Communication

A Dominant-negative Receptor for Type β Transforming Growth Factors Created by Deletion of the Kinase Domain*

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To prove the postulated role of type β transforming growth factors (TGFβ) in cardiac development and other events, specific inhibitors of TGFβ signal transduction are needed. We truncated the type II TGFβ receptor cDNA (ΔTβRII), to delete the predicted serine/threonine kinase cytoplasmic domain. ΔTβRII was co-transfected into neonatal cardiac myocytes, together with reporter constructs for two cardiac-restricted genes that are regulated antithetically by TGFβ. ΔTβRII impaired activation of the skeletal α-actin promoter by TGFβ1, -2, and -3 and, conversely, impaired TGFβ inhibition of α-myosin heavy chain expression. Thus, a kinase-defective TβRII blocks signaling by all three mammalian TGFβ isoforms, and can disrupt both positive and negative control of transcription by TGFβ.

The biological importance of TGFβ has largely been inferred from the intricate spatial and temporal program that governs this family of growth factors during development, the impact of exogenous TGFβ on gene expression or growth (1), and, recently, the use of neutralizing antibodies (2) or decorin, a TGFβ-binding proteoglycan (3), to substantiate in vivo effects. A role for TGFβ in differentiation and disease of the heart is deemed likely (4, 5), given the abundance of TGFβ in myocardium (6), its up-regulation by infarction (7), mechanical load (8), or adrenergic agonists (9), and its ability to protect myocardium from ischemic injury (10), to sustain contractility in culture (11), and to control the expression of at least six cardiac-restricted genes (12, 13). Unlike the global suppression of differentially regulated gene expression by TGFβ in skeletal muscle (14-16), neonatal cardiac myocytes possess a continuum of responses to TGFβ1: up-regulation of a gene ensemble, including skeletal α-actin (SkA), expressed preferentially in fetal myocardium, concurrent with down-regulation of genes including α-myosin heavy chain (aMHC) that are associated with adult ventricular muscle (12, 13), dichotomous responses which correspond to the generalized "fetal" phenotype produced by mechanical load (4, 17). Positive and negative control of developmentally regulated genes thus exist in this system, making the cardiac myocyte particularly intriguing as a model for studies of TGFβ signal transduction. Investigations of pluripotent cell lines (18), amphibian cardiac progenitor cells (19), and avian cardiac endothelium (20) also suggest that TGFβ-related peptides might regulate cardiac organogenesis itself. However, mechanistic tests of this hypothesis require a suitable inhibitor of the TGFβ signaling cascade and TGFβ-dependent gene expression.

Neonatal cardiac myocytes possess all three of the characteristic cell surface receptors for TGFβ (TβR) visualized by receptor cross-linking (21). Expression cloning proved the type II TβR, a 75-kDa glycoprotein, to possess an intracellular domain distinct from the four classes of tyrosine kinase found in the receptors for platelet-derived, epidermal, insulin-like, and fibroblast growth factors (22). Instead, TβRII resembles the type II receptor for activin, a distant member of the TGFβ superfamily, and Daf-1, a protein controlling larva formation in Caenorhabditis elegans; all three constitute a novel class of transmembrane protein with a consensus serine/threonine kinase as the predicted cytoplasmic signaling domain (22-25). TβRII is fully functional in the absence of the type III receptor (β-glycan), illustrated by the absence of this proteoglycan from L6 myoblasts (14, 26, 27). TβRII is competent to bind TGFβ in the absence of the type I TβR, a 53-kDa protein whose structure has not yet been defined, but signal generation apparently requires a heteromeric protein complex involving both receptors I and II (28).

Kinase-defective mutations of receptor tyrosine kinases are known to inhibit the function of wild-type receptors, possibly by a block to the intracellular autophosphorylation that follows ligand-induced dimerization (29-31). Although the corresponding initial aspects of TGFβ signal transduction are less completely understood, we reasoned that a truncated TβRII, lacking the serine/threonine kinase domain, would function as a dominant inhibitor of TGFβ-dependent transcription. We have used the cardiac myocyte model to demonstrate that the truncated TβRII resists control of TGFβ-dependent transcription of developmentally regulated cardiac genes.

