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Alternative splicing within the TGF-β type I receptor gene (ALK-5) generates two major functional isoforms in vascular smooth muscle cells

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Abstract We have identified in rat vascular smooth muscle cells (SMCs) the simultaneous expression of two TGF-β type I receptor (ALK-5) cDNAs, occurring as a consequence of alternate usage of AG splice acceptor motifs separated by 12 nucleotides located at an intron-exon junction. When translated the resultant full length proteins differ from each other only by the in-frame presence or absence of Gly-Pro-Phe-Ser residues adjacent to their transmembrane domain. Stable expression of these alternate ALK-5 isoforms in ALK-5-deficient cells demonstrated that both were competent in signaling TGF-β-induced growth inhibition and gene transcription, but with an apparently distinct potency. Our data suggest that alternate splicing within the ALK-5 gene is an important mechanism whereby SMCs may regulate their response to TGF-β.

Key words: Type I transforming growth factor-β receptor (ALK-5); Splice variant; Vascular smooth muscle

1. Introduction

Transforming growth factor-β (TGF-β) is a multifunctional cytokine/growth factor which has been implicated in a variety of vascular pathologies, including vessel remodeling after angioplasty [1], the development of atherosclerotic lesions [2], and the development of vascular hypertrophy in hypertensives [3]. Three closely related TGF-β isoforms, -β1, -β2, and -β3, are present in vascular smooth muscle cells (SMCs) [4] and are capable of either inhibiting or stimulating the proliferation of these cells [5], such effects elicited through interaction with cell surface receptors that possess serine/threonine kinase activity [6]. Initially, TGF-β binds to the type II receptor (TβR-II), a constitutively autophosphorylated kinase, and then this ligand-receptor complex recruits and transphosphorylates an appropriate type I receptor (mostly TβR-I/ALK-5), thereby initiating intracellular signaling [6,7].

The TβR-II:TGF-β:ALK-5 complex formed has been proposed to exist as a heterotetramer in which two molecules of TβR-II and ALK-5 are each present [8], and their association involves interactions between both extracellular and intracellular domains of each receptor [9]. The mechanisms by which such complexes initiate diverse TGF-β signaling patterns are still unclear, but may involve interactions between different functional receptor variants possibly arising as a consequence of alternate splicing events. Evidence that such processes occurring within the ALK-5 gene could generate multiple isoforms of this receptor has been provided by our recent description [10] of the expression of an ALK-5 cDNA in rat SMCs distinct from the initially isolated ALK-5 cDNA [11] in that it harbored an in-frame 12 nucleotide deletion in the extracellular region encoding the Gly-Pro-Phe-Ser motif adjacent to the transmembrane domain, a region rich in proline residues in both the rat and human ALK-5 proteins [11,12]. Deletion or insertion of proline residues/motifs in proteins via naturally occurring nucleotide substitution or aberrant splicing events is known to markedly affect conformational constraints, and therefore biological activity, of such proteins [13]. Thus, a mutation within the extracellular region of the growth hormone receptor causing a change from glutamine to proline not only alters the normal conformation of the receptor, but also prevents growth hormone binding [14]. Proline residues that introduce kinks into transmembrane helices of G protein-coupled receptors are also critical determinants of ligand binding, and receptor signal transduction [15].

Thus, because of the importance of proline residues in regulating receptor structure and signal transduction, our aims in the current study were to: (i) elucidate whether SMCs were capable of expressing both 'long' and 'short' ALK-5 isoforms, differing from each other by the presence or absence of the 12 nucleotides encoding the amino acids Gly-Pro-Phe-Ser, (ii) confirm whether these isoforms arise as a consequence of alternate splicing events within the ALK-5 gene, (iii) determine the functional significance of this alternate ALK-5 isoform expression. Our data indicate that SMCs simultaneously express both the long and short ALK-5 isoforms via alternate usage of AG splice acceptor motifs separated by 12 nucleotides located at an intron-exon junction. Both ALK-5 isoforms are processed in a similar manner intracellularly, and are competent in transducing growth inhibition and gene transcription in response to TGF-β1. However, a non-redundant physiological role for these isoforms appears to exist given that their capacity to elicit such responses is not strictly equipotent, suggesting that their tissue-specific expression levels may provide an important mechanism whereby the cellular response to TGF-β is regulated.

