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REVIEW

Smad linker region phosphorylation in the regulation of extracellular matrix synthesis

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Abstract The canonical TGF-β signalling pathway involves Smad transcription factors through direct serine phosphorylation of the carboxy termini, nuclear translocation and regulation of transcription by receptor-regulated (R)-Smad complexes. Smads can also be phosphorylated in the linker region most prominently by the action of mitogen-activated protein (MAP) kinases, which in turn have been activated by TGF-β or a multitude of other growth factors and hormones. Linker region phosphorylation can prevent nuclear translocation of Smads and inhibit TGF-β signalling, potentially leading to oncogenesis. However, some evidence has revealed that linker region phosphorylated Smads can be translocated to the nucleus where they regulate transcription particularly of the synthesis of extracellular matrix molecules. Matrix molecules such as collagen and proteoglycans are involved in diseases such a fibrosis and atherosclerosis, respectively, and the involvement of linker region phosphorylation may represent a new therapeutic target.

Keywords Transforming growth factor-β · Smads · Phosphorylation · Signalling · Vascular smooth muscle · Collagen · Proteoglycans

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Introduction

Transforming growth factor (TGF)-β is a pleiotropic growth factor, and its signalling pathway involves multiple cell surface receptors leading to phosphorylation and ultimate gene regulation by Smad transcription factors [1, 2]. TGF-β is associated with broad aspects of cellular physiology and also in diverse disease states such as cancer, atherosclerosis, and cardiac and renal fibrosis [3–8]. TGF-β signals through cell surface receptors, which are recognised for their serine/threonine kinase activity; however, the cytoplasmic kinase domain also has weaker tyrosine kinase activity, which strictly classifies the receptors as dual specificity kinases [9]. As serine/threonine kinases, they are the largest group of such cell surface receptors. This is in contrast to the overall situation in which most cell surface receptors demonstrate tyrosine kinase activity and intracellular kinases are well divided between serine/threonine and tyrosine kinases [10]. TGF-β receptors consist of an N-terminal extracellular ligand-binding domain, a transmembrane domain and a C-terminal kinase domain. TGF-β receptors form heteromeric complexes in the presence of dimeric ligands. There are five type II receptors (TβRII) and seven type I receptors (TβRI) (also known as activin like kinase 5 or Alk 5) in humans and other mammals. Type II receptors phosphorylate the cytoplasmic domain of

the type I receptor, which initiates downstream signalling activity [7, 11].

TGF-β signalling has been a major area of research over the last 2 decades, and most work has focused on Smads and the canonical Smad transcription factor pathway [7, 11]. Smad proteins are homologs of both the *C. elegans* protein SMA (small) and the drosophila protein, mothers against decapentaplegic (MAD), and the name Smad arises as a combination of the two [12]. Smads are comprised of three domains—firstly the MH1 domain, the amino (N) terminal phosphorylation domain associated with nuclear translocation; secondly the central linker region domain that interacts with prolyl isomerases and ubiquitin ligases and is enriched in serine and threonine residues that can be phosphorylated [7]; thirdly, the carboxy (C) terminal MH2 domain that binds to type I receptors and can interact with some other proteins and that also mediates Smad homo and hetero oligomerisation and the transcriptional activity of the nuclear Smad complexes [7]. Receptor-regulated Smads (R-Smads) are phosphorylated by TβRI in their extreme carboxy termini, which is the initiating step for translocation of phosphoSmads and associated chaperone molecules to the nucleus where they regulate transcription [7, 11]. TGF-β is also known to be a modest, relative to tyrosine kinase agonists, activator of MAP kinases; for example, PDGF is a more efficacious activator of extracellular signal-regulated kinases 1 and 2 (Erk 1/2) phosphorylation compared to TGF-β (see Fig. 3E in [7]). In contrast to the carboxy terminal phosphorylation by the receptor-mediated kinase activity, MAP kinase activation leads to phosphorylation of the R-Smads in their linker region, a peptide sequence occurring between the MH1 DNA binding domain and MH2 C terminal phosphorylation domain [7, 13] (Fig. 1). The linker domains of Smad2 and Smad3 can act as sensors of non-Smad or Smad-independent signal transduction cascades. It is worth noting that in this context "non-Smad pathways" or "Smad-independent" generally refers to signalling that is not via carboxy terminal phosphorylation mediated directly by the TGF β receptor (TβRI/Alk5), but the signalling nevertheless does involve Smads through indirect (i.e. MAP kinase) phosphorylation of the linker region of Smads. However, where TGF-β activates MAP kinases, there are downstream pathways that are completely independent of any R-Smad involvement [14].

