Smad Signal Transduction

PROTEINS AND CELL REGULATION

Volume 5

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Aims and Scope

Our knowledge of the ways in which a cell communicates with its environment and how it responds to information received has reached a level of almost bewildering complexity. The large diagrams of cells to be found on the walls of many a biologist's office are usually adorned with parallel and interconnecting pathways linking the multitude of components and suggest a clear logic and understanding of the role played by each protein. Of course this two-dimensional, albeit often colourful representation takes no account of the three-dimensional structure of a cell, the nature of the external and internal milieu, the dynamics of changes in protein levels and interactions, or the variations between cells in different tissues.

Each book in this series, entitled "*Proteins and Cell Regulation*", will seek to explore specific protein families or categories of proteins from the viewpoint of the general and specific functions they provide and their involvement in the dynamic behaviour of a cell. Content will range from basic protein structure and function to consideration of cell type-specific features and the consequences of disease-associated changes and potential therapeutic intervention. So that the books represent the most up-to-date understanding, contributors will be prominent researchers in each particular area. Although aimed at graduate, postgraduate and principle investigators, the books will also be of use to science and medical undergraduates and to those wishing to understand basic cellular processes and develop novel therapeutic interventions for specific diseases.

Smad Signal Transduction

Smads in Proliferation, Differentiation and Disease

Edited by

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Cover Legend: Phylogenetic tree of the SMAD protein family: members from human, Drosophila melanogaster (fruit fly) and Caenorhabditis elegans (nematode) are shown. The topology of the tree is the same as the tree shown in Chapter 1 by Newfeld and Wisotzkey (Figure 1); see the figure legend for details. Here each of the seven subfamilies is shown in a different color. Note that human and fly proteins are clustered together in four subfamilies while three subfamilies contain only nematode sequences.

Dedicated to the memory of Dr. Anita Roberts who sadly passed away
on May 25, 2006, at the age of 64. Dr. Roberts was a leader in the field
of TGF-β research, and through her remarkable personality
she was an inspiration to us all.

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PREFACE

THE SMAD FAMILY

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1. INTRODUCTION

About 10 years ago, our understanding of how signals from transforming growth factor-β (TGF-β) family members and their specific serine/threonine kinase receptors are transduced from the plasma membrane to the nucleus, was a black box. Yeast two-hybrid screening approaches with the intracellular domain of TGF-β superfamily receptors as baits, were initiated by several laboratories, but failed to identify critical intracellular downstream effectors. A breakthrough came through genetic studies in *Drosophila*; in screens for dominant enhancers of weak *dpp* alleles (dpp is the TGF-β homolog in *Drosophila*) *Mothers against dpp (Mad)* and *Medea* were discovered (Raftery et al., 1995; Sekelsky et al., 1995). Homozygous *Mad* and *Medea* mutants are phenotypically similar to dpp mutants. In *C. elegans, daf-4* encodes a serine/threonine kinase receptor and *daf-4* mutants are dauer-constitutive and smaller than wild-type. Screening for mutants with the same small *daf-4* phenotype revealed three genes, *sma-2, sma-3* and *sma-4* (Savage et al., 1996). Mad, Medea and Sma proteins were found to be essential components downstream of TGF-β receptor signaling pathways in these lower invertebrates (Newfeld et al., 1996; Wiersdorff et al., 1996) (see Chapters 2 and 3). Shortly thereafter, homologous *Mad* and *sma*-related genes were identified in *Xenopus*, mouse and man, and shown to function as principal effectors downstream of serine/threonine kinase receptors in vertebrates (Eppert et al., 1996; Graff et al., 1996; Hoodless et al., 1996; Liu et al., 1996; Thompson et al., 1996) (see Chapter 1). The designation Smad was then suggested for the vertebrate homologues of Sma and Mad (Derynck et al., 1996).

After the discovery of Smads, several laboratories independently, and at about the same time, identified additional members of the Smad gene family through their

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homology with Sma and Mad genes by PCR cloning and/or mining of expressed sequence tags (EST) databases. Since then intensive work has been devoted to elucidate which Smads are activated by specific type I serine/threonine kinase receptors, the subcellular localization of Smads before and after ligand stimulation, the role of Smads as transcriptional factors, modulation of Smad function by interacting proteins, and the *in vivo* function and role in disease of Smads. These efforts led to a remarkably quick progress in our understanding of the mechanism of action of Smads; their role as principle intracellular downstream effectors for TGF-β family members is now firmly established (Attisano and Wrana, 2002; Derynck and Zhang, 2003; Shi and Massagué, 2003; ten Dijke and Hill, 2004) (see Chapters 2-8). Smad activation, subcellular distribution and stability have been shown to be intricately regulated (see Chapters 9-16), and Smads have been found to function as signal integrators within an extensive intracellular network (see Chapters 14-18).