2. Materials and methods

2.1. Cell culture, DNA extraction and polymerase chain reaction (PCR), and RNA extraction and reverse transcription-PCR

Primary cultures of SMCs were prepared from aortas of Wistar-Kyoto rats [5], and maintained in DMEM supplemented with 10% FCS containing 60 μg/ml penicillin G; CHO cells were maintained in similar medium. DNA [16] and DNA-free RNA [10] were extracted from SMCs or aortic tissue and used, respectively, in PCR [16] utilizing primers that span the region 194–217 to 426–455 in the ALK-5

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cDNA (GenBank accession number L26110), or in reverse transcription-PCR (RT-PCR) [10], utilizing primers that span the region 289–317 to 407–435 in the ALK-5 cDNA. Amplified PCR and RT-PCR fragments were electrophoresed through either 1% or 2.5% agarose/ethidium bromide gels, gel purified, then cloned into pGEM and sequenced as previously described [10].

2.2. Construction of full length 'long' and 'short' ALK-5 cDNA expression vectors

Full length rat ALK-5 cDNAs containing the entire coding region either with (1506 bp) or without (1494 bp) the 12 nucleotides GACCTTTTTCAG were constructed in the expression vector *pCI-neo* (Promega) by a multistep procedure. Using a plasmid containing nucleotide sequence upstream of the rat ALK-5 cDNA initiation codon [11], the 5′ end of the cDNA was purified as a 285 bp fragment (containing 40 nucleotides upstream of the initiation codon required for appropriate expression of ALK-5 protein) after digestion with *Eco*RI and *Alw*441. Internal fragments of either 714 bp (containing the 12 nucleotides) or 702 bp (missing the 12 nucleotides) were gel purified from *Alw*441 and *Kpn*I digestion of RT-PCR products obtained using primers spanning the regions 1–28 to 967–992 in the ALK-5 cDNA. The 3′ end of ALK-5 cDNA was prepared by amplifying a fragment 576 bp in size using primers spanning 931–959 to 1477–1506 in the cDNA. After an intermediate cloning step, in which a *Bst*ZI restriction site was placed at the 3′-terminus of this fragment, it was digested with *Kpn*I and *Bst*ZI, and gel purified as a 600 bp fragment containing the ALK-5 termination codon TAA (nucleotides 1504–1506 in the cDNA). In two separate reaction tubes the 5′ (285 bp), internal (714 or 702 bp), and 3′ (600 bp) fragments were then ligated into *pCI-neo* that had been digested with *Eco*RI and *Not*I, to yield the respective full length 'long' (ALK-5L) and 'short' (ALK-5S) ALK-5 isoform expression vectors. The integrity of each construct was confirmed by sequencing of their entire coding region, and Qiagen midi-prep systems were then used to prepare plasmid DNA for transient and stable transfections.

2.3. Transient and stable expression of ALK-5 isoform cDNA

Transient transfection of the two ALK-5 cDNAs into CHO cells was carried out using lipofectamine essentially as described by the manufacturer (Gibco BRL). Cell lysates were prepared 48 h later by solubilization in lysis buffer (0.05% Triton X-100, 120 mmol/l Tris–HCl, pH 8.7, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and clearing by centrifugation at $10\,000\times g$ for 20 min. Western blot analysis of protein extracts (50 μg) in 7.5% polyacrylamide gels with an ALK-5 antibody (SantaCruz Biotechnology, #sc-398) and the appropriate horseradish peroxidase-linked secondary antibody (Amersham, ECL kit) was carried out essentially as described by us previously [17]. For stable expression into ALK-5-deficient mink lung epithelial cells (R1-B/L17 cells, [18]) transfected cell clones were established after exposure to 10% FCS supplemented with 1 mg/ml geneticin (G-418) for 12–14 days, expanded and screened for restoration of TGF-β responses.