Smad linker region phosphorylation modulates the outcome of cellular TGF-β stimulation by determining the propensity of the hetero-complex to translocate to the nucleus, mostly inhibiting translocation, and the transcriptional activity of the complex if it undergoes translocation to the nucleus [7, 15]. Nuclear Smad complexes ultimately bind to chromatin to regulate gene transcription [16]. Intriguingly, as mentioned above,

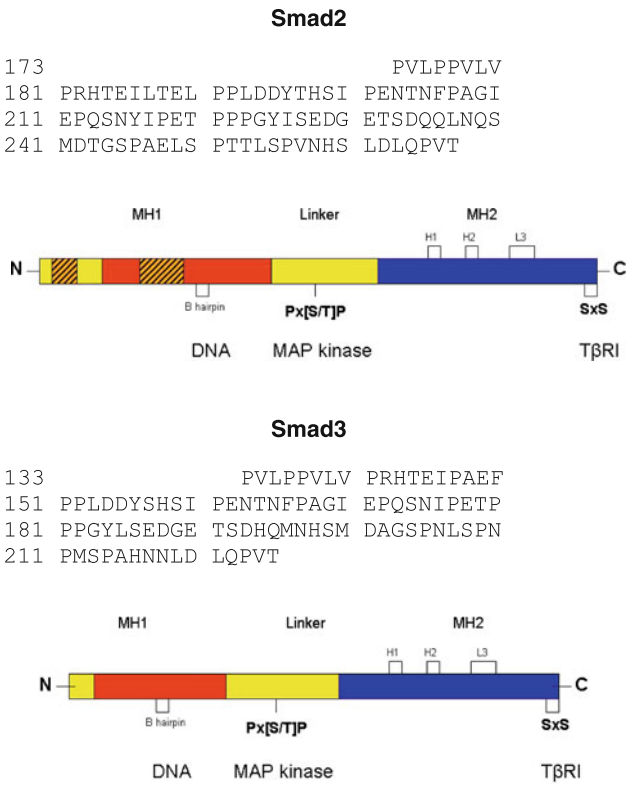

Fig. 1 Amino acid and domain structure of human Smad2 and Smad3 (SwissProt accession numbers Q15796 and P84022, respectively) showing the MH1 and MH2 domains and the central linker region. Both Smads contain nine serine residues in their linker regions. See text for details of biochemistry and role in signalling

activation of MAP kinases and potentially other kinases by growth factors and hormones can lead to phosphorylation of the linker region of R-Smads notwithstanding that these agents do not mediate phosphorylation of the carboxy terminal of the Smad, which is generally considered essential for nuclear translocation [17]. The meaning and implications for this phosphorylation remain unknown. Due to prominent early work in the major area of carcinogenesis, the dominating paradigm of linker region phosphorylation provides a view that it is a negative regulatory event preventing TGF-β transcriptional regulation [15]. For example, after C terminal phosphorylation, nuclear GSK3β and cyclin-dependent kinase phosphorylate three distinct Smad3 linker region residues, which in accord with the dominant paradigm negatively regulates gene transcription [18]. However, evidence is emerging that linker region phosphorylation may not only be a negative regulator of TGF-β signalling, but may indeed be a point of regulation in determining the transcriptional activity of the oligomeric Smad complexes. Some of this evidence has emerged in the context of the synthesis of extracellular matrix. Thus, the mechanism leading to the phosphorylation of the linker region of the R-Smads,

Smad2 and Smad3 and its implications for the regulation of transcription and cellular responses, with a focus on extracellular matrix, are the subject of this review.

TGF-β family polypeptides

The TGF-β family of growth and differentiation factors (GDF) has 33 members [2, 19]. They are dimeric, secreted polypeptides and include bone morphogenic proteins (BMP), GDF, activins and nodal. TGF-β most commonly inhibits but sometimes stimulates cell growth [20]. TGF-β is also implicated in many disease processes such as cancer, atherosclerosis, and cardiac and renal fibrosis [8, 11, 21]. All TGF-β ligands are synthesized as precursor proteins with a longer N terminal pro-peptide followed by a shorter C terminal mature polypeptide [22].

Canonical Smad signalling by TGF-β and TβRI

Canonical TGF-β/Smad signalling refers to the agonist-stimulated receptor-mediated extreme carboxy terminal serine phosphorylation of R-Smads, predominantly Smad2/3 for TGF-β and Smad1/5/8 for BMP, the formation of heteromeric complexes with co-Smad, the translocation of the phosphoSmad complex to the nucleus and the regulation of gene transcription.

