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Dominant Negative Mutants of Transforming Growth Factor-β1 Inhibit the Secretion of Different Transforming Growth Factor-β Isoforms

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Transforming growth factor-β (TGF-β) is a secreted polypeptide factor that is thought to play a major role in the regulation of proliferation of many cell types and various differentiation processes. Several related isoforms have been structurally characterized, three of which, TGF-β1, -β2, and -β3, have been detected in mammalian cells and tissues. Each TGF-β form is a homodimer of a 112-amino-acid polypeptide which is encoded as a larger polypeptide precursor. We have introduced several mutations in the TGF-β1 precursor domain, resulting in an inhibition of TGF-β1 secretion. Coexpression of these mutants with wild-type TGF-β1, -β2, and -β3 results in a competitive and specific inhibition of the secretion of different TFG-β forms, indicating that these mutated versions act as dominant negative mutants for TGF-β secretion. Overexpression of dominant negative mutants can thus be used to abolish endogenous secretion of TGF-β and structurally related family members, both in vitro and in vivo, and to probe in this way the physiological functions of the members of the TGF-β superfamily.

Transforming growth factor-β (TGF-β) is a growth and differentiation factor that is secreted by a large variety of cells and modulates the growth of many cell types (for reviews, see references 24 and 33). While TGF-β stimulates the proliferation of selected cell types, mostly of mesenchymal origin, it inhibits the proliferation of many cell types, especially hematopoietic cells, epithelial cells, and endothelial cells. Besides its action as a growth factor, TGF-β is also a potent chemotactic factor for macrophages and fibroblasts. In addition, TGF-β is able to induce the synthesis of various extracellular matrix proteins, decrease the synthesis of matrix-degrading enzymes, and increase the synthesis of cell adhesion receptors, thus resulting in an increased adhesion of the cells with the matrix. Finally, experimental evidence suggests that TGF-β plays an important role in mediating and directing cellular differentiation. Despite this complex array of biological activities, largely established on the basis of cell culture data, the physiological role of TGF-β, especially in vivo, is still ill defined. The documentation of the physiological role of this factor is further compounded by the recent realization that there exist several structurally closely related isoforms of TGF-β, of which at least three (TGF-β1, -β2, and -β3) are expressed by mammalian tissues and cells (7, 18, 37). These three TGF-β species each have a distinct spatial and temporal expression pattern during development, and their expression is differentially controlled (9, 30, 31, 33). Various other polypeptide factors, including the bone morphogenic proteins, the activins, and certain other proteins, are structurally related to TGF-β and form, together with the different TGF-β forms, the TGF-β superfamily (5, 6, 20, 21, 23, 24, 33, 39). Very little is as yet known about the physiological role of most of these TGF-β-related factors, although it is believed that most TGF-β superfamily members play a role in cell-matrix interaction and in differentiation. An exploration of the physiological role of these TGF-β related factors would obviously be aided if it would be possible to specifically abolish their functional expression by cells that produce these factors.

All members of the TGF-β superfamily are dimers, usually homodimers, in their mature, fully processed form. The monomeric mature polypeptide is 110 to 130 amino acids long and is derived as the C-terminal segment of a 390- to 500-amino-acid precursor polypeptide. The structural relationship between the different TGF-β superfamily members is most obvious in the C-terminal mature sequence, in which the positions of seven cysteines are conserved in all of these factors (5, 6, 20, 21, 23, 24, 33, 39). In the specific case of the TGF-β isoforms, the mature monomer sequences are 112 amino acids long and their structural identity of 75 to 85% includes nine cysteine residues. The remainder of the precursor polypeptides, the proregions, are only about 25% identical, but there are highly conserved regions, including N-glycosylation sites, cysteines, and other short sequences (7, 18, 37). Biochemical analysis has shown that TGF-β, the disulfide-linked homodimer of the C-terminal precursor segment, is secreted by the cells in a complex consisting of the active TGF-β dimer in a noncovalent interaction with two pro-region polypeptides (10, 26, 27). This complex formation results in an inactivation of the mature TGF-β, thus rendering the complex latent. This interaction with the pro regions is necessary for the secretion of the TGF-β dimer, indicating that the pro regions act as chaperones in the secretion process of TGF-β (14). Similarly to other chaperones, these

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pro regions may also be needed for inducing the proper conformation of the TGF-β dimer. In analogy with TGF-β, we can assume that the pro regions of the other TGF-β related factors also play an obligatory role as binding proteins needed for secretion of the properly folded factor.