EXPERIMENTAL PROCEDURES

Plasmids—To generate the truncated human TβRII by PCR amplification, each 100-μl reaction mixture contained 10 ng of TβRII clone H2–3FF (22), 600 ng of the primers shown, 200 μM of each dNTP, 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.0, and 5 units of Taq polymerase (Promega). Amplification comprised 5 min of initial denaturation at 94 °C, then 30 cycles (1 min at 94 °C, 1.5 min at 72 °C, and 1 min at 60 °C) using a Perkin-Elmer Cetus DNA thermal cycler. The final extension reaction was for 7 min at 72 °C. The resulting PCR product was analyzed on an 8% polyacrylamide gel and had the expected size of 883 nucleotides. For directional subcloning, the products of three PCR reactions were combined, purified with a Centricon 100 spin column, digested with EcoRI and HindIII, and loaded on a 1.2% agarose gel. The DNA band was excised, and DNA was isolated with the Quiaex gel slurry.
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To construct the truncated TβRII (ΔTβRII), we subjected the human TβRII cDNA H2-3FF (22) to PCR amplification, using primers that correspond to nucleotides 306–326 and 1153–1172 and incorporated asymmetric linkers for directional cloning (Fig. 1). The resultant fragment, encoding the nominal extracellular and transmembrane domains of TβRII and 272 amino acids of the cytoplasmic domain, was identical to the corrected sequence of Lin et al. (22) and excludes 264 of 298 amino acids in the serine/threonine kinase motif. For expression in eukaryotic cells, ΔTβRII was subcloned between the EcoRI and HindIII sites of pSV-Sport1, under the transcriptional control of the cytomegalovirus (CMV) immediate-early promoter (34).

Cell Culture and Transfection—Neonatal cardiac myocytes were isolated as previously described from 1-2-day-old rats (12, 13). Myocytes were purified by density centrifugation through a Percoll step gradient (22). The resultant fragment, encoding the nominal amino acids of the cytoplasmic domain, was identical to the receptor. By contrast, as shown in Fig. 3, exogenous full-length TβRII is contingent on truncation of the cytoplasmic domain. One stringent test for the specificity of dominant-negative mutations is whether exogenous wild-type protein can rescue the mutant phenotype. Increasing the amount of full-length TβRII cDNA progressively restored the responsiveness of cardiac muscle cells to TGFβ1, despite a constant amount of the expression vector encoding ΔTβRII. As was true for the truncated activin receptor (37), complete rescue required less than stoichiometric amounts of the wild-type receptor.

Materials and Methods

FIG. 2. ΔTβRII suppresses up-regulation of the Ska promoter by TGFβ in cardiac muscle cells. Transfected ventricular myocytes were cultured in absence or presence of TGFβ for 36 h and then were assayed for activity of the Ska-luciferase and CMV-lacZ reporter genes. Levels of luciferase expression, corrected for transfection efficiency, are expressed relative to that of the Ska promoter in the vehicle-treated, vector-transfected cells. Mean values ± S.E. are shown. Open bar, pSV-Sport1; solid bar, ΔTβRII; gray bar, ΔAcRII.
Biological actions of the TGFβ isoforms, while often similar, differ drastically in some systems. As one illustration, only TGFβ3 is implicated in the epithelial-mesenchymal transformation required for creation of cardiac valves (20). Therefore, to ascertain whether ΔK TpRII might disrupt signaling by more than one form of TGFβ, we first tested if neonatal rat cardiac myocytes were transfected with ΔK TpRII and full-length TpRII cDNA in the amounts shown and were analyzed for the activity of the SkA-luciferase and CMV-lacZ reporter genes. For this experiment, ΔK TpRII was subcloned into the CMV-driven expression vector, pcDNA, to ensure direct comparability with the wild-type TpRII as provided by Lin and colleagues (22). Results are shown for TGFβ1-treated cells; full-length TpRII had no effect on basal transcription of the SkA promoter in cardiac cells. Luciferase activity (mean ± S.E.) is expressed relative to that of the SkA construct in vehicle-treated, vector-transfected cells. Solid bar, 1 μM T3; open bar, minus T3.

To facilitate analysis of a TGFβ-inhibited pathway in cardiac muscle cells, we generated an αMHC-luciferase construct, since the cardiac gene whose expression, at the mRNA level, is repressed most completely by TGFβ1 (12, 13). As shown in Fig. 4, αMHC-luciferase activity was highly dependent on thyroid hormone (1.000 ± 0.117 versus 0.993 ± 0.028 at 1 and 0 nM; p = 0.0017) and was inhibited nearly 70% by TGFβ1 (0.344 ± 0.088; p = 0.0110). ΔK TpRII specifically abolished down-regulation by TGFβ1, with no effect on up-regulation by T3. Thus, ΔK TpRII impairs TGFβ-dependent signals for both negative and positive control of gene expression, without spurious effects on a TGFβ-independent pathway. In agreement with this evidence that ΔK TpRII specifically disrupts TGFβ-dependent transcription, activity of the CMV-driven lacZ gene was indistinguishable in ΔK TpRII- and vector-transfected cells.