This volume provides an in-depth review of the rapidly developing field of Smad research, in which structures are integrated with *in vivo* functions (see Chapter 11). Moreover, the impact of functional genomics and systems biology approaches on Smad signaling (see Chapters 17 and 18), links between alterations in Smad signaling and disease (see Chapters 19 and 20) and how this knowledge may come to be applied in the clinic (see Chapters 21 and 22), will be discussed. In this preface, we will start by reviewing the TGF-β family and their specific type I and type II serine/threonine kinase receptors, and will subsequently introduce the Smad family.

2. TGF-β FAMILY MEMBERS AND THEIR SIGNALING RECEPTORS

2.1 TGF-β Family Members are Multifunctional Cytokines

TGF-β family members, which include TGF-βs, Activins, and bone morphogenetic proteins (BMPs)/growth and differentiation factors (GDFs), are structurally related secreted dimeric cytokines (Roberts and Sporn, 1990). They are produced by cells as larger precursor proteins that are processed within the Golgi apparatus by endoproteases of the convertase family (*e.g.* furin) (Dubois et al., 1995) (Fig. 1A). Upon cleavage, the amino-terminal remnant, also termed latency-associated peptide (LAP), remains non-covalently associated to the carboxy-terminal part that contains the mature protein. LAP prevents binding of mature ligand to the receptor and thus keeps the ligand inactive (Annes et al., 2004). The mature TGF-β can be released from the inactive complexes by several mechanisms, including cleavage of LAP by proteases, such as plasmin (Lyons et al., 1988), and through action of LAP binding proteins, such as thrombospondin (Crawford et al., 1998). This mechanism of latency imposed by LAP has been mainly investigated and demonstrated for TGF-βs; whether it also occurs for the many other TGF-β family members remains to be investigated.

The TGF-β family members share most similarity in their mature domains that have a characteristic cystine knot motif. At least 34 family members have been

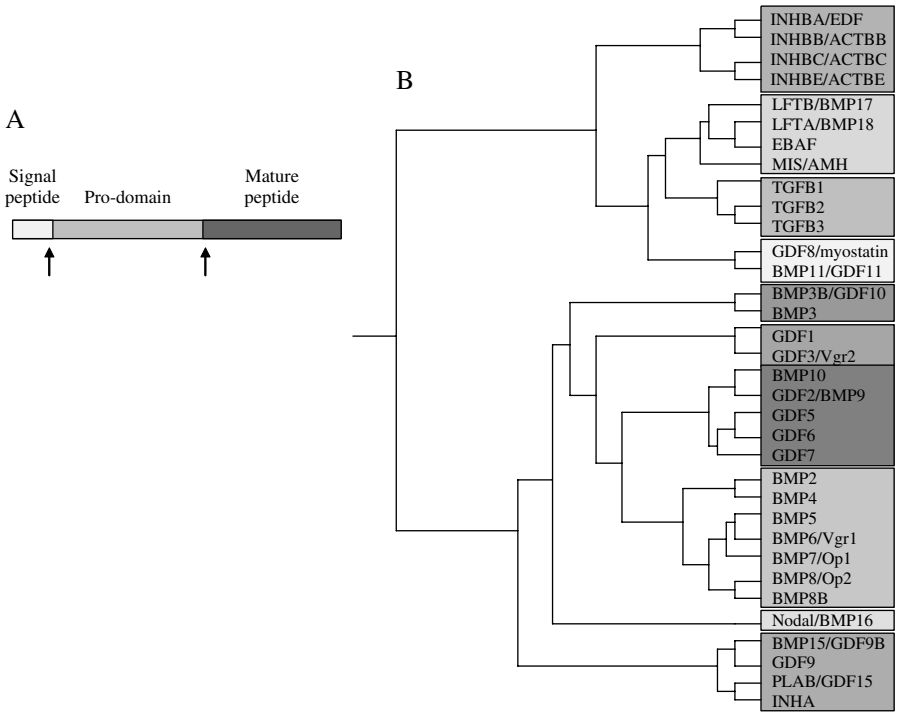

Figure 1. TGF-β family members: multiple cytokines with pleiotropic functions. (A) Schematic structure of TGF-β family members. The precursor of TGF-β family members is composed of a signal peptide, an amino-terminal propeptide (also termed latency-associated peptide) and a carboxy-terminal mature ligand. (B) Phylogenetic analysis of the TGF-β family. Reproduced with permission and copyright © of the Britisch Editorial Soceity of Bone and Joint Surgery (ten Dijke et al., J. Bone Joint Surg 2003, 85-B:34-8) (A color version of this figure is freely accessible via the website of the book: http://www.springer.com/1-4020-4542-5)