To define the physiological roles of TGF-β and the TGF-β-related factors in vitro and in vivo, it would be advantageous to abolish TGF-β, e.g., by using antisense RNA techniques (17) or by gene targeting (22, 36). Although these approaches may be feasible, no successful experiments resulting in an efficient inhibition of TGF-β synthesis using overexpression of antisense RNA have been reported. In addition, the abolition of synthesis of the protein by using specific gene targeting is quite complex and necessitates the use of several rounds of gene targeting when applied to cells in culture. Moreover, the interpretation of such abolition experiments may be quite difficult because of a possible compensation effect by other TGF-β species when the synthesis of only one specific TGF-β species is blocked. An alternative approach would be to block the generation of active molecules by using dominant mutants that block function. Such an approach with dominant negative mutants, originally proposed by Herskowitz (16), could in principle be used in various systems in which interaction of several protein entities is needed to obtain a functional complex. In the case of secreted growth and differentiation factors, this approach could be used at the level of receptors and ligands. In the former case, dominant negative mutants have been used to inactivate the function of dimeric receptors. This approach has been used to suppress the biological response through receptors for epidermal growth factor (19), platelet-derived growth factor (38), and fibroblast growth factor (1). In the latter case, dominant negative mutants have been developed only for platelet-derived growth factor (25). These mutants had an abolished proteolytic cleavage site in the precursor, thus preventing release of the mature growth factor and, as a consequence, functional ligand-induced activation of the receptor. Since TGF-β and related factors constitute dimers in their active forms, the dominant negative approach could in principle be used for these proteins.

We have explored the effects of several specific mutations in the proregion of the TGF-β precursor on the secretion of TGF-β and found that some mutations blocked the secretion of TGF-β. Overexpression of these mutants strongly inhibited the secretion of different TGF-β forms derived from their unmodified precursor, indicating that they act as dominant negative mutants. These dominant negative mutants can be used to block the secretion of the endogenous TGF-β, both in cultured cells and in vivo. A similar approach of suppression by overexpression of dominant negative mutants could be designed and used to abolish the functional synthesis of other members of the TGF-β superfamily.

MATERIALS AND METHODS

Site-directed mutagenesis. Site-directed mutagenesis was performed as described previously (40) on single-stranded M13mp18 derivatives containing the TGF-β1 precursor encoding *Bam*HI-*Hin*dIII restriction fragment from plasmid pRK5-β1E (2). Following mutagenesis, the mutated sequences were verified by dideoxynucleotide sequencing (35). The oligonucleotides for mutagenesis incorporated the modified codons flanked at both sides by a 12-nucleotide-long sequence complementary to the single-stranded template. The mutations in the cDNA gave rise to the following mutations in the translated TGF-β precursor. The charged sequence which is identical in all three human TGF-β precursors (7, 37), i.e., residues 42 to 63 (7), was removed in the ΔRK mutant. The ΔR mutant had only the first four amino acids of this same sequence removed. The RKKR cleavage site preceding the TGF-β1 mature monomer sequence is replaced by the sequence QNQG in the Mut S mutation. The Asn residues that are normally N glycosylated are replaced by Gln residues in mutants ΔN1 (residue 82), ΔN2 (residue 136), and ΔN1N2 (residues 82 and 136). The specifically mutated cDNAs were then introduced into the expression vector pRK5 (15) such that the resulting expression vector was identical to plasmid pRK5-β1E except for the mutated codons. The expression plasmid pRK5-β1E contains the unmodified TGF-β1 precursor cDNA under the control of the human cytomegalovirus promoter (2).

Transfections. The human embryonic kidney cell line 293S (13) was maintained in F12 Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 20 μM glutamine. Transfections were carried out by the calcium phosphate precipitation technique as previously described (11). Briefly, 293S cells were seeded 2 to 3 days prior to transfection and allowed to grow to reach 60 to 70% confluency in 60-mm dishes. Cells were then transfected with the plasmid DNA of interest, and the precipitate was allowed to incubate at 37°C for 6 h in a 7% CO_2 humidified incubator. The cells were exposed to a 15% glycerol shock for 45 s at room temperature and then placed in DMEM for 48 h. Initial experiments were done without the addition of a simian virus 40 T-antigen expression plasmid. Later experiments were modified to include 2.5 μg of an expression vector encoding the simian virus 40 T antigen under the transcriptional control of the Rous sarcoma virus promoter (12).