2.4. [^{3}H]thymidine incorporation assay, and Northern blot analysis of plasminogen activator inhibitor-1 (PAI-1) mRNA levels

The effect of TGF-β1 on DNA synthesis in R1-B cells stably transfected with either ALK-5 cDNA was determined by measuring the extent to which the cytokine inhibited [^{3}H]thymidine incorporation stimulated by 10% FCS. Briefly, cells were cultured in 10% FCS-1 mg/ml G-418 until they reached semi-confluence (cell densities ~ 1–2×10^5 cells/cm^2), serum deprived for 48 h, then triplicate wells were exposed to 10% FCS either alone or with the addition of varying concentrations of TGF-β1. After 24 h exposure, the cells were washed with DMEM, incubated for 2 h with [^{3}H]thymidine, and then the amount of radioactivity incorporated into their DNA was determined as we have previously described [5]. For analysis of the effects of TGF-β1 on PAI-1 mRNA levels, total RNA was extracted from stably transfected quiescent cells (60 mm dishes) after exposure to 1 ng/ml of the cytokine for 30 min, 1 h, 2 h, 4 h and 24 h, and then 15 μg was electrophoresed in 1% agarose/(2.2 mol/l) formaldehyde gels. Northern blot detection of PAI-1 mRNA was carried out essentially as previously described by us [5], using a [^{32}P]dCTP-labelled (1 to 1.5×10^6 cpm/ml) PAI-1 cDNA fragment (389 base pairs) amplified from mink lung epithelial cell RNA using primers that span the region 667–696 to 1026–1055 in the cDNA [19]. Autoradiographs were analyzed by laser densitometry at 600 nm, and the intensity of ribosomal 28S and 18S bands visualized under UV light was used to monitor equal loading, and transfer, of RNA.

3. Results

3.1. Two ALK-5 isoforms are simultaneously co-expressed in SMCs and arise from alternate usage of AG splice acceptors at an intron-exon junction

Whilst we have previously shown the existence of an ALK-5 isoform in SMCs that differs from the initially characterized ALK-5 cDNA in that it harbors an in-frame deletion of 12 nucleotides (GACCTTTTTCAG, nucleotides 326–337 in the cDNA) adjacent to its transmembrane domain, the precise molecular basis for the existence of these two ALK-5 forms is not known. In addition, because SMCs exist in two phenotypes, *contractile* in normal arteries and *synthetic* in culture, we also elucidated the extent to which the two ALK-5 isoforms were co-expressed within these SMC phenotypes. RT-PCR utilizing primers that flank the region (nucleotides 289–435 in the cDNA) in which the 12 nucleotides are present or absent demonstrated the simultaneous co-expression of two ALK-5 cDNA fragments ('long' ALK-5, *ALK-5L*; 'short' ALK-5, *ALK-5S*) in both intact aortic tissue (*contractile* phenotype) and in cultured, serum-stimulated aortic SMCs (*synthetic* phenotype) (Fig. 1); nucleotide sequencing (data not shown) confirmed that the larger of these fragments was 147 bp in size, and was identical in sequence to the smaller 135 bp fragment except that it contained the 12 nucleotides GACCTTTTTCAG. These analyses indicated that of the two isoforms, ALK-5S was a more abundant cDNA species in both synthetic and contractile SMC phenotypes than was ALK-5L.

