COMMENTARY

Linking Smads and transcriptional activation

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TGF-β1 (transforming growth factor-β1) is the prototypical member of a large family of pleiotropic cytokines that regulate diverse biological processes during development and adult tissue homoeostasis. TGF-β signals via membrane bound serine/threonine kinase receptors which transmit their signals via the intracellular signalling molecules Smad2, Smad3 and Smad4. These Smads contain conserved MH1 and MH2 domains separated by a flexible linker domain. Smad2 and Smad3 act as kinase substrates for the receptors, and, following phosphorylation, they form complexes with Smad4 and translocate to the nucleus. These Smad complexes regulate gene expression and ultimately determine the biological response to TGF-β. In this issue of the *Biochemical Journal*, Wang et al. have shown that, like Smad4, the linker domain of Smad3 contains a Smad transcriptional activation domain. This is capable of recruiting the p300 transcriptional co-activator and is required for Smad3-dependent transcriptional activation. This study raises interesting questions about the nature and regulation of Smad-regulated gene activation and elevates the status of the linker domain to rival that of the much-lauded MH1 and MH2 domains.

Key words: co-activator, Smad, Smad activation domain (SAD), transcription, transforming growth factor-β (TGF-β).

There is considerable interest in delineating the molecular mechanisms of action of TGF-β (transforming growth factor-β), as TGF-β signalling has been linked with a plethora of human conditions, including cancer, fibrosis and autoimmune disease [1].

TGF-β signals transduced from the plasma membrane to the nucleus ultimately result in an alteration of the gene expression programme, with each step in this process acting as a point of control and a potential target for therapeutic intervention. Following ligand activation, TGF-β binds to the high-affinity constitutively active type II receptor (TβRII), which then recruits the type I receptor (TβRI/ALK5). TβRII phosphorylates and activates ALK5, which in turn phosphorylates downstream substrates. Several signalling pathways are known to operate downstream of TGF-β receptors, but by far the best characterized is the Smad pathway [2]. The receptor-regulated Smads (R-Smads) Smad2 and Smad3 are direct targets of ALK5. Phosphorylation occurs on the two C-terminal serine residues in the SSXS (Ser-Ser-Xaa-Ser) motif at the extreme C-termini of Smads 2 and 3. Following phosphorylation, the R-Smads form heterodimeric or heterotrimeric complexes with Smad4 via their MH2 domains and translocate to the nucleus [3,4]. These Smad complexes are involved directly in transcriptional regulation of target genes, usually in association with other sequence-specific DNA-binding transcription factors [3].

Smad3 and Smad4 can bind directly to the SBE (Smad-binding DNA element), which contains only four base pairs (5′-GTCT-3′ or its reverse complement, 5′-AGAC-3′), via their N-terminal MH1 domains. These interactions are weak and insufficient to convey promoter selectivity [2], hence Smad3–Smad4 complexes synergize with other transcription factors, such as members of the AP-1 family, TFE3 and FoxG1 [3,5] to regulate gene expression.

Despite being highly homologous with Smad3, Smad2 is unable to bind to DNA directly owing to the insertion of an extra 30 amino acids immediately before the DNA-binding hairpin. Smad2–Smad4 complexes do not bind DNA alone, but require other transcription factors to target them to specific sequences (reviewed in [6]). TGF-β signal transduction results in the initiation of a new program of gene expression. It is therefore essential to determine how the Smads are regulated and how they in turn regulate transcription. Fundamental to the understanding of these processes is a detailed characterization both of the TGF-β-induced Smad heteromeric complexes, and of the complexes that assemble at promoter elements with sequence-specific transcription factors, co-activators and/or co-repressors. Consequently, there has been a flurry of papers and a vigorous scientific effort devoted to characterizing the molecular mechanisms of Smad-mediated transcriptional regulation. Recent data indicate that Smads may repress gene expression by indirect recruitment of transcriptional co-repressor complexes via proteins such c-Ski, SnoN, TGIF, E2F4/5 and ATF2 (reviewed in [2,3]).

It is becoming increasingly clear that co-activators function either directly or indirectly to provide enzymatic activities that are absolutely required to alter chromatin structure from a quiescent non-permissive to an active transcriptionally permissive state (see [7] and references cited therein). Transcriptional co-activators can be broadly sorted into three classes. Multi-component complexes of the SWI/SNF family contain ATP-dependent DNA-unwinding activities which are required for efficient gene transcription *in vivo*. A second class of activators are members of the TRAP–DRIP–Mediator–ARC complex. These complexes contain proteins which recruit RNA polymerase II directly and interact with the general transcription apparatus. A third class of proteins modify histones and alter the so-called ‘histone code’ to facilitate the access of transcriptional regulators to DNA. Included in this class are the HATs (histone acetyltransferases) p300 and CBP (cAMP-response-element-binding-protein-binding protein), and methyltransferases.

Recent work has demonstrated that Smads 2, 3 and 4 can recruit the Mediator complex via an MH2-dependent interaction with ARC105, possibly facilitating recruitment of RNA polymerase II to TGF-β immediate early target genes [8]. Earlier work demonstrated that Smads 2 and 3 can also recruit the p300 and CBP

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HATs to activate transcription in an MH2-dependent fashion [3]. Similarly MH2-dependent interaction of Smad4 with p300 and CBP via SMIF and MSG1 has been reported (see [2]). Previous studies have also demonstrated that Smad4 contains an intrinsic 48-amino-acid p300-dependent SAD (Smad activation domain) present in the linker [9].