In the specific case of TGF-β, the growth factor binds to a type II receptor that recruits a type I receptor to form hetero-tetrameric complexes [23]. In TβRI/TβRII complexes, the TβRII phosphorylates a serine/threonine-rich region called the GS region, an immediately cytoplasmic domain on the TβRI, enabling it to serine phosphorylate R-Smads and hence initiate the canonical Smad pathway of nuclear translocation and transcriptional regulation [23]. When TβRI and TβRII are not complexed, the molecules FKB12 and FKBP12.6 bind the GS region of TβRI to inhibit any non-TGF-β signalling [24]. The activated (phosphorylated) TβRI phosphorylates Smad2/3 through direct serine kinase activity. The Smad proteins are brought into contact with the activated receptor by cytosolic retention factors, most notably the protein SARA (Smad anchor for receptor activation). SARA contains a Smad-binding domain (SBD) that connects to the hydrophobic corridor of the Smad MH2 domain, as well as an FYVE (Fab1p, YOTB, Vac1p, EEA1) phospholipid-binding domain that anchors it to a plasma membrane [25]. The activated Smad 2/3 conjugates with the Co-Smad, Smad 4, in the cytosol to form Smad 2(3)/4 complexes. The stoichiometry of the interaction of R- and co-Smads is such that either one or two molecules of (phospho) Smad2 or Smad3 bind to a single Smad4 molecule. The Smad heterocomplex moves to the nucleus where it further associates with co-activators such as CBP/P300, which regulate their activities through acetylation [26] as well as different transcription factors to form transcriptional complexes.

Biochemistry of Smad transcription factors

Smad proteins are divided into three classes based on functionality. Co-mediator Smads (co-Smads) namely Smad4 participates in signalling by multiple agonists; R-Smads including Smad1, -2, -3, -5 and -8 are involved in ligand-induced signalling and inhibitory Smads (I-Smads), including Smad6 and -7, negatively regulate signalling. The subjects of this review, Smad2 and Smad3, differ by the insertion of an extra 30 amino acids immediately before the DNA-binding hairpin in Smad2, and the consequences of this are that Smad2 is unable to bind to DNA directly (Fig. 1). It follows as a consequence of course that Smad2 can be distinguished from Smad3 by its higher molecular weight. Smad2/Smad4 complexes do not bind to promoter regions and require other transcription factors to target then to specific sequences to affect gene transcription and cellular functions. Smad interacting transcription factors have different intrinsic affinities for Smad complexes and target them to specific genes. Although these two transcription factors, phosphoSmad2 and phosphoSmad3, are sometimes considered together, they have quite distinct features and functions and should be considered individually in the context of cell biology.

Smad3 and Smad4 can bind directly to the Smad binding element (SBE) of the promoter region of genes. The SBE contains only four base pairs (5′-GTCT-3′) or its reverse compliment. Such interactions with promoter regions are generally not strong enough to regulate transcription, so these heterodimers complex and synergise with other factors including members of the AP-1 family, TFE3 and FoxG1 [27]. Co-activators of Smad transcription factors provide enzymatic activities that are absolutely required to alter chromatin structure from the quiescent non-permissive to the active transcriptional state. Smad4 contains an intrinsic 48 amino acid p300-dependent SAD (Smad activation domain) in its linker region. Smad3 also contains a SAD.

Smad linker region phosphorylation

The linker region is by definition the peptide region of variable sequence and length that lies between the MH1 and MH2 domains of Smads (Fig. 1). A Smad3 deletion mutant without the linker region does not mediate TGF-β transcriptional activation of a reporter gene despite its ability to be (carboxy termini) phosphorylated and to form complexes

with Smad4 [27]. This suggests that phosphorylation of the linker region of Smad3 is essential for the biological activities of TGF-β actions that are mediated via Smad3.

There are at least four potential consensus sequence phosphorylation sites in the linker region of Smads 2 (Fig. 1a) and 3 (Fig. 1b). Kinases that are known to phosphorylate these sites include the MAP kinases p38, Erk1/2, Jnk, rho-associated kinase (ROCK), cyclin-dependent kinase (CDK) 2, CDK4 and calcium-calmodulin dependent (CAM) kinase [27, 28]. The serine residues at 110, 240 and 260 of Smad2 are targets for the CAM kinase, the result of which is nuclear exclusion and antagonism of TGF-β signalling [29]. Mutation of the target sites or inhibition of these kinases leads to cell-type-specific effects on the transcriptional activity of the Smads. These kinases regulate Smad transcriptional activity by modulating the recruitment of transcriptional co-activators, which alters the translocation potential of the complex to the nucleus or its ability to act as a transcriptional regulator within the nucleus. It is thus prudent to consider the linker region, along with the carboxy terminal phosphorylation region and the DNA-binding domain, as the third important structural region of Smad transcription factors.

Linker region phosphorylation sites are of the PXSP sequence type, which is a MAP kinase consensus phosphorylation site [30, 31]. It is apparent from the sequences shown in Fig. 1 that there are some of these sites, but also others that are similar but not identical in the linker regions of Smad 2 and 3.