To elucidate the molecular basis for the occurrence of these two ALK-5 isoforms we used primers to amplify the region in the ALK-5 gene that encompasses the 12 nucleotides present or absent in either ALK-5L cDNA or ALK-5S cDNA, in which based on the homologous region in the human ALK-5 gene an intron is located [20]. By comparison to the human exon/intron structure we utilized a sense primer that would theoretically be present within exon 2 of the rat ALK-5 gene (bases 194–217 of the cDNA), and an antisense primer that would theoretically be present within exon 3 (bases 426–455 of the cDNA). Using rat genomic DNA we amplified an ~ 2.5 kb fragment with these primers, indicating the presence of a

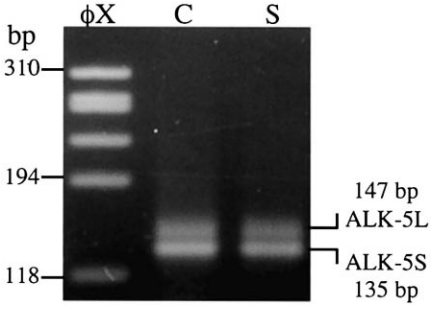

Fig. 1. Agarose gel depicting the simultaneous co-expression of long (147 bp fragment, ALK-5L) and short (135 bp fragment, ALK-5S) ALK-5 cDNA isoforms in contractile aortic tissue (C) and synthetic cultured SMCs (S). φX represents φX174 DNA digested with *Hae*III as molecular weight markers.

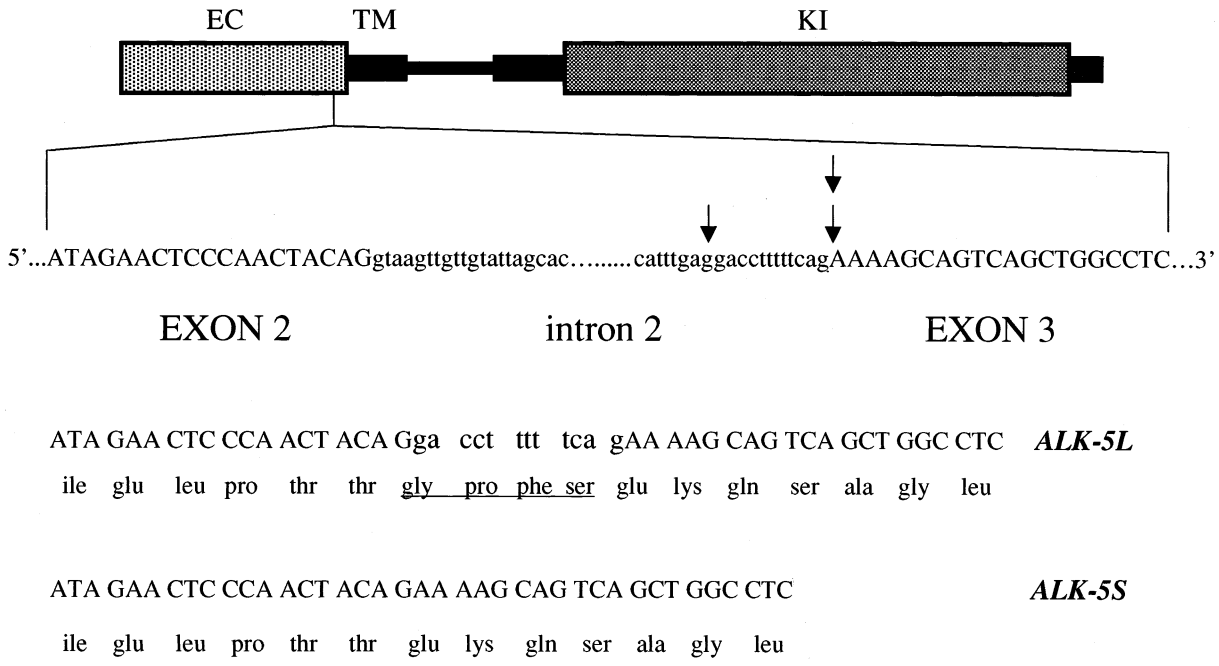

Fig. 2. Nucleotide sequence of exon (nucleotides in upper case)-intron (nucleotides in lower case) junctions, indicating the origin of the long (*ALK-5L*) and short (*ALK-5S*) ALK-5 cDNA isoforms arising from alternative splicing at AG splice acceptor motifs, designated by single- and double-down arrows, respectively. The location of the alternate splicing in relation to the mature ALK-5 protein is shown (EC, extracellular; TM, transmembrane; KI, kinase). The junctions between exon 2/intron 2/exon 3 are based on sequence homology to the human ALK-5 gene [20]. Amino acid residues for each ALK-5 isoform are shown below nucleotides, with the deleted Gly-Pro-Phe-Ser motif in ALK-5S underlined.

