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Review article

Integration of the TGF-β pathway into the cellular signalling network

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Abstract

Transforming growth factor-βs (TGF-βs) regulate pivotal cellular processes such as proliferation, differentiation and apoptosis. After ligand binding, the signals are transmitted by two types of transmembrane serine/threonine kinase receptors. The type I receptor phosphorylates Smad proteins, intracellular effectors which upon oligomerization enter the nucleus to regulate transcription following assembly with transcriptional co-factors and co-modulators.

The cellular distribution of TGF-β receptors along with their oligomerization mode and their complex formation with different cell surface receptors represent crucial steps in determining the initiation of distinct signalling cascades.

In addition, the broad array of intracellular proteins that influence the TGF-β pathway demonstrates that signal transduction does not proceed in a linear fashion but rather comprises a complex network of cascades that mutually influence each other.

The present review describes the intricate control of TGF-β signal transduction on various levels of the cascade with particular focus (i) on the assembly of different receptor subtypes and (ii) on the multitude of crosstalk with signal transducers from other pathways. Integration of the TGF-β/Smad pathway into the signalling network has taken on added importance as it substantially contributes to elicit the plethora of cell- and tissue-specific effects of TGF-β.

Keywords: Transforming growth factor; Receptors; Signal transduction; Smads; Crosstalk; Receptor oligomerization

1. Introduction

Transforming growth factor-β (TGF-β) is a secreted cytokine that exerts an amazing diversity of biological effects including proliferation, differentiation, migration and apoptosis. The superfamily of TGF-β proteins comprises more than 30 members, the most prominent being TGF-β itself, bone morphogenetic proteins (BMPs), activins and growth and differentiation factors (GDFs) [1,2].

Considering the limited assortment of transmembrane receptors and downstream signalling molecules, the pleiotropic nature of TGF-β asks for flexibility and variability of the signalling processes. Flexibility is achieved by mechanisms that control and fine-tune TGF-β signalling on all levels, ranging from regulating activation of TGF-β in the extracellular space to modulation of transcriptional activation in the nucleus (Fig. 1). Substantial events that generate variability are first, the cell- and tissue-specific composition and interaction of receptors, signal transducers and DNA-binding partners and second, the crosstalk with regulators from other pathways.

Elucidation of these diverse regulatory processes and the complex interactions within the signalling network are currently in the centre of TGF-β signal transduction research and will be compiled in this review.

2. Basic mechanism of TGF-β signal transduction

All three human isoforms of TGF-β (TGF-β1, -β2 and -β3) are synthesized as a large precursor proprotein which is processed to yield the mature protein. Bioactive TGF-β homodimers signal through transmembrane serine/threonine kinase receptors designated as TGF-β type I (TβRI) and type II (TβRII) receptors [3–5]. Initial ligand binding to the constitutively active TβRII is followed by recruitment of TβRI into a heteromeric complex [6]. Subsequent transphosphorylation of TβRI at the juxtamembrane glycin/serine-rich region, the so-called GS-box, is mediated by TβRII and leads to activation of TβRI. Interestingly, TβRI

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