Metabolic labeling and immunoprecipitation. Transfected 293S cells were allowed to grow for 48 h in DMEM, washed twice, and labeled overnight in 1 ml of cysteine- and methionine-free serum-free DMEM containing [^{35}S cysteine and [^{35}S]methionine (100 μCi/ml each). The conditioned medium was then collected and clarified by centrifugation. The cells were rinsed twice with phosphate-buffered saline and lysed in 1 ml of lysis buffer (20 mM Tris-HCl [pH 7.5], 10 mM ethanolamine, 10 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 10 μg of leupeptin per ml, 2 μg of aprotinin per ml) and spun for 10 min. Lysates and secreted proteins were stored at −20°C until use. At the time of analysis, 100-μl aliquots of the secreted proteins in the conditioned medium were incubated overnight at 4°C with mouse monoclonal antibody 4A11, which was raised against recombinant human TGF-β1 but recognizes all three TGF-β types. Immunoprecipitations of the antigen-antibody complexes were then done by using protein A-Sepharose (Pharmacia) or Tachisorb (CalBiochem). The immunoprecipitates were resuspended, denatured, reduced in 2× Laemmli loading buffer (125 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% mercaptoethanol), and electrophoresed in SDS–15% polyacrylamide gels. Gels were permeated with Amplify (Amersham) and exposed for autoradiography at −70°C.

RESULTS

Lack of secretion of TGF-β mutants. Various site-specific mutations were introduced in the cDNA encoding the 390-amino-acid TGF-β1 precursor in order to modify sequences

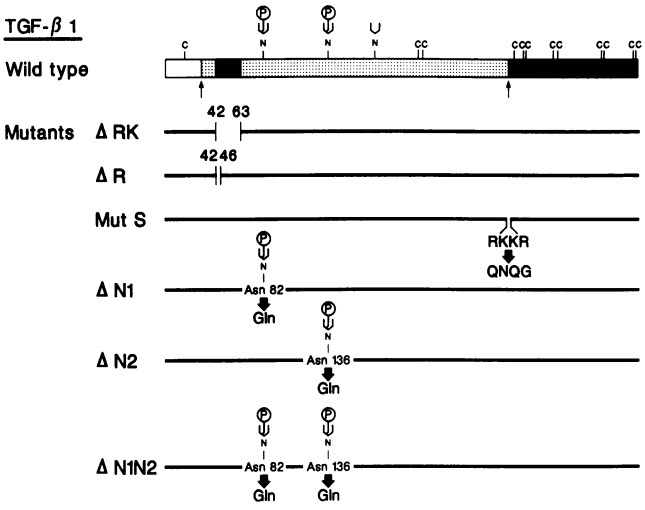

FIG. 1. Schematic representation of the TGF-β precursor and the introduced mutations. The mature TGF-β monomer with its nine cysteine residues (C) corresponds to the C-terminal segment of the precursor and is proteolytically cleaved from the precursor (arrow). Another cleavage site follows the signal peptide. The precursor segment has three Asn-based N-linked carbohydrates, two of which are mannose-6-phosphorylated (P). The striated section of the precursor segment is a charged sequence (residues 42 to 63) which is identical in TGF-β1, -β2, and -β3. This sequence is either entirely or partially removed in the ΔRK or ΔR mutation, respectively. The RKKR cleavage site preceding the TGF-β1 mature monomer sequence is replaced with the sequence QNQG in the Mut S mutation. The Asn residues, which normally carry the N-linked carbohydrate groups, are replaced by Gln residues in mutants ΔN1, ΔN2, and ΔN1N2. The expression plasmid encoding the unmodified TGF-β1 precursor sequence under the control of the human cytomegalovirus promoter is named pRK5-β1E.

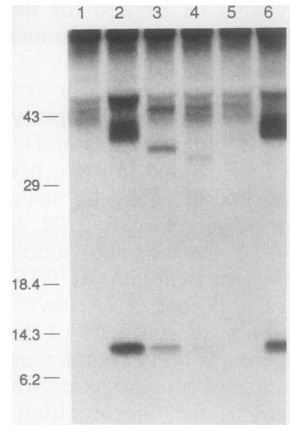

FIG. 2. Immunoprecipitations of secreted TGF-β encoded by expression vectors containing various mutations in the TGF-β precursor cDNAs. Transfected 293S cells were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine (100 μCi/ml) for 18 h, and conditioned medium was immunoprecipitated with the TGF-β-specific monoclonal antibody 4A11. Aliquots were fractionated on a denaturing 15% polyacrylamide under reducing conditions, and fluorography was performed. Lanes: 1, pRK5-β1E ΔR; 2, pRK5-β1E ΔN1; 3, pRK5-β1E ΔN2; 4, pRK5-β1E ΔN1N2; 5, pRK5-β1E ΔRK; 6, pRK5-β1E C2S2. Molecular weight markers are indicated in kilodaltons at the left. Plasmid pRK5-β1E C2S2 is identical to pRK-5-β1E except that the cysteines in the precursor at positions 223 and 225 are replaced by serines (2).