MAP kinase signalling

The MAP kinases are a ubiquitously expressed group of intracellular signalling molecules that play important roles in many cellular responses and are stimulated by a variety of biochemical and environmental factors. The most well known and characterised are the Erk1/2, c-Jun N-terminal kinase (Jnk 1,2 and 3) and p38 (α, β, γ and δ). In its simplest paradigm Erk is recognised as a growth factor signalling pathway, and p38 and Jnk are recognised as stress-activated pathways, although this distinction is tending to break down as more information becomes available on the cellular functions of these kinase families [32]. MAP kinases relay their signal through triple kinase amplification cascades that involve a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and ultimately the respective MAP kinase [33, 34]. The initial activation of the MAPKKK occurs at the level of the cell surface receptor, tyrosine or serine/threonine kinases, which when phosphorylated have docking sites for peptides, such as Shc and Grb-2 [35, 36], which recruit further peptides which lead to activation of MAPKKKSs [37]. MAP kinases also transduce the signalling from non-kinase G protein coupled receptors (GPCR) to kinase-dependent signalling, which explains the growth factor-like activity of well-known agents such as angiotensin II, endothelin and thrombin (for review see [33]). GPCR agonists such as endothelin and angiotensin II elicit classical GPCR responses such as calcium mobilisation [38, 39], but they can also, under some conditions, elicit a full mitogenic response [40, 41]. GPCR activation of cell proliferation pathways can arise from epidermal growth factor (EGF) receptor transactivation where the hijacking of the kinase receptor by the GPCR ultimately recruits MAP kinase signalling cascades and a classic cell growth response [42, 43]. MAP kinases have been implicated in several cardiovascular disorders including cardiac hypertrophy, cardiac remodelling after myocardial infarction, vascular restenosis and atherosclerosis [34].

Modes of Smad linker region phosphorylation

TGF-β/TβRI-mediated cytosolic and nuclear linker region phosphorylation

The current paradigm of so-called non-Smad signalling invokes the signalling from the TβRI receptor to the activation of MAP kinases in the cytosol and linker region phosphorylation. With regard to Erk MAP kinase, it was recently revealed that upon ligand stimulation, TβRI phosphorylates SchA at its tyrosine and serine residues at as early as 2 min. This renders the TβRI a dual specificity kinase as it possesses tyrosine kinase ability in addition to its well-characterised serine/thronine kinase ability. The activated SchA propagates signals via recruitment of the adapter proteins Grb2 and Sos, which leads to Ras-mediated cytosolic Erk activation [35]. It is reasonable to suggest that the activation of Erk by TβRI occurs simultaneously with Smad2 activation and that the synergistically activated Erk is free to phosphorylate Smad2 in the linker region. This notion is supported by the fact that inhibition of TβRI completely blocks linker region phosphorylaton, indicating that the response originates from the receptor complex [35, 46]. In different cell types each of the three main MAP kinases, Erk 1/2, p38 and Jnk, have been demonstrated to phosphorylate Smads in the cytosol. As mentioned above, Smads 2 and 3 can be phosphorylated in the linker region, and this has a profound affect on their function. However, several aspects of this response are presently controversial.

Smads can also be phosphorylated in the nucleus by nuclear MAP kinases and also by cyclin-dependent kinases (CDK)s [44]. Nuclear phosphorylation of the linker also primes the Smad for degradation, and it is an emerging

paradigm that factors that activate transcription factors such as Smads also prime the activated factors for degradation [45]. The impact of this step in limiting signalling is potentially very beneficial for the cell in modulating and terminating the actions of the transcription factors. In this work we describe several examples, related to the extracellular matrix, in which the cytosolic MAP kinase-mediated phosphorylation of the linker region of Smads augments gene transcription and expression for extracellular matrix molecules.

Smad linker region phosphorylation independent of TβRI activation

TGF-β through its receptors leads to moderate activation of MAP kinases and subsequent phosphorylation of Smads in the linker region. Agonists of other receptors, for example, PDGF and GPCRs, can activate MAP kinases so the question arises if activation of MAP kinases via pathways other than TGF-β and TβRI can lead to phosphorylation of R-Smads in the linker region? We have recently observed that in human VSMCs, PDGF stimulates Erk1/2 phosphorylation more strongly than TGF-β [46]. We recently examined if PDGF stimulation leads to phosphorylation of Smad2 in the linker region under the same conditions. PDGF stimulation leads to clear phosphorylation of Smad2 in its linker region, and our preliminary data also show that endothelin-1 and thrombin, both of which stimulate proteoglycan synthesis in human VSMCs [47, 48], also lead to linker region phosphorylation of Smad2 (unpublished observations). PDGF also stimulates Jnk, leading to linker region phosphorylation of Smad2/3 in rat hepatic stellate cells [28].