Abbreviations: ACT, Activin; BMP, bone morphogenetic protein; GDF, growth and differentiation factor; INH, inhibin; MIS/AMH, müllerian inhibiting substance/anti-müllerian hormone; OP, osteogenic protein; TGF-β, transforming growth factor β

identified in the human genome (Fig. 1B). TGF-β, the founding member of this family, was discovered in the late 70's/early 80's as a factor produced by virus-transformed cells with the ability to induce the growth of normal rat kidney cells in soft agar (DeLarco and Todaro, 1976; Roberts et al., 1981). Subsequent studies demonstrated that TGF-β has a potent growth inhibitory activity (Tucker et al., 1984), and, in fact, is a pleiotropic molecule that regulates cell proliferation, differentiation, apoptosis, migration, adhesion of many different cell types (Moses and Serra, 1996; Roberts and Sporn, 1990). Activin was originally identified as a factor that stimulates the secretion of follicle stimulating hormone from the pituitary gland (Mason et al., 1985), and as a stimulator of erythroid differentiation (Murata et al., 1988). BMP was first known for its ability to induce cartilage and bone (Wozney

et al., 1988). Subsequent studies on Activins and BMPs revealed that these, like TGF-β, also are multifunctional proteins (Massagué, 1990). Certain members of the TGF-β family, such as müllerian inhibiting substance (MIS)/anti-müllerian hormone (AMH), nodal, myostatin, and GDF-9, appear to have a restricted expression pattern and have been implicated in specific biological responses. However, it is likely that future studies will ascribe additional functions to these factors.

Often TGF-β family members act as homodimers, but heterodimers between different isoforms can also occur. In the case of Activins, four β chains (βA through βE) have been identified, of which βA and βB can form homo- as well as heterodimers. Moreover, inhibins that antagonize the activity of Activins, are heterodimers of inhibin α chains and Activin β chains (Mathews, 1994). In addition, a BMP2/7 heterodimer has been isolated from bone (Sampath et al., 1990) and shown to be more potent in bone induction than their respective homodimers (Israel et al., 1996).

TGF-β family members, and their downstream signaling components, can be found in species as diverse as nematodes, fruit flies, frogs, fish and mammals (see Chapters 1-3). Gene targeting approaches of TGF-β family ligands have revealed their pivotal roles in embryogenesis and in maintaining tissue homeostasis (Chang et al., 2002). Disruption of TGF-β signaling has been linked to various developmental disorders and numerous human diseases, including cancer, fibrosis and autoimmune diseases (see Chapters 19-22) (Blobe et al., 2000; Siegel and Massagué, 2003) (Fig. 2).

The multifunctional characteristics of TGF-β family members imply the need for tight control of their activities. Such control is exerted at different levels. TGF-β is synthesized as an inactive precursor form and is activated in a controlled manner (see above). In addition, the activity of TGF-β family members is kept in check by

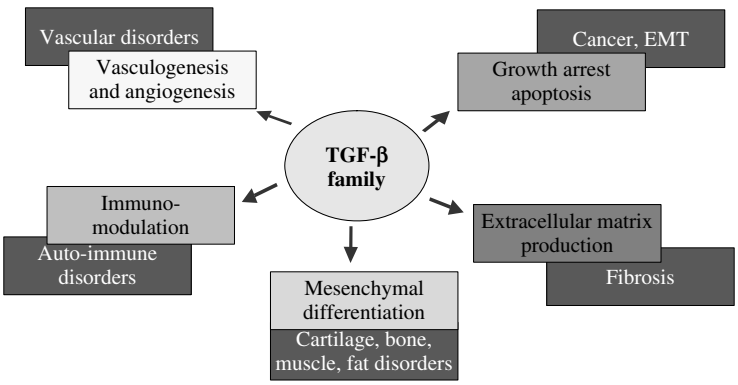

Figure 2. TGF-β family members are multifunctional proteins with crucial roles in embryonic development and in maintaining tissue homeostasis. For example, TGF-β inhibits proliferation of epithelial, endothelial and immune cells, stimulates mesenchymal cell proliferation and extracellular matrix production, regulates the migration and differentiation of many different cell types. Deregulation of their signaling has been implicated in several developmental disorders and in various human diseases including cancer, fibrosis, connective tissue diseases, auto-immune diseases and vascular diseases (A color version of this figure is freely accessible via the website of the book: http://www.springer.com/1-4020-4542-5)