encoding structural features in the precursor which are highly conserved in the TGF-β1, -β2, and -β3 species (Fig. 1). One mutation (Mut S) removes in the precursor polypeptide the tetrabasic cleavage site (amino acids 275 to 281) preceding the mature TGF-β1 sequence. A second mutation, ΔRK, lacks the highly charged sequence that follows closely the signal peptide at amino acids 41 to 62 and is identical in the precursor polypeptides for all three TGF-β sequences. The mutation ΔR results in the removal of only the first four residues of this same conserved sequence. We also modified the Asn codons at two N-glycosylation sites at positions 82 (ΔN1) and 136 (ΔN2) that are conserved in all three TGF-β precursor species and are mannose-6-phosphorylated on the carbohydrate moiety (32). As a result, the translated precursor polypeptide cannot undergo N glycosylation at these single sites or at both sites (mutation ΔN1N2). The mutated cDNAs were introduced in expression vectors under the transcriptional control of the human cytomegalovirus promoter (15), and the resulting plasmids were transfected in human 293S cells (12) to evaluate the effects of these mutations on the TGF-β expression. These cells secrete low levels of TGF-β1 (2), which cannot be detected by immunoprecipitation. Analysis of the secreted protein in the conditioned medium indicates that the presence of these mutations resulted in a decreased secretion of TGF-β, the extent of which depended on the nature of the mutation (Fig. 2). The ΔRK deletion was most effective in inhibiting TGF-β secretion, whereas the ΔR deletion also effectively blocked TGF-β secretion. The Mut S mutation, which removed the tetrabasic cleavage site, also inhibited TGF-β secretion (data not shown). Finally, removal of the second N-glycosylation site strongly decreased the level of secreted TGF-β synthesis, whereas removal of the first N-glycosylation site (ΔN1) had only little effect. The double mutation ΔN1N2 was much more effective than the single mutations. This inhibition in the absence of these N glycosylations is consistent with previous results that documented inhibited secretion of TGF-β in the presence of tunicamycin, an inhibitor of N glycosylation (3a, 4). The inhibition of TGF-β secretion was not related to lower mRNA levels, since transfection of all expression vectors, containing either the unmodified or mutated coding sequences, yielded about equally high levels of plasmid-derived TGF-β mRNA (data not shown).

Dominant negative mutants of TGF-β. Considering the strong inhibitory effect of these mutations on the secretion of TGF-β (Fig. 2), we evaluated whether overexpression of these mutants would inhibit the secretion of TGF-β derived from the unmodified precursor cDNA. It would indeed be possible that overexpression of the mutated precursor results in heterodimer formation of a mutated with an unmodified monomer in a competitive fashion. These heterodimers would, similarly to the mutated homodimers, not be secreted, and thus heterodimer formation would efficiently decrease the levels of wild-type, unmodified TGF-β precursor dimers and the resulting level of secreted TGF-β. Because of the competitive nature of the inhibition process, the efficiency of the inhibition would increase as the ratio of the expressed mutated form versus the unmodified form increases. To test the possibility that the mutated forms could act as dominant negative mutants in this process, we performed cotransfection experiments, in which expression plasmids for the mutated precursor polypeptide were transfected together with unmodified TGF-β expression vectors. Cotransfection of both plasmids in the 293S cells at a ratio 4:1 (mutated/wild type) resulted in a strong suppression of

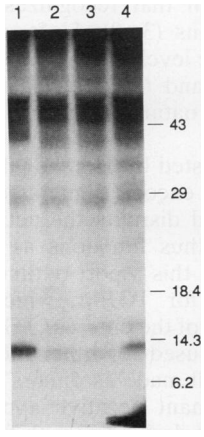

FIG. 3. Competition of a dominant negative TGF-β mutant with wild-type TGF-β. Transfections of 293S cells were performed with two different quantities of mutant pRK5-β1E ΔRK. 293S cells were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine (100 μCi/ml) for 18 h, and conditioned medium was immunoprecipitated with the TGF-β-specific monoclonal antibody 4A11. Lanes: 1, pRK5-β1E (10 μg); 2, pRK5-β1E/pRK5-β1E ΔRK (10 μg/30 μg); 3, pRK5-β1E/pRK5-β1E ΔRK (10 μg/40 μg); 4, pRK5-β1E/pTGF-α (10 μg/40 μg). Molecular weight markers are indicated in kilodaltons at the right.