The stimulation of linker region phosphorylation, secondary to activation of MAP kinases by a plethora of factors, all of which bar TGF-β, cannot stimulate C terminal phosphorylation of Smads, leads to several interesting questions around what is the purpose and function of this phosphorylation? On the general assumption that phosphorylation of Smad2 in the carboxy terminal is required for both translocation to the nucleus and for it to undertake its role as a transcriptional regulator, is there any function for linker phosphorylation of Smads by agents that cannot mediate carboxy terminal phosphorylation? Phosphorylation of Smad2 in the linker region by non-TGF-β and TβRI pathways is a clear example of pathway cross-talk. As phosphorylation of the linker region has functional consequences such as mediating the TGF-β activation of collagen [13] and proteoglycan [46] synthesis, then the cell is pre-programmed by this phosphorylation to determine the outcome should the cell be then stimulated by TGF-β, which will lead to phosphorylation of the Smad carboxy terminal and translocation to the nucleus.

Linker region phosphorylation by agents other than TβRI primes or pre-programs the cell, through the Smad, for the response to TGF-β and TβRI (Fig. 2). When the particular phosphorylation in the linker region prevents nuclear translocation, then the agents causing linker region phosphorylation program the cell to prevent TGF-β signalling. Alternatively, when the linker region phosphorylation leads to a specific transcription mediated cellular response then it is possible to speculate that the cell is favourably programmed to express this activity when stimulated by TGF-β. Receptor tyrosine kinase agonists such as PDGF and fibroblast growth factor (FGF)-2 elicit very strong activation of MAP kinases and again speculatively stronger linker region phosphorylation. The stronger linker region phosphorylation may be manifest as either or both of qualitative and qualitative changes in phosphorylation. It is possible that linker region phosphorylation downstream of TGF-β may elicit one response, whereas this may be overwhelmed by cell receptor tyrosine kinase derived phosphorylation. Aortic valve myofibroblasts respond to TGF-β in the classic manner and concomitant treatment with FGF-2 prevents the nuclear translocation of Smad3, and this inhibits the fibrotic action of TGF-β [15]. This response is blocked by an inhibitor of Erk1/2. Thus, the origin and extent of MAP kinase activation and its ultimate action on Smad2/3 linker region phosphorylation are a very interesting area for further work to understand the cross talk from cell surface serine/threonine and tyrosine kinase receptors mediated via phosphorylation of the linker region of R-Smads.

The manner in which TGF-β and other agonists result in the activation of MAP kinases and the C terminal and linker region phosphorylation of Smads leading to the subsequent cellular effects in this case on the synthesis of extracellular matrix is depicted schematically in Fig. 2.

Non-linker region interactions and regulations of Smads and MAP kinases

TGF-β stimulation of MAP kinases is not physiologically restricted to linker region phosphorylation of Smads. The MAP kinases are known to have actions and interactions on Smads that do not involve linker region phosphorylation.

Jnk has been shown to signal indirectly through the TGF-β receptor independent of Smads during apoptosis [28]. Interestingly, c-jun, the downstream substrate of Jnk, has been implicated in Smad repression by stabilising a complex involving the Smad repressor, Ski [29]. This complex inhibits any Smad basal activity in the absence of TGF-β; however, stimulation by TGF-β causes dissociation of c-jun from the complex and hence allows Smad activation. In addition, activation of the Jnk cascade overwhelms the ability of TGF-β to induce c-jun