interaction of specific extracellular inhibitors that prevent ligands from binding to signaling receptors (Balemans and Van Hul, 2002). For example, noggin strongly interacts with BMPs with affinities that resemble BMP binding to BMP receptors (Groppe et al., 2002), and follistatin sequesters Activins and prevents them from binding to Activin receptors (de Winter et al., 1996). Moreover, the TGF-β family members are rapidly cleared through the action of scavenger proteins, such as α2-macroglobulin (O'Connor-McCourt and Wakefield, 1987), which results in a short biological half-life of TGF-β family members (Wakefield et al., 1990).

2.2 TGF-β Serine/threonine Kinase Receptors

TGF-βs transduce their signals across the plasma membrane into the cell by inducing heteromeric complexes of type I and type II receptors with intrinsic serine/threonine kinase activity (Attisano and Wrana, 2002; Derynck and Zhang, 2003; Shi and Massagué, 2003; ten Dijke and Hill, 2004). The receptor types are structurally similar with short cysteine-rich extracellular domains, single transmembrane spanning regions, and intracellular parts with serine/threonine kinase domains (Fig. 3A). At least two type II receptors and two type I receptors are needed for signaling (Luo and Lodish, 1996; Weis-Garcia and Massagué, 1996), and probably form a heterotetrameric receptor complex (Yamashita et al., 1994). Five type II receptors and seven type I receptors, also termed Activin receptor-like kinases (ALKs), are present in the human genome (Fig. 3B). The TGF-β type II receptor, MIS type II receptor and BMP type II receptors only bind TGF-β, MIS/AMH and BMPs/GDFs, respectively, but Activin type IIA and type IIB receptors bind Activins, nodal as well as BMPs. Different members of the BMP family thusbind to different type II receptors. The type II receptor has constitutive kinase activity and upon ligand-induced heteromeric complex formation, the type II receptor kinase phosphorylates the type I receptor on particular serine and threonine residues in the juxtamembrane region (also termed GS-domain) (Wrana et al., 1994). Thus, type I receptors act downstream of type II receptors; consistent with this notion, type I receptors have been shown to determine the specificity of the heteromeric receptor complex (Cárcamo et al., 1995).

In most cells, ALK4 and ALK5 are type I receptors for Activin and TGF-β, respectively. Recently, GDF9 and myostatin have been shown to bind to ALK5 in cooperation with BMP type II receptor and Activin type II receptor, respectively (Mazerbourg et al., 2004; Rebbapragada et al., 2003). ALK4 and ALK7 are nodal type I receptors. BMPs (and possibly also MIS/AMH) generally signal via ALK2, ALK3 and ALK6 (Miyazono et al., 2005), but surprisingly, BMP3 has been shown to signal via Activin type II and ALK4 receptors (Daluiski et al., 2001). Thus, homodimeric and heterodimeric forms of individual BMPs are capable of recruiting different type I and type II receptors in the signaling receptor complex; moreover, individual receptors can bind several different ligands. Furthermore, ALK1, in addition to ALK5, is a signaling type I receptor for TGF-β in endothelial cells and neurons (Goumans et al., 2002; Konig et al., 2005). Interestingly, TGF-β signaling