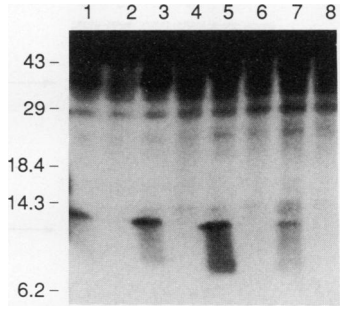

FIG. 4. Specificity of the dominant negative mutants in their competition with TGF-β1, -β2, and -β3. Transfections of 293S cells were performed with expression vectors for the three human TGF-β species, TGF-β1, -β2, and -β3, and with the mutated TGF-β1 C2S2 mutant. Competition was with the dominant negative mutant expression vector pRK5-β1E ΔRK or with the parent control vector pRK5. Cells were metabolically labeled, and conditioned medium was immunoprecipitated with a TGF-β-specific monoclonal antibody. Aliquots were fractionated on a denaturing 15% polyacrylamide gel, and fluorography was performed. All plasmid combinations indicated were transfected in a ratio of 10 μg/40 μg. Lanes: 1, pRK5-β1E/pRK5; 2, pRK5-β1E/pRK5-β1E ΔRK; 3, pRK5-β1E C2S2/pRK5; 4, pRK5-β1E C2S2/pRK5-β1E ΔRK; 5, pRK5-β2/pRK5; 6, pRK5-β2/pRK5-β1E ΔRK; 7, pRK5-β3-7/pRK5; 8, pRK5-β3-7/pRK5-β1E ΔRK. Plasmid pRK5-β3-7 has been previously described (15). Molecular weight markers are indicated in kilodaltons at the left.

the secretion of the unaltered TGF-β complex (Fig. 3). The most efficient suppressor mutation was ΔRK. The mutations ΔR and Mut S were less efficient in this type of competitive inhibition (data not shown). No or little suppression of the secretion of TGF-β from the unmodified TGF-β precursor expression vector was observed when the mutated TGF-β plasmid was replaced by a control plasmid which lacked a cDNA or which directed the expression of an unrelated factor such as TGF-α (Fig. 3). Gel electrophoretic analysis of ^{35}S-labeled secreted proteins in the medium indicated that there was no detectable inhibition of the secretion of other proteins.

Specificity of the dominant negative effect. As outlined above, there are several TGF-β species which are all highly conserved in their mature sequence but have a more relaxed conservation in their precursor sequences. The different mature TGF-β species are usually homodimers, but the existence of heterodimers has been documented. We thus constructed expression vectors for TGF-β2 and TGF-β3 in order to evaluate whether the dominant negative TGF-β1 mutants specifically suppress only TGF-β1 secretion or whether they are also effective in inhibiting TGF-β2 and -β3 secretion. Competition experiments in which the expression plasmid for the ΔRK mutant of TGF-β1 was cotransfected with the unmodified precursor expression plasmids for either TGF-β1, -β2, or -β3 showed that the secretion of other TGF-β species was considerably inhibited (Fig. 4). However, titration experiments revealed that the efficiency to block TGF-β secretion was higher for TGF-β1 than for other TGF-β species (data not shown). Thus, the dominant negative effect was not restricted to TGF-β1 but also occurred with the two other TGF-β species.

We also wanted to verify whether the TGF-β dominant negative mutants had the ability to inhibit the secretion of the more distantly related members of the TGF-β superfamily. We thus constructed an efficient expression vector containing the full-size vgr-1 cDNA (6, 19a, 21). This cDNA for vgr-1 (6), which also has been named BMP-6 (6), encodes a polypeptide which is more related to BMP-5 and OP-1/BMP-7 (6, 28) than to any other members of the TGF-β superfamily. Analysis by gel electrophoresis of the secreted translation product of the vgr-1 cDNA allowed the detection of two polypeptides in the medium; the larger one corresponds to the precursor segment from which the mature vgr-1 peptide has been cleaved, whereas the smaller one represents the mature polypeptide itself. The latter band, however, is usually barely detectable following autoradiography, presumably due to the very sticky nature of the mature factor and the resulting interaction with cells, matrix, and cell culture substrate (19a). This stickiness was also apparent with the *dpp* gene product, the BMP-2/4 homolog in *Drosophila melanogaster* (29). The efficiency of vgr-1 secretion is thus best evaluated by measuring the level of secretion of the vgr-1 precursor segment, which unfortunately cannot be carried out by using antibody-specific detection. We thus performed competition experiments in which the expression vector for the dominant negative TGF-β1 mutant ΔRK was cotransfected with the vgr-1 cDNA expression plasmid. Comparison of the result of this cotransfection with the cotransfection of the vgr-1 plasmid with a control plasmid revealed that the TGF-β1 dominant negative mutant did not affect or only slightly suppressed the secretion of the vgr-1 polypeptides (Fig. 5). Thus, the dominant negative effect was restricted to the different TGF-β species and did not affect or only minimally influenced the secretion of more distantly related or unrelated proteins.