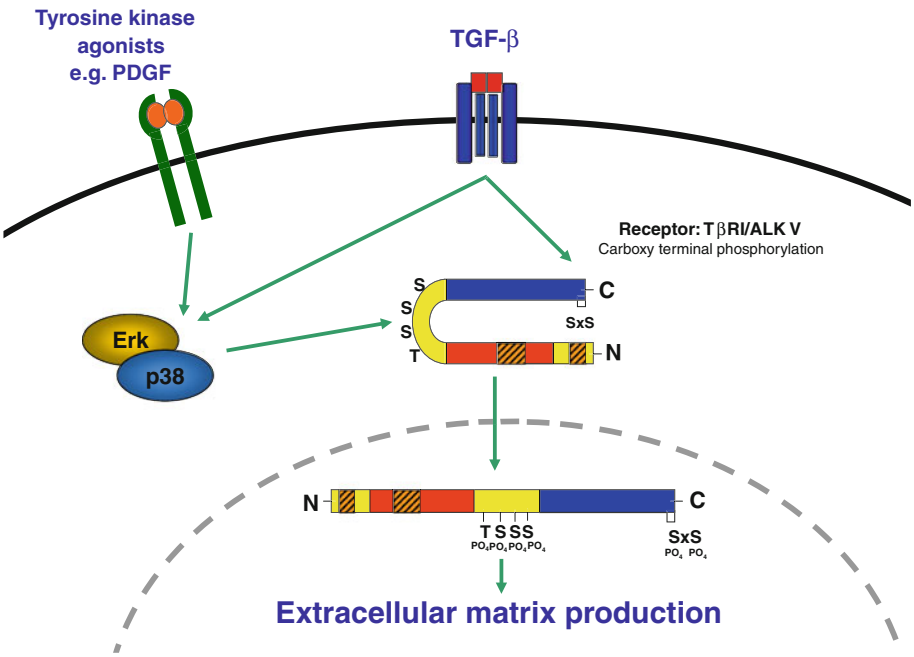

Fig. 2 Stylised schematic depicting the current hypothesis for growth factor-mediated extracellular matrix production via phosphorylation of the linker region of Smad2. The linker region of Smad2 is phosphorylated by the MAP kinases Erk and/or p38 (in the case of proteoglycan synthesis) stimulated by either TGF-β or other agonists capable of activating MAP kinases (e.g., PDGF). This pre-programs Smad2 for carboxy terminal phosphorylation and activation by Alk5. The fully activated Smad2 translocates to the nucleus where it controls transcriptional activity of extracellular matrix molecules (i.e., collagen and the proteoglycan, biglycan)

dissociation [29], suggesting a possible repressive role for Jnk in TGF-β signalling.

Conversely Erk has been shown to enhance the transcriptional ability of Smad2 due to additional phosphorylation of the Smad protein at Thr8 in the MH1 domain. This was shown using cells transgenic for mutated Smad2 that lack this Erk binding site, which resulted in reduced transcription [30].

p38 MAP kinase activation has been shown to occur downstream of Smad signalling. The Smad pathway activates the transcription and translation of GADD45, which then activates the MAPKKK, MKK6, an upstream activator of p38. This causes a delay in the activation of p38 after the initial TGF-β receptor activation [32, 33]. The Smad/GADD45/p38 pathway is responsible for the upregulation of the interstitial proteoglycan biglycan expression in pancreatic carcinoma cells [49]. The TGF-β pathway involving activation of p38 has also been implicated in the potent growth arrest of mouse primary VSMCs. Pharmacological inhibition of both Alk 5 and p38 attenuates the ability of TGF-β to induce these anti-proliferative effects [34].

MAP kinase regulation of TGF-β mediated Smad signalling through Smad linker region phosphorylation

The current dogma, supported by evidence, suggests that linker region phosphorylation of Smads prevents translocation of the phosphorylated Smads to the nucleus and thus blocks TGF-β signalling [30, 50]. As a classic example, Kretzschmar et al. [30] showed that activation of Ras/MAP kinase leads to phosphorylation of the linker region of Smads, which results in nuclear exclusion of the phosphorylated Smad and resistance to or inhibition of the action of TGF-β. A further recent example is the effect of TGF-β on activation of aortic valve myofibroblasts [15]. In these myofibroblasts TGF-β induces increased expression of smooth muscle α-actin and prevents the exit of cells from the cell cycle. Both of these responses are inhibited by FGF-2 [15]. TGF-β stimulates accumulation of Smads in the nucleus of these cells, and this translocation is prevented by co-treatment with FGF-2. The actions of FGF-2 are blocked by the Erk inhibitor UO126, and cells are activated in the presence of FGF-2 and the Erk inhibitor. The mechanism is that FGF-2 induces Erk-dependent phosphorylation of the linker region of Smad2/3, which prevents translocation to the nucleus and prevents the profibrotic action of TGF-β [15].

The nuclear translocation—importation and exportation—of phosphoSmad2 and phosphoSmad3 is mediated by Nup 215, Nup 153 and importin β [50]. Smad subcellular localisation is controlled by cytoplasmic and nuclear retention factors. Thus, linker region phosphorylation could result in cytoplasmic retention (as opposed to inhibited nuclear translocation) by blocking the interaction of the polyphosphorylated Smad with the nuclear pore translocation complex. Alternatively, linker region phosphorylation could prevent nuclear translocation by increasing the affinity of binding of the phosphorylated Smad2 complex to a cytoplasmic nuclear anchor.

The fate and function of linker region phosphorylated Smads can depend on further factors. Kretzschmar et al. [30] showed that linker region phosphorylation of Smads only occurs at sub-maximal doses of TGF-β. This is an

interesting example of the phenomena whereby, non-classically, the response to complex agonists such as growth factors can vary not only quantitatively but also are qualitatively different throughout the dose response curve of a particular agonist [51]. Classically it is expected that the response to agonist varies only quantitatively; however, for agonists with complex signalling pathways such as TGF-β, the response at a signalling and even functional level can vary across the dose response relationship.

In contrast to popular dogma, there are examples of MAP kinase activation that does not lead to nuclear exclusion. Sustained activation of Raf, the upstream mediator of Erk, has been shown to block cells from apoptotic signals, allowing them to respond to TGF-β. This indicates that Smads and the Erk pathway work in tandem in some circumstances [52], albeit not directly related to linker region phosphorylation. Recent evidence is emerging that under certain circumstances, MAP kinase-mediated phosphorylation of the linker region of Smad2 actually enhances nuclear translocation, transcriptional regulation and control of the synthesis of extracellular matrix molecules [46, 56] (discussed in detail in the next section) and may provide cell- and context-specific roles for Smad linker region phosphorylation.