large intron in this region. This was confirmed by nucleotide sequencing analysis, with the intron being in the identical position to that within the homologous region of the human ALK-5 gene. The sequence of the intron 2/exon 3 junction was characterized by the presence of two AG splice acceptor motifs separated by 12 nucleotides (Fig. 2); alternate usage of the leftward AG motif would give rise to the ALK-5L isoform (1506 bp as a full length cDNA) whereas usage of the rightward AG motif would give rise to the ALK-5S isoform (1494 bp as a full length cDNA) (Fig. 2).

3.2. Cellular processing of alternate ALK-5 isoforms

In a previous study it has been demonstrated that a splice variant of the type II receptor for the TGF-β superfamily member anti-mullerian hormone (AMH), which contains a four amino acid insertion in its extracellular domain, is inappropriately processed within the cell, thereby implicating it in altered AMH responses [21]. Therefore, to begin to elucidate the potential functional significance of SMC alternate ALK-5 isoform expression involving the presence or absence of the four amino acids Gly-Pro-Phe-Ser in the extracellular domain, we initially characterized the intracellular processing of the two ALK-5 isoforms. In transient transfection experiments into CHO cells using full length expression vectors containing either the ALK-5L or ALK-5S cDNAs, immunoblotting with an ALK-5 antibody demonstrated that in both cases a full length ALK-5 protein was expressed (Fig. 3). Thus, unlike the AMH receptor, it appears that the presence or absence of the four amino acid Gly-Pro-Phe-Ser motif in ALK-5L or ALK-5S, respectively, has no effect on their intracellular processing, suggesting that both isoforms have the potential to be involved in transducing TGF-β responses.

3.3. Ability of alternate ALK-5 isoforms to transduce TGF-β growth inhibitory and gene transcription responses

An ideal cellular model exists in which the functional significance of expression of the alternate ALK-5 isoforms at the cell surface can be ascertained, which involves their stable expression in Mv1Lu mink lung epithelial cells deficient for ALK-5 function (R1-B/L17 cells); because of this ALK-5 deficiency these R1-B/L17 cells, unlike their parental Mv1Lu cell line, are not growth inhibited by TGF-β and do not display specific gene transcription responses to the cytokine [22,23]; restoration of these responses can only occur in the setting of

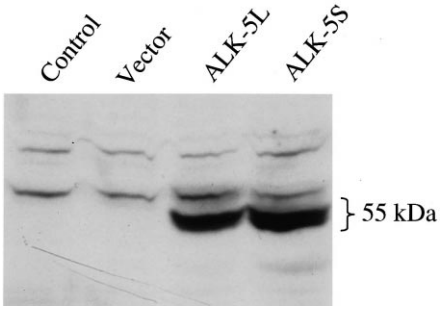

Fig. 3. Expression of ALK-5L and ALK-5S cDNAs in CHO cells, demonstrating that the intracellular processing of these alternate isoforms is identical. 1 μg of either empty *pCI-neo* vector ('Vector') or *pCI-neo* containing each of the respective ALK-5 cDNAs ('ALK-5L', 'ALK-5S') were transiently transfected into CHO cells, then samples of total lysates extracted 48 h later were resolved on 7.5% SDS–polyacrylamide gels ('Control' denotes untransfected CHO cells). Immunobinding was performed using an ALK-5-specific primary antibody. The transiently expressed ALK-5 forms are indicated by the bracket as ~55 kDa proteins.