DISCUSSION

These experiments have shown that transfection of an expression vector encoding a TGF-β1 precursor, mutated in some specific, highly conserved areas of the pro segment, resulted in an inhibition of the secretion of the TGF-β derived from the modified TGF-β precursor. Following the completion of our study, Sha et al. (34) reported that other

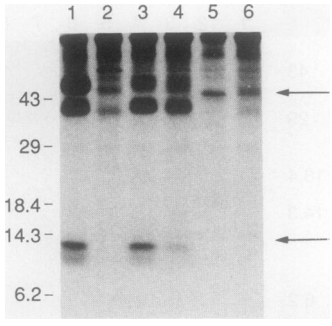

FIG. 5. Competition of a dominant negative TGF-β1 mutant with TGF-β1 and with vgr-1, a not so closely related TGF-β superfamily member. Transfections of the dominant negative mutant plasmid pRK5-β1E ΔRK into 293S cells were performed in the presence of pRK5-β1E, encoding the wild-type TGF-β1 precursor, pRK5-β1E C2S2, encoding a mutated and activated TGF-β1 form, and pRK5-vgr-1, encoding the TGF-β superfamily member vgr-1. Control competition experiments were done with the parent vector pRK5. Cells were metabolically labeled, aliquots of the conditioned medium were fractionated on a denaturing 15% polyacrylamide gel, and fluorography was performed. In lanes 1 to 4, the samples were immunoprecipitated with a TGF-β-specific antibody. No immunoprecipitations were carried out in lanes 5 and 6 because of the unavailability of an antibody. All plasmid combinations indicated were transfected in a ratio of 10 μg/40 μg. Lanes: 1, pRK5-β1E/pRK5; 2, pRK5-β1E/pRK5-β1E ΔRK; 3, pRK5-β1E C2S2/pRK5; 4, pRK5-β1E C2S2/pRK5-β1E ΔRK; 5, pRK5-vgr-1/pRK5; 6, pRK5-vgr-1/pRK5-β1E ΔRK. Molecular size values are indicated in kilodaltons at the left. The top arrow indicates the size of the vgr-1 precursor (lanes 5 and 6), whereas the lower arrow shows the position of mature TGF-β1 (lanes 1 to 4).

mutations of the TGF-β precursor similarly prevented TGF-β secretion. We have also shown that transfection of an expression vector for such mutant TGF-β1 cDNA resulted in an inhibition of the secretion of TGF-β derived from the unmodified precursor. This inhibition not only was restricted to TGF-β1 but was also effective for TGF-β2 and -β3. However, there is no inhibition of the secretion of unrelated proteins, such as TGF-α or human growth hormone (data not shown), or of more distantly related members of the TGF-β superfamily, such as the vgr-1 polypeptide. These TGF-β1 precursor mutants thus act as dominant negative mutants. What is the basis for this specific inhibition? It is to be expected that overexpression of the mutant will result in a heterodimer formation between the mutated derivative and the wild-type, unmodified monomer. When the mutants are expressed at high levels, most endogenous TGF-β derived from the unmodified template will be titrated out by heterodimer formation. Previous studies have shown that the proregion is needed for the secretion of TGF-β, thus suggesting that the precursor segment functions as a chaperone, analogously with several other well-studied protein complexes (14). The precursor is thus needed for intracellular transport and secretion and may be needed for correct folding of the mature TGF-β dimer. It is possible that structural disruption of some highly conserved sequences in the pro segment confer an anomalous configuration to this polypeptide, which is then no longer able to function properly. Such structurally abnormal complexes, either in mutated homodimers or in heterodimers of the mutated and wild-type form, will then be retained and degraded intracellularly, e.g., in the endoplasmic reticulum. This could perhaps take place as a result of the action of the hsp70-based scanning mechanism that recognizes and removes incorrectly folded proteins (3, 8). Unfortunately, experimental determination of the levels of correctly or incorrectly folded TGF-β complexes and their fate inside the cells was not possible due to the nature of the antibodies used (data not shown).

As initially suggested by Herskowitz (16), a cloned gene can be mutated to encode a mutated polypeptide, which when overexpressed disrupts the activity of the wild-type gene product and thus functions as a dominant negative mutant. We have in this report outlined a strategy to make dominant mutants for TGF-β, which specifically inhibit functional secretion of the different TGF-β species. A similar approach could be used for other members of the TGF-β family, which are all made as dimers derived from a larger precursor. A dominant negative approach has also been designed for platelet-derived growth factor, another type of dimeric growth factor (25). Dominant negative mutants of TGF-β or TGF-β-related factors could be used to specifically block secretion of these factors in cells and thus open a new strategy to reveal the physiological functions of these complex growth and differentiation factors. In addition, overexpression of these dominant negative mutants in transgenic mice can reveal the functions of these factors in a tissue-specific way, depending on the specificity of the promoter used, and represents an alternative to gene inactivation by homologous recombination.