There is considerable variation in the ability of linker region phosphorylation to cause nuclear exclusion. Many of the factors are discussed above, but further work is required to define the factors that determine the fate of linker region phosphorylated Smads.

Smad linker region phosphorylation regulation of extracellular matrix synthesis

Collagen synthesis in fibroblasts

The collagen family are an abundant group of large extracellular matrix molecules that play important roles in development and the physiology of all body tissues. In addition, due to excessive production, collagens are also involved in a myriad of disorders, termed fibrosis, that effect many organs and tissues of the body including the skin, lung, kidney and cardiovascular system [53, 54]. TGF-β plays a pivotal role in the synthesis of collagen [55], but the signalling pathway(s) through which it elicits this response has not been completely elucidated, and it remains a topic of interest because of its potential as a therapeutic target. Initially it was suggested that TGF-β may regulate collagen synthesis via the interaction of Erk with the canonical Smad pathway. Inhibition of Erk2 using small interfering RNA (siRNA) was sufficient to significantly diminish basal and TGF-β induced type I and type III collagen synthesis in rat joint adhesion tissue fibroblasts [56]. In human mesangial cells TGF-β causes a marked increase in phosphorylation of the SSXS, extreme C-terminal of Smad2/3, which is unresponsive to pharmacological inhibition of Erk [57]. Interestingly, pharmacological blockade of Erk caused inhibition of total serine phosphorylation of immuno-precipitated Smad2/3, indicating an effect on phosphorylation sites outside of the receptor-mediated SSXS domain [57]. Further to this, over-expression of a Smad3 mutant with C-terminal serine residues converted to alanine still presented with serine phosphorylation. To evaluate a functional role for the interaction of Erk with Smad signalling, the authors examined α2(1) collagen promoter activity. Over-expression of a constitutively active upstream mediator of Erk, caMEK1 increased basal and TGF-β-treated promoter reporter levels. This effect was abolished with over-expression of another Smad3 mutant in which the linker region serine phosphorylation sites were converted to alanines [57]. This work demonstrated the possibility that Erk, acting on the linker region of Smad2/3, plays a role in TGF-β-mediated collagen synthesis. Very recently, this finding was verified and extended upon in NIH/3T3 fibroblasts. Li et al. [13] showed that RNA interference of Erk2, but not Erk1 attenuated TGF-β stimulation of Smad2 linker region phosphorylation, but had no effect on the phosphorylation of C-terminal SSXS motif. The protein levels of type 1 (COL1) and type 3 (COL3) collagen were also abolished using Erk2, but not Erk1 siRNA. Introduction of a Smad2 linker region mutant, similar to that of the Smad3 mutant used by Hayashida et al. [57], blocked TGF-β linker region phosphorylation, which was associated with inhibition of TGF-β stimulated COL1 and COL3 mRNA expression and protein levels [13]. Understanding the mechanisms through which TGF-β mediates collagen synthesis in basal and fibrotic conditions may provide a therapeutic strategy for the treatment of fibrotic disorders.

Proteoglycan synthesis in vascular smooth muscle

Growth factor-mediated proteoglycan synthesis by VSMCs is of importance because it has been implicated as an initiating event in atherosclerosis [58, 59]. The response to retention hypothesis states that atherosclerosis is initiated by the binding of atherogenic lipoproteins to extracellular matrix molecules particularly proteoglycans [60]. Further, the hyperelongated glycosaminoglycan (GAG) chains CS/DS proteoglycans such as biglycan occurring when VSMCs are stimulated with growth factors bind lipoproteins with increased affinity and capacity [61]. Cardiovascular drugs and metabolic and pharmacological agents block GAG hyperelongation as an indicator of a potential therapeutic activity [48, 62, 63]. The signalling pathways that regulate proteoglycan and GAG synthesis

have been proposed as therapeutic targets for the prevention of atherosclerosis [64, 65]. Recently proof of principle for this proposal has been provided in a murine model of atherosclerosis in which treatment with a protein tyrosine kinase inhibitor which prevents PDGF mediated GAG hyperelongation on biglycan attenuated lipid deposition on the vessel wall of high fat fed ApoE$^{-/-}$ mice [66]. Notwithstanding our finding in the murine model it is widely appreciated that the role of lipid trapping by proteoglycans is more prominent and important in human atherosclerosis relative to animal models, the latter of which is biased towards the later phase inflammatory response that leads to the development of complex atherosclerotic plaques [67].