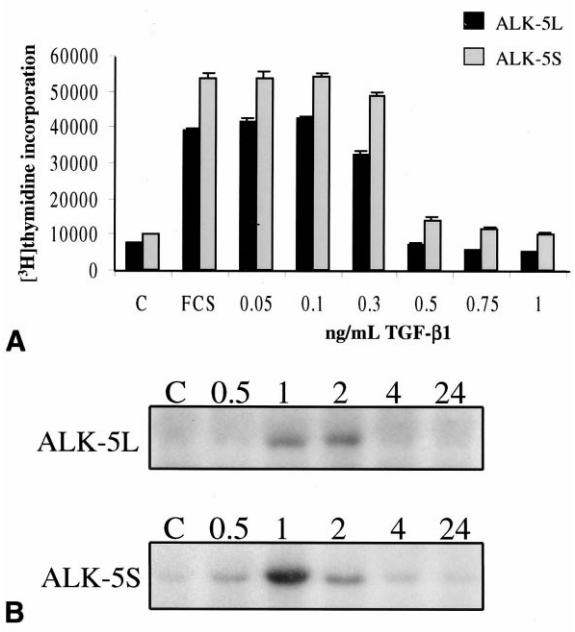

Fig. 4. A: Restoration of TGF-β1 growth inhibitory responses in ALK-5-deficient R1-B/L17 cells after stable transfection with ALK-5L or ALK-5S isoform cDNA. Quiescent cells were exposed to 10% FCS either alone or with the indicated concentrations of TGF-β1 for 24 h, and the growth inhibitory effects were ascertained by assaying for reductions in FCS-stimulated [^{3}H]thymidine incorporation (cpm) in triplicate wells. Results represent the mean ± S.E.M. of three independent experiments; C represents quiescent cells not exposed to either 10% FCS or TGF-β1. B: Comparison of PAI-1 mRNA induction profile stimulated by TGF-β1 in ALK-5-deficient (R1-B/L17) cells stably transfected with either the ALK-5L or ALK-5S isoform cDNA. Quiescent cell cultures were treated with 1 ng/ml of TGF-β1 for the indicated times in h (C represents no TGF-β1 addition), then total RNA was isolated. Northern blot analysis was performed using a [^{32}P]dCTP-labelled PAI-1 cDNA fragment.

exogenously restored ALK-5 function. Thus, we generated R1-B cells stably expressing either ALK-5L or ALK-5S, and determined the extent to which growth inhibitory and gene transcription occurred after TGF-β exposure. In such stably transfected cells cultured in 10% FCS, and exposed to varying concentrations of TGF-β1 for 24 h, significant inhibition of FCS-stimulated [^{3}H]thymidine incorporation occurred with TGF-β1 concentrations higher than 0.3 ng/ml (Fig. 4A). Interestingly, the ALK-5L isoform exhibited a slightly higher potency in eliciting this inhibition than did the ALK-5S isoform (EC_{50} values of 0.34 ng/ml TGF-β1 and 0.41 ng/ml TGF-β1, respectively).

There is some evidence to suggest that specific structural alterations within either ALK-5 or the type II TGF-β receptor (TβR-II) are able to independently affect cellular proliferation and gene expression [24,25]. Therefore, we next determined the capacity of the ALK-5L and ALK-5S isoforms to transduce specific gene transcription responses, by exposing stably transfected cells to TGF-β1 over a 24 h time period and assaying for increases in mRNA levels for plasminogen activator inhibitor 1 (PAI-1). Although both ALK-5 isoforms were able to increase PAI-1 mRNA levels in response to TGF-β1, the extent of induction was greater with ALK-5S, reaching a peak of 7-fold induction over basal levels at 1 h after cytokine addition, as compared to the 3-fold induction for the same exposure time with ALK-5L (Fig. 4B); after 4 h exposure to TGF-β1, PAI-1 mRNA levels had returned to basal in both the ALK-5L and ALK-5S transfected cells.