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REFERENCES

1. **Amaya, E., T. J. Musci, and M. W. Kirschner.** 1991. Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. Cell **66**:257–270.
1a. **Arrick, B. A., and R. Derynck.** Unpublished data.
2. **Arrick, B. A., A. R. Lopez, F. Elfman, R. Ebner, C. H. Damsky, and R. Derynck.** Submitted for publication.
3. **Beckmann, R. P., L. A. Mizzen, and W. J. Welch.** 1990. Interaction of hsp70 with newly synthesized proteins: implications for protein folding and assembly. Science **248**:850–854.
3a. **Bringman, T., and R. Derynck.** Unpublished data.
4. **Brunner, A. M., L. E. Gentry, J. A. Cooper, and A. F. Purchio.** 1988. Recombinant type 1 transforming growth factor β precursor produced in Chinese hamster ovary cells is glycosylated and phosphorylated. Mol. Cell. Biol. **8**:2229–2232.
5. **Cate, R. L., R. J. Mattaliano, C. Hession, R. Tizard, N. M. Farber, A. Cheung, E. G. Ninfa, A. Z. Frey, D. J. Gash, E. P. Chow, R. A. Fisher, J. M. Bertonis, G. Torres, B. P. Wallner, K. L. Ramachandran, R. C. Ragin, T. F. Manganaro, D. T. MacLaughlin, and P. K. Donahoe.** 1986. Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. Cell **45**:685–698.
6. **Celeste, A. J., J. A. Iannazzi, R. C. Taylor, R. M. Hewick, V. Rosen, E. A. Wang, and J. M. Wozney.** 1990. Identification of transforming growth β family members present in bone-inductive protein purified from bovine bone. Proc. Natl. Acad. Sci. USA **87**:9843–9847.
7. **Derynck, R., P. B. Lindquist, A. Lee, D. Wen, J. Tamm, D. M. Miller, R. J. Coffey, H. L. Moses, and E. Y. Chen.** 1988. A new