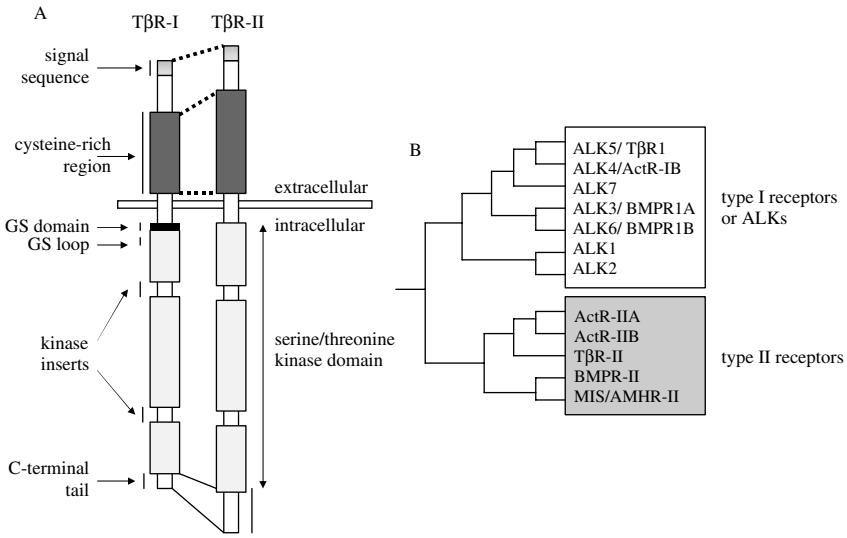

Figure 3. TGF-β family type I and type II receptors. (A) Schematic representations of human TGF-β type I and type II serine/threonine kinase receptors. TβR-I and TβR-II are single transmembrane proteins with short cysteine-rich extracellular domains and intracellular serine/threonine kinase domains with two short kinase inserts. The carboxy-tail of TβR-II is longer than that of TβR-I, and TβR-I contains a domain rich in glycine and serine amino acid residues (termed GS domain) in which particular serine and threonine residues are phosphorylated by TβR II kinase. The L45 loop in TβR I is an exposed nine-amino acid residue region within the kinase domain that is an important determinant for R-Smad interaction. Modified with permission from Figure 1A from Cardiovascular Research, 65(3):599-608, Lebrin, et al. © 2005 European Society to Cardiology. (B) Phylogenetic analysis of human type I and type II receptors of two distinct subfamilies. Five type II receptors and seven type I receptors (also termed ALKs) have been identified in humans (A color version of this figure is freely accessible via the website of the book: http://www.springer.com/1-4020-4542-5)

Abbreviations: ActR, Activin receptor; ALK, Activin receptor-like kinase; MIS/AMH, MIS/AMH receptor; BMPR, BMP receptor; TβR, TGF-β receptor

via ALK1 was shown to be dependent on the kinase activity of ALK5, thereby providing a lateral mode of signaling (Goumans et al., 2003). Whether similar lateral signaling occur for other ALKs or other ligands, needs to be investigated. The availability of more and more ligands as recombinant proteins, will allow a detailed determination of their preferences for type I and type II receptor partners.

3. THE SMAD FAMILY OF SIGNAL TRANSDUCERS

3.1 Nomenclature and Structure of Smads

The Smad family can be divided into three distinct subfamilies: receptor-regulated (R)-Smads, common partner (Co)-Smads and inhibitory (I)-Smads. Activated type I receptor kinases transiently interact with and phosphorylate particular R-Smads at

their extreme C-terminal serine residues (see Chapter 12) (Fig. 4A). Whereas Smad2 and Smad3 act downstream of ALK4, ALK5 and ALK7, Smad1, Smad5 and Smad8 are phosphorylated by ALK1, ALK2, ALK3 and ALK6 (Fig. 4C). The L45 loop in the type I receptor kinase domain determines the specificity of Smad isoform activation (see Chapter 11). Phosphorylated Smads form heteromeric complexes with Co-Smads that are shared components in signal transduction by TGF-β family members. Whereas one Co-Smad, *i.e*. Smad4, has been identified in mammals, two C-Smads, *i.e*. Smad4α and Smad4β (also termed Smad10), have been identified in *Xenopus*. Smad complexes accumulate in the nucleus (see Chapter 10), where they can bind to DNA directly or indirectly through other DNA binding proteins (see Chapter 15), and thus control the expression of target genes in a cell type-specific manner through interaction with co-activators and co-repressors (see Chapter 14).

R-Smads and Co-Smads have two highly similar regions at their amino terminal and carboxy terminal regions, termed Mad homology 1 (MH1) domain and MH2 domain, respectively (Fig. 4B). The two MH domains are separated by a less conserved linker region of variable length that is rich in proline residues. The MH1 domain of R-Smads, except Smad2, can bind through a protruding 11-residue β-hairpin directly to specific DNA sequences (Fig. 4B). The MH1 regions in R- and Co-Smads contain a nuclear localization signal-like (NLS-like) sequence (Fig. 4B), which in Smad3 and Smad4 has been shown to interact with importin β and α, respectively. Mutation of these NLS sequences prevent Smad nuclear accumulation

