, W. L. (1994). Genomic structure and cloned cDNAs predict that four variants in the kinase domain of serine/threonine kinase receptors arise by alternative splicing and poly(A) addition. *Proc. Natl. Acad. Sci. USA* **91:** 7957–7961.

Zhang, Y., Feng, X.-H., Wu, R.-Y., and Derynck, R. (1996). Receptor-associated Mad homologues synergize as effectors of the TGF-β response. *Nature* **383:** 168–172.