type of transforming growth factor β, TGF-β3. EMBO J. **7**:3737–3743.
8. **Gatenby, A. A., and R. J. Ellis.** 1990. Chaperone function: the assembly of ribulose biphosphate carboxylase-oxygenase. Annu. Rev. Cell Biol. **6**:125–149.
9. **Gatherer, D., P. ten Dijke, D. T. Baird, and R. J. Akhurst.** 1990. Expression of TGF-β isoforms during first trimester human embryogenesis. Development **110**:445–460.
10. **Gentry, L. E., N. R. Webb, G. J. Lim, A. M. Brunner, J. E. Ranchalis, D. R. Twardzik, M. N. Lioubin, H. Marquardt, and A. F. Purchio.** 1987. Type 1 transforming growth factor beta: amplified expression and secretion of mature and precursor polypeptides in Chinese hamster ovary cells. Mol. Cell. Biol. **7**:3418–3427.
11. **Gorman, C., R. Padmanabhan, and B. H. Howard.** 1983. High efficiency DNA-mediated transformation of primate cells. Science **221**:551–553.
12. **Gorman, C. M., D. Gies, and G. McCray.** 1990. Transient production of proteins using adenovirus transformed cells. DNA-Protein Eng. **2**:3–10.
13. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–77.
14. **Gray, A. M., and A. J. Mason.** 1990. Requirement for activin A and transforming growth factor β1 pro-regions in homodimer assembly. Science **247**:1328–1330.
15. **Graycar, J. L., D. A. Miller, B. A. Arrick, R. M. Lyons, H. L. Moses, and R. Derynck.** 1989. Human transforming growth factor β3: recombinant expression, purification, and biological activities in comparison with transforming growth factors-β1 and -β2. Mol. Endocrinol. **3**:1977–1986.
16. **Herskowitz, I.** 1987. Functional inactivation of genes by dominant negative mutations. Nature (London) **329**:219–222.
17. **Izant, J., and H. Weintraub.** 1985. Constitutive and conditional suppression of exogenous and endogenous gene expression by antisense RNA. Science **229**:345–352.
18. **Jakowlew, S. B., P. J. Dillard, P. Kondaiah, M. B. Sporn, and A. B. Roberts.** 1988. Complementary deoxyribonucleic acid cloning of a novel transforming growth factor β messenger ribonucleic acid from chick embryo chondrocytes. Mol. Endocrinol. **2**:747–755.
19. **Kashles, O., Y. Yarden, R. Fisher, A. Ullrich, and J. Schlessinger.** 1991. A dominant negative mutation suppresses the function of normal epidermal growth factor receptors by heterodimerization. Mol. Cell. Biol. **11**:1454–1463.
19a. **Kobrin, M., and K. Derynck.** Unpublished data.
20. **Lee, S. J.** 1990. Identification of a novel member of the transforming growth factor β superfamily. Mol. Endocrinol. **4**:1034–1040.
21. **Lyons, K., J. L. Graycar, A. Lee, S. Hashmi, P. B. Lindquist, E. Y. Chen, B. L. M. Hogan, and R. Derynck.** 1989. Vgr-1, a mammalian gene related to *Xenopus* vg-1, is a member of the transforming growth factor-β superfamily. Proc. Natl. Acad. Sci. USA **86**:4554–4558.
22. **Mansour, S. L., K. R. Thomas, and M. R. Capecchi.** 1988. Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature (London) **336**:348–352.
23. **Mason, A. J., J. S. Hayflick, N. Ling, F. Esch, N. Ueno, S.-Y. Ying, R. Guillemin, H. Niall, and P. H. Seeburg.** 1985. Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming grwoth factor β. Nature (London) **318**:659–663.
24. **Massagué, J.** 1990. The transforming growth factor-β family. Ann. Rev. Cell Biol. **6**:597–641.
25. **Mercola, M., P. L. Deininger, S. M. Shamah, J. Porter, C. Wang, and C. D. Stiles.** 1990. Dominant negative mutants of a platelet derived growth factor gene. Genes Dev. **4**:2333–2341.
26. **Miyazono, K., U. Hellman, C. Wernstedt, and C.-H. Heldin.** 1988. Latent high molecular weight complex of transforming growth factor-β1. J. Biol. Chem. **263**:6407–6415.
27. **Miyazono, K., A. Olofsson, P. Colosetti, and C.-H. Heldin.** 1991. The role of the latent TGF-β1-binding protein in the assembly and secretion of TGF-β1. EMBO J. **10**:1091–1101.
28. **Ozkaynak, E., D. C. Rueger, E. A. Drier, C. Corbett, R. J. Ridge, T. K. Sampath, and H. Opperman.** 1990. OP-1 cDNA encodes an osteogenic protein of the TGF-β family. EMBO J. **9**:2085–2093.
29. **Panganiban, G. E. F., K. E. Rashka, M. D. Neitzel, and M. Hoffmann.** 1990. Biochemical characterization of the *Drosophila dpp* protein, a member of the transforming growth factor β family of growth factors. Mol. Cell. Biol. **10**:2669–2677.
30. **Pelton, R. W., M. E. Dickinson, H. L. Moses, and B. L. M. Hogan.** 1990. In situ hybridization analysis of TGF-β3 RNA expression during mouse development: comparative studies with TGF-β1 and -β2. Development **110**:609–620.
31. **Pelton, R. W., B. L. M. Hogan, D. A. Miller, and H. L. Moses.** 1990. Differential expression of TGF-β1, -β2 and -β3 during murine palate formation. Dev. Biol. **141**:456–460.
32. **Purchio, A. F., J. A. Cooper, A. M. Brunner, M. N. Lioubin, L. E. Gentry, K. S. Kovacina, R. A. Roth, and H. Marquardt.** 1988. Identification of mannose-6-phosphate in two asparagine-linked sugar chains of recombinant TGF-β1 precursor. J. Biol. Chem. **263**:14211–14215.
33. **Roberts, A. B., and M. B. Sporn.** 1990. The transforming growth factor-betas, p. 419–472. *In* M. B. Sporn and A. B. Roberts (ed.), Peptide growth factors and their receptors. I. Springer-Verlag, Berlin.
34. **Sha, X., L. Yang, and L. E. Gentry.** 1991. Identification and analysis of discrete functional domains in the pro regions of pre pro-transforming growth factor-β1. J. Cell Biol. **114**:827–839.
35. **Smith, A. J. H.** 1980. DNA sequence analysis by primed synthesis. Methods Enzymol. **65**:560–580.
36. **Smithies, O., R. G. Gregg, S. S. Boggs, M. A. Koralewski, and R. S. Kucherlapati.** 1985. Insertion of DNA sequences into the human chromosomal beta globin locus by homologous recombination. Nature (London) **317**:230–234.
37. **ten Dijke, P., P. Hanson, K. K. Iwata, C. Pieler, and J. G. Foulkes.** 1988. Identification of a new member of the transforming growth factor β gene family. Proc. Natl. Acad. Sci. USA **85**:4715–4719.
38. **Ueno, H., H. Colbert, J. A. Escobedo, and L. T. Williams.** 1991. Inhibition of PDGF β receptor signal transduction by coexpression of a truncated receptor. Science **252**:844–848.
39. **Wozney, J., V. Rosen, A. J. Celeste, L. M. Mitsock, M. J. Whitters, R. W. Kriz, R. M. Hewick, and E. A. Wang.** 1988. Novel regulators of bone formation: molecular clones and activities. Science **242**:1528–1534.
40. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA **3**:479–488.