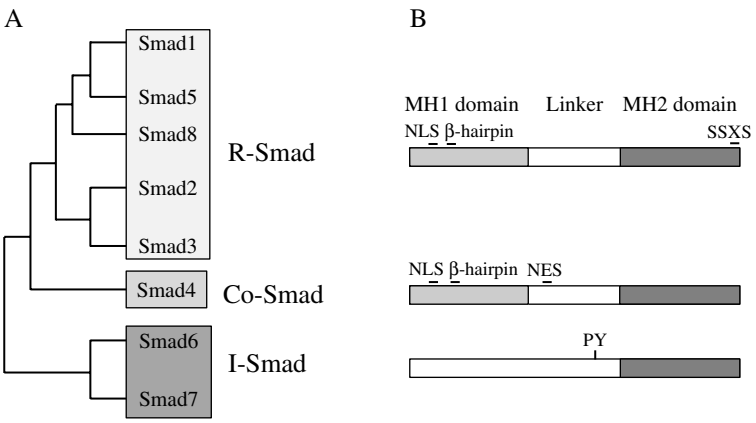

Figure 4. The Smad family. Phylogenetic analysis of human Smads and schematic representations of human Smad structures. The Smad family can be divided into three distinct subfamilies: Receptor-regulated (R)-Smads (*i.e*. Smad1, Smad2, Smad3, Smad5 and Smad8), Common-partner (Co)-Smad (*i.e*. Smad4) and Inhibitory (I)-Smads (*i.e*. Smad6 and Smad7). Conserved Mad Homology (MH)1 and MH2 domains are indicated. The β hairpin and the PPxY motif (PY) that mediates binding to DNA and Smad ubiquitin regulatory ligases (Smurfs), respectively, are indicated. Nuclear localization signal (NLS) and nuclear export signal (NES) important for nuclear-cytoplasmic translocations are also shown. The serines in the C-terminal SXS motif of R-Smads can be phosphorylated by type I receptor kinases (A color version of this figure is freely accessible via the website of the book: http://www.springer.com/1-4020-4542-5)

in response to TGF-β. Smad4 contains a nuclear export signal (NES) in the linker region (Fig. 4B), which interacts with the nuclear exporter CRM1. Formation of homomeric and heteromeric complexes among R- and Co-Smads are mediated via their MH2 domains (see Chapter 11). The MH2 domains of R- and Co-Smads can also recruit transcriptional co-activators and co-repressors (see Chapter 14).

I-Smads have a conserved MH2 domain, but their amino-terminal regions show only weak similarity to the MH1 domains of R- and Co-Smads (see Chapter 19) (Fig. 4B). I-Smads interact via a PY-motif with WW-domain containing HECT-domain ubiquitin ligases (Smurfs) (Fig. 4B). Upon recruitment of an I-Smad-Smurf complex to the activated receptor, Smurf induces receptor degradation via proteosomal and lysosomal pathways (Kavsak et al., 2000). Additional mechanisms by which I-Smads antagonize signaling have been described and are discussed in Chapter 19.

3.2 Activation and Regulation of Smad Function

The recruitment of Smads to activated TGF-β receptor complexes is carefully controlled. Several proteins with scaffolding, anchoring and/or chaperone activity have been identified. Smad anchor for receptor activation (SARA) is localized in early endosomes and, by interacting with non-activated Smads and receptor complexes, presents Smad2 or 3 for the type I receptor and promotes their phosphorylation and activation (see Chapter 9). In their non-activated state, the MH1 and MH2 domains interact and inhibit each others functions, *i.e.* the MH1 domain represses MH2-domain-mediated recruitment of transcriptional co-activators and the MH2 domain inhibits MH1-domain-mediated DNA binding. Upon C-terminal phosphorylation, R-Smads form homo- and hetero-oligomeric complexes of different stoichiometry with each other and with Smad4. Upon Smad complex formation, nuclear import sequences may become exposed and nuclear export sequences shielded, thereby inducing the nuclear accumulation of these complexes (see Chapter 10) (Fig. 5). The affinity of R- and Co-Smads for binding to DNA is relatively low and Smads therefore require other DNA sequence-specific binding factors to bind efficiently to promoters of target genes. Some of these Smad-interacting transcription factors are expressed in a cell-type specific manner and their activation state is subject to specific stimuli, thereby providing integration with other signaling pathways (see Chapter 14-18). In addition, signal integration is achieved through various post-translation modifications of Smads (in addition to the C-terminal phosphorylation by the activated type I receptor), including phosphorylation, poly- and mono-ubiquitination, sumoylation and acetylation. These modifications were found to change interaction of Smads with partner proteins or DNA, stability and/or subcellular localization (see Chapter 12 and 13). The transactivation or repression properties of Smads are mediated through interaction with co-activators and co-repressors that recruit, or contain intrinsic, histone acetyltransferase (HAT) or histone deacetylase (HDAC) activities, respectively, and thereby regulate chromosome condensation and accessibility of Smads with the basal transcription machinery (see Chapter 14).