**DISCUSSION**

Identification of the cDNA sequence for TpRII has provided a critical opportunity to construct a truncated receptor variant, as a reagent to interdict TGFβ signal transduction at the level of the receptor itself. These experiments indicate that deletion of the serine/threonine kinase domain generates a trans-dominant inhibitor of TGFβ signal transduction. In overall agreement with this conclusion, Melton and colleagues (37) have recently shown that a kinase-defective form of the homologous type II activin receptor can block activin-dependent events in early Xenopus embryos. Thus, alteration of the cytoplasmic signaling domain may be a generic strategy for producing loss-of-function mutations, not only in receptor tyrosine kinases but also in those whose action depends on a serine/threonine kinase domain. An additional inference to be drawn from both studies is that mutation of the respective type II receptors is sufficient to repress signal transduction with no need for concomitant mutation of other proteins in the ligand-binding complex (25, 28).

An additional caveat is the potential, for which credible support exists (38), that unexpected tyrosine kinase activity could also be inherent to this class of transmembrane protein.

The ability of all three isoforms of TGFβ to activate the SkA promoter in neonatal rat ventricular myocytes concurs with their shared ability to antagonize depressive effects of interleukin-1β on beating rate and equivalence for binding to cardiac cells (11). Analogously, the fact that ΔK TpRII blocks gene activation by all three peptides agrees with their equal potency for inhibition of DNA synthesis in receptor-defective DR-27 mink lung cells transfected with the full-length human TpRII (28). Thus, our results with the dominant-negative TpRII corroborate the conclusion that TpRII acts as a receptor for all three mammalian TGFβ isoforms (28).

In contrast to other TGFβ-regulated genes, activation of SkA transcription by TGFβ1 appears to be mediated largely via a proximal serum response factor (SRF)-binding element (SRE) and a potential TEF-1 site, which are indispensable as well for basal, tissue-restricted expression. However, the 3' arm of this SRE possesses an overlapping recognition site for a second SRE-binding protein, the bifunctional transcription factor YY1 (39), a competitive antagonist for SRF at this location (40, 41). It is unknown whether TGFβ acts through up-regulation of SRF, modification of SRF, or, conceivably, decreased YY1 activity. cis-Acting sequences for TGFβ repression of αMHC have not yet been delineated, but candidate elements within the 5′-flanking region that was required for cardiac-specific expression in vivo include consensus sites both for SRF and the SRF-related MADS box protein, MEF-2 (33, 42).

Genetic methods to obtain a mechanistic understanding of growth factor signal transduction can be confused by ambiguous results from conventional techniques used to create gain- or loss-of-function mutations. For example, induction of endogenous Fos and Jun by mechanical stress is...
associated with up-regulation of atrial natriuretic factor in ven-
tricular muscle cells (17), not repression as seen with forced expression (43). Similarly, despite the importance of homolo-
gous recombination, an increasingly encountered shortcoming of this strategy is the risk of a misleading or false-negative outcome after disrupting only one member of a redundant multi-gene family. This may be the case for knock-out muta-
tions in TGFβ (44); other recent examples include Fos (45), MyoD (46), and E2A proteins (47). Thus, dominant-inhibitory
genes such as ΔkTβRII offer a crucial alternative to other pro-
cedures for generating loss-of-function mutations. Indeed, there exist corresponding dominant-negative forms of TGFβ itself (48). The finite growth capacity of cardiac muscle cells in culture precludes stable transfection as a means to uniformly modify ventricular myocytes, which would be required to assess the impact of ΔkTβRII on other aspects of the cardiac pheno-
type such as endogenous genes and gene products, signaling intermediaries, or DNA synthesis. This limitation can be over-
come using replication-defective recombinant adenovirus to achieve efficiencies for gene transfer that approach 100% in neonatal and even adult ventricular muscle cells. However, cell culture model systems substitute only partially for investiga-
tions of cardiac organogenesis itself. By analogy to their use in Xenopus oocytes (31, 37), dominant-negative genes like ΔkTβRII might serve as a generic approach, complementary to gene ablation, to create loss-of-function mutations in trans-
genric mammals.

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