TGF-β treatment of VSMCs regulates the synthesis and structure of proteoglycans and such proteoglycans show increased binding to LDL [68, 69]. We initially showed that TGF-β invoked the canonical Smad2/phosphoSmad2 pathway and thus carboxy terminal phosphorylation of Smad2 [69]. In the VSMCs, all three MAP kinases are phosphorylated and thus activated by treatment of VSMCs with TGF-β, but only p38 and Erk1/2 are involved in proteoglycan synthesis [46]. Watanabe et al. [70] had earlier shown that in confluent undifferentiated chondrocytes in which TGF-β mediates phosphorylation of Erk and p38 (but not Jnk), the involvement of the MAP kinases acting through phosphorylation of Smads is required for the increased expression of the proteoglycan, aggrecan. Using a pharmacological approach it was demonstrated that the phosphorylation of the linker region of Smad2 induced by treatment of cells with TGF-β was mediated by p38 and Erk 1/2 MAP kinases but not by Jnk [46]. Inhibition of Erk and p38 but not Jnk MAP kinase inhibits TGF-β stimulated linker region phosphorylation, but inhibitors of all three MAP kinases have no effect on TGF-β stimulated C terminal phosphorylation, which occurs as a direct action of Alk 5 on R-Smads (see Fig. 3) [46]. Linker region phosphorylation can be inhibitory to TGF-β signalling by blocking the translocation of Smad2/3 to the nucleus but we demonstrated appreciable levels of linker region phosphorylated Smad2 in a nuclear fraction isolated from the TGF-β treatment VSMCs [46]. This led us to suggest that linker region phosphorylation leads to the activation of the genes that ultimately mediate GAG elongation in these cells [61, 71]. We have demonstrated that GAG hyperelongation by a range of growth factors requires gene transcription and de novo protein translation [72], although the nature of the genes and their subsequent downstream mediators presently remains unknown [71]. Our finding of a high level of linker region phosphorylated Smad in the nucleus of VSMC treated with TGF-β is consistent with linker region phosphorylation determining GAG hyperelongation, which is related to increased binding to LDL as an initiating factor in atherosclerosis [61].

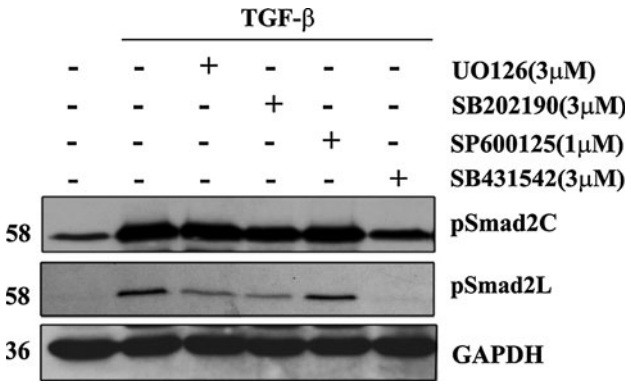

Fig. 3 An example of the distinct pathways leading to the phosphorylation of R- Smad transcription factor in the carboxy terminal and the linker region. Figure (*upper line of blots*) shows the TGF-β-mediated phosphorylation of the carboxy terminal of Smad2 (pSmad2C), which is completely unaffected by inhibitors of MAP kinases (Erk1/2: UO126; p38: SB202190 and Jnk: SP600125), but is blocked by the Alk 5 antagonist SB431542. The linker region phosphorylation (pSmad2L) (*middle line*) is less strongly induced compared to the carboxy terminal phosphorylation, and it is reduced by Erk and p38, but not Jnk inhibitors. Note that both responses are blocked by SB431542, indicating that they originate with the TβRI/Alk 5 receptor (reprinted from Burch et al. 46] with permission of Springer Basel AG)

Summary and conclusions

The study of the actions of TGF-β has yielded considerable new information directly related to the role of the growth factor in health and disease, but also in more general regards to the area of cell signalling. TGF-β signalling involves the phosphorylation of ubiquitous Smad transcription factors and the exquisite regulation of transcription. R-Smad transcription factors are phosphorylated directly by the TGF-β receptor TβRI in their carboxy terminal to affect cell regulation. R-Smads have a linker domain that also contains serine and threonine residues susceptible to phosphorylation. These residues can be phosphorylated by MAP kinases and other kinases. MAP kinases can be activated by TGF-β, but also by a multitude of other agonists that activate MAP kinases. Initial data indicated that linker region phosphorylation was antagonistic to TGF-β signalling, but there has been a slow increase in examples of where linker region phosphorylation promotes TGF-β signalling, and much of this has occurred in the context of the regulation of the synthesis of extracellular matrix. As multiple agonists can mediate linker region phosphorylation, then this is potentially an important integrating pathway for the determination of the effects of agonists on cells. Extracellular matrix molecules play a somewhat under-recognised role in the physiology and pathology, and a pathway that integrates and regulates the cell signalling leading to the control of the synthesis of extracellular matrix represents an important area of

investigation to understand the regulation of matrix synthesis and also potentially as a therapeutic target for manipulation of matrix synthesis in disease states.

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