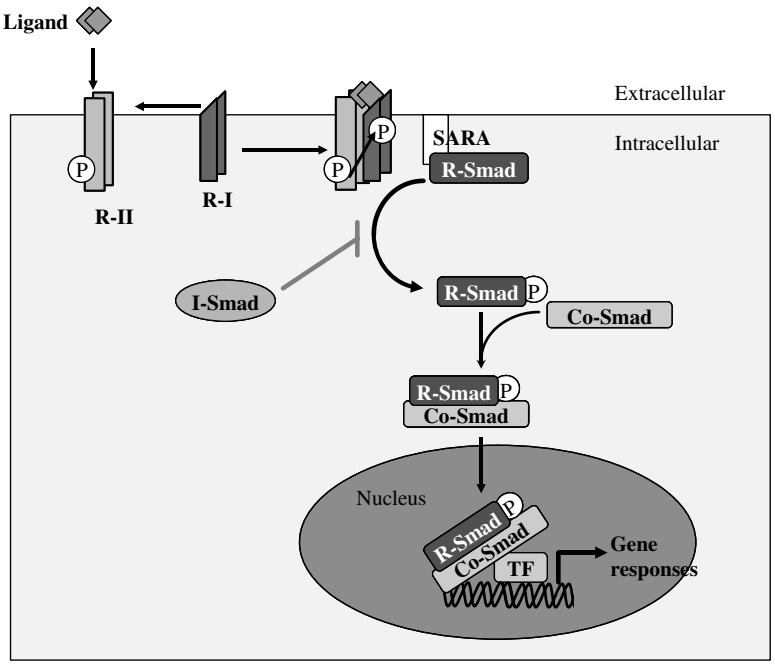

Figure 5. The canonical TGF-β/Smad signaling pathway. TGF-β binds to and stabilizes heteromeric complexes of type I and type II serine/threonine kinase receptors. The type II receptor is endowed with constitutively active kinase activity and phosphorylates the type I receptor on specific serine and threonine residues in the juxtamembrane region (also termed GS domain). Upon this activation, the type I receptor propagates the signal inside the cell through phosphorylation of R-Smads at two C-terminal serine residues found in an SXS motif. R-Smads can be recruited to the activated type I receptor through auxiliary proteins, such as Smad anchor for receptor activation (SARA). Activated R-Smads form heteromeric complexes with Smad4 that in combination with transcription factors can bind to promoters of target genes. These complexes regulate, together with co-activators and co-repressors, specific transcriptional responses (A color version of this figure is freely accessible via the website of the book: http://www.springer.com/1-4020-4542-5)

3.3 The Future of Smad Research

The field of Smad research has diversified enormously as is exemplified by the many aspects of Smad research covered in this book. The multifunctional character of TGF-β family members is reflected in the many positive and negative modes of regulation of Smads. The investigation of cross-talk with other signaling pathways will be a recurring theme in future studies. While the TGF-β/Smad pathway has been implicated in many responses, an important issue that largely remains to be explored is the requirement of particular Smad isoforms in these responses, *e.g.* whether responses require specific R-Smads and/or Smad4. In addition, the recent results which have demonstrated transcription-independent functions of Smads, such as recruitment, sequestration and enzyme activation, need to be further investigated (ten Dijke and Hill, 2004).

Studies in genetically accessible model organisms, such as *C. elegans*, *Drosophila* and the vertebrate Zebrafish, will continue to be important in elucidating the mechanisms that underlie TGF-β/Smad signaling. The combination of these efforts with unbiased large scale genetic, biochemical and/or proteomic interaction screens (Vidal, 2005), functional genomic approaches using siRNAs (Paddison et al., 2004) or morpholino's (Ekker, 2000), and transcriptional profiling using micro arrays (Ideker, 2004) (see Chapters 2, 3, 17 and 18), will be particularly powerful. It will also be important to validate the patho-physiological significance of the identified biochemical and genetic interactions between TGF-β signaling components using transgenic mouse models.

An important challenge for the future will be to translate our current knowledge into clinical applications. Specific TGF-β receptor kinase inhibitors have recently been generated, and shown to block ligand-induced Smad-dependent responses (see Chapters 21 and 22). However, like TGF-β, Smads are multifunctional proteins; they have been implicated in the anti-proliferative response of TGF-β (see Chapter 4), but also in TGF-β-induced invasion and metastasis of tumor cells (see Chapter 7 and 20) and in TGF-β-induced extracellular matrix formation leading to fibrosis (see Chapter 22). Further dissection of Smad-driven responses, and identification of specificity determinants for these various responses, may allow for specific intervention of diseases with perturbed TGF-β/Smad signaling.