4. Discussion

In most cell types the broad diversity of responses elicited by TGF-β has been shown to occur via signaling through a receptor complex in which ALK-5, as the major type I receptor for this ligand, is activated through transphosphorylation by the type II receptor, TβR-II. In SMCs, TGF-β can invoke a number of opposing effects, either stimulating or inhibiting cellular proliferation [5] and/or gene expression [26]. However, the basis for these differential effects has remained largely unknown. Clearly, one mechanism that could generate such diversity in TGF-β responses involves alternative splicing in the ALK-5 gene giving rise to multiple functional isoforms with differential signaling capacities. The data we have obtained in the current study suggest that such a mechanism occurs in SMCs. Nucleotide sequencing of the ALK-5 gene encompassing the region in which the long (ALK-5L) and short (ALK-5S) ALK-5 isoforms differ in structure due to the absence or presence of 12 nucleotides encoding the Gly-Pro-Phe-Ser motif adjacent to the transmembrane domain indicates the presence of a large intron and the occurrence of AG splice acceptor motifs located 12 nucleotides apart at an intron/exon junction. The ALK-5L isoform in SMCs corresponds to the ALK-5 isoform initially cloned from a rat brain cDNA library [11], and originally postulated to be the 'normally' spliced receptor. The ALK-5S isoform exhibits greater homology to the human ALK-5 cDNA [20], which has a similar motif of 12 nucleotides missing in the corresponding region. Whilst it remains uncertain as to which of the two ALK-5 isoforms is the 'normally' spliced or the 'alternatively' spliced form of this TGF-β type I receptor in rat SMCs, their high simultaneous co-expression in SMCs, either in the contractile or synthetic phenotype, suggests that they fulfil non-redundant physiological roles, perhaps in regulating the TGF-β response. Support for this possibility is provided from our observations that the ALK-5L and ALK-5S isoforms were not strictly equipotent in their capacity to transduce specific TGF-β responses, with ALK-5L displaying a lower EC_{50} value for TGF-β1 inhibition of [^{3}H]thymidine incorporation into cellular DNA, and ALK-5S displaying a greater ability to induce PAI-1 mRNA levels in response to TGF-β1.

While the precise basis for the apparently differential signaling capacity of the ALK-5L and ALK-5S isoforms remains to be fully elucidated, it may involve a mechanism in which the alternate forms exhibit differential conformations within the TβR-II:TGF-β:ALK-5 signaling complex, arising through the presence (ALK-5L) or absence (ALK-5S) of the proline residue in the Gly-Pro-Phe-Ser motif located 11 amino acid residues upstream of the transmembrane-spanning domain, respectively, in each of these isoforms. Theoretically, the absence or presence of the proline residue contained in this four amino acid motif might thereby alter the interaction between the particular ALK-5 isoform and TβR-II in the heteromeric signaling complex. This possibility is further suggested from recent findings demonstrating that the transmembrane domains of ALK-5 and TβR-II play pivotal roles in receptor association and activation, most likely by influencing the relative orientation of each receptor within

the signaling complex activated by TGF-β [27]. In this scenario, both long-term inhibitory cellular proliferation responses and shorter-term gene transcription responses would be transduced by both ALK-5 isoforms, but in a quantitatively differential manner. In this regard, our data are consistent with those of Pasche et al. [28], who identified in human tissue a slightly shorter ALK-5 variant arising from the deletion of three GCG motifs occurring in the NH2-terminus of the receptor, and resulting in the in-frame absence of three alanine residues from the normal cluster of nine alanine residues. These workers found that transfection of the shorter variant ALK-5 cDNA into ALK-5-deficient cells (the same R1-B/L17 cell line utilized in our study) resulted in the restoration of both TGF-β growth inhibitory and gene transcription responses; however, a tendency was seen for the shorter variant ALK-5 to be more potent in some cases at restoring these TGF-β responses than the corresponding normally processed ALK-5.

In conclusion, our study demonstrates that SMCs are capable of generating alternate functional ALK-5 isoforms that appear, however, not strictly equipotent in their capacity to transduce growth inhibitory or gene transcription responses for TGF-β1. The relatively high simultaneous co-expression of these isoforms in either contractile or synthetic SMCs suggests that their expression may be involved in regulating the response of such cells to the presence of TGF-β.

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