In an incisive report in this issue of the *Biochemical Journal*, Wang et al. [10] shed further light on Smad-dependent transcriptional activation. Wang et al. [10] performed a similar analysis to that undertaken by de Caestecker et al. [9] when studying Smad4, and demonstrate that Smad3 also contains a SAD. Fusion of the linker domain of Smad3 to the heterologous GAL4 DNA-binding domain revealed a ligand-independent transcriptional activation potential comparable with the Smad4 SAD. This activity could be blocked by wild-type adenovirus E1a protein, but not by an E1a mutant that fails to bind p300. p300 expression could partially rescue E1a-mediated repression, and immunoprecipitation analysis revealed that the Smad3 linker could bind p300. Importantly, Wang et al. [10] went on to demonstrate that, in the context of full-length Smad3, a deletion mutant that deletes the linker can no longer support TGF-β-mediated transcriptional activation of reporter genes, despite maintaining the ability to be phosphorylated and form complexes with Smad4 in solution. Further experiments employing the GAL4 system demonstrated that, like Smad4, the MH2 domain and the SAD domains of Smad3 can both activate transcription and co-operate together. The results reported by Wang et al. [10] clearly demonstrate that Smad3, as well as Smad4, may provide SAD function in the regulation of TGF-β target genes. This study potentially sheds light on several previous findings, and suggests many further avenues for important future research.

The biological outcome of a TGF-β signal depends on the dose and duration of the signal, as well as the type, state and environment of the target cell. Smad-interacting transcription factors have different intrinsic affinities for Smad complexes, and target them to distinct genes. The availability of active Smad complexes dictates which genes are activated, and ultimately determines the biological response to TGF-β. Studies of the stoichiometry of Smad complexes in TGF-β treated *in vitro* and *in vivo* have demonstrated that Smad2–Smad4 and Smad3–Smad4 complexes may be either dimeric or trimeric in nature, containing one Smad4 [2,4,11,12]. Previous dogma has stated that the transcriptional activity of these complexes is dependent on Smad4 and its SAD domain. The discovery of a SAD in Smad3 now suggests that all these Smad complexes are not created equal, and may contain different numbers of transcriptional co-activator molecules conferring varying transcriptional rates on different target genes. It will be important in the future to determine the exact stoichiometries of all of the components of Smad transcriptional complexes, which will require considerable crystallographic analyses. These have so far been hampered by the 'flexible' nature of the Smad linkers when studied in the context of isolated Smads, and, intriguingly, these need to be tryptically cleaved off to successfully crystallize Smad hetero-oligomers [12]. Addition of DNA and the SAD-associated components may be required to reveal the nature of Smad transcriptional regulating complexes.

The observation of Wang et al. [10] that p300 can efficiently, but not completely, rescue the E1a-dependent repression of SAD activity indicates that other transcriptional regulatory components may interact with the SADs to activate gene expression. Indeed, several other cellular proteins have been shown to associate with the N-terminus of E1a that are involved in transcriptional regulatory processes [e.g. TBP (TATA-box-binding protein) and RAP30 (30 kDa RNA polymerase II-associated protein); see the E1a database at http://www.geocities.com/jmymryk.geo/ and references therein]. Investigation into which cellular proteins interact with SADs should shed light on how Smads regulate transcription. It is likely that Smad complexes will utilize SWI/SNF-related and methyltransferase complexes to regulate gene expression.

In addition to these newly ascribed roles of the linker domain of Smad3 in transcriptional activation, the linker domains of Smads 2 and 3 also act as sensors of Smad-independent signal transduction cascades. There are at least four potential phosphorylation sites in the linker region of Smads 2 and 3 which can be phosphorylated by p38 MAPK (mitogen-activated protein kinase), ERK1/2 (extracellular-signal-regulated kinase 1/2), ROCK (Rho-associated kinase), and CDK (cyclin-dependent kinase) 2 and CDK4 [13–16]. Mutation of these sites and/or inhibition of these pathways leads to cell-type-specific effects on the gene regulatory potential of the Smads [13–16]. Given the results of Wang et al. [10], it is tempting to speculate that these kinase pathways regulate Smad transcriptional potential by modulating the recruitment of transcriptional co-activators. Furthermore, the identification of the Smad3 SAD suggests that Smad4-independent gene regulation [17] may take place utilizing this domain. Formation of homo- or hetero-oligomers of Smad4-deficient Smad complexes can occur *in vitro*, and is possible *in vivo* [12]. The identification of Smad4-independent target genes may reveal Smad3-dependent targets that are associated with Smad4-deficiency-associated pathology.

The straightforward approach of Wang et al. [10] potentially paves the way for many more fruitful investigations. Theirs and the recent studies of others enhance the importance of the divergent Smad linker regions. This divergence, coupled with the controversial role of linker phosphorylation and the intractability of the linker to crystallization has led to a perception of the linker region acting as a flexible hinge to allow the more important MH1 and MH2 domains to go about their business. It is probably time to consider the linker as a third important structural domain in Smad biology.

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Received 23 December 2004/10 January 2005; accepted 11 January 2005
Published on the Internet 8 February 2005, DOI 10.1042/BJ20042133