ACKNOWLEDGEMENTS

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CHAPTER 1

MOLECULAR EVOLUTION OF SMAD PROTEINS

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Abstract: To date, Smad family members have been found only in eumetazoan animals. To understand the evolutionary relationship between family members we conducted a phylogenetic analysis. To simplify the analysis but retain its explanatory power, we focused on Smad proteins from organisms in three distinct phyla: human, fly, and nematode. Overall, we found that human and fly proteins always cluster together in four subfamilies while three subfamilies contain only nematode proteins. Sequence alignments of distinct regions of were also analyzed. Data from the alignments confirmed that the MH1 (DNA-binding) and MH2 (protein-protein interaction) domains are highly conserved family-wide. The linker region between these domains is also highly conserved but only within subfamilies. Conservation in the C-terminal receptor phosphorylation region provides new insight into a unique subfamily containing three interacting nematode proteins that signal for DAF-7. From a larger perspective, our analysis strongly supports the traditional view that flies are more closely related to humans than to nematodes

Keywords: multigene family; SMAD proteins; phylogeny; amino acid alignments; evolutionary conservation; developmental-evolution; signal transduction

1. INTRODUCTION

The evolutionary relationships between members of a multigene family are ascertained through a phylogenetic analysis involving three steps. First, one must calculate the amount of amino acid similarity between each family member by aligning the protein sequences (Thompson et al., 1997). Second, one applies an amino acid similarity matrix and the extent of similarity between each protein and all of the others are prioritized with the most similar proteins clustered together. These clusters are depicted as the familiar phylogenetic tree (Kumar et al., 2001). Third, the relationships between pairs of proteins are tested for robustness using statistical methods such as bootstrap analysis (Felsenstein, 1985).

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Here we describe a new phylogenetic analysis of the Smad protein family. In order to simplify the analysis but retain its explanatory power, we focus on Smad sequences from organisms in three distinct phyla: human (deuterostome), fly (protostome) and nematode (pseudocoelomate; see Raff 1996, for example, for a taxonomic description of these phyla). We include other species as necessary to add confidence to individual results. Our studies of the MH1 and MH2 domains support the long-standing view that they are highly conserved. Our analysis of the linker region between the MH1 and MH2 domains, previously dismissed as a highly divergent and potentially non-functional part of the protein, reveals surprising levels of sequence conservation within Smad subfamilies. This suggests the hypothesis that distinct functions associated with each subfamily involve linker sequences. An analysis of the receptor phosphorylation domain provides new insights into a unique subfamily containing only nematode proteins that signal for the TGF-β/Activin subfamily member DAF-7.

Recently it has become possible to test phylogenetically derived hypotheses using an approach known as functional genomics. In this technique, interspecies experiments are conducted that evaluate the ability of a family member from one species to mimic the activity of another family member either by rescuing mutant phenotypes (*e.g*. Padgett et al., 1993) or in parallel over-expression experiments (*e.g*. Marquez et al., 2001). We have conducted a number of such tests and review those results here.

2. SMAD FAMILY MEMBERS

To date, Smad family members have been found only in animals. Within the animal kingdom they have been identified in eumetazoans (multicellular organisms with many types of cells) but not yet in metazoans such as sponges (multicellular organisms with very few cell types). However, several transmembrane receptors with similarity to both type I and type II TGF-β receptors have been identified in a freshwater sponge (Suga et al., 1999). A phylogenetic analysis showed that the sponge receptors are very similar to the unusual *C. elegans* receptors DAF-1 and SMA-6 that also fall between receptor types (Herpin et al., 2004). The similarity between sponge and nematode receptors suggests that Smad-like proteins will eventually be found in sponges. Thus, ancestral TGF-β family members and their signaling pathways predate the metazoan/eumetazoan divergence roughly 1.5 billion years ago (Hedges and Kumar, 2003).

The simplest eumetazoans with definitive Smad family members are cnidarians (animals with two germ layers – diploblasts). A sequence similar to Smad1/Mad in the BMP signaling subfamily has been identified in coral (Samuel et al., 2001) and in hydra. The simplest eumetazoan with Smad proteins similar to both Smad1/Mad and Smad2/3 is the blood fluke *Schistosoma mansoni* – an acoelomate with three germ layers but no digestive cavity (Beall et al., 2000). From this it is reasonable to conclude that BMP signaling Smads, and by extension their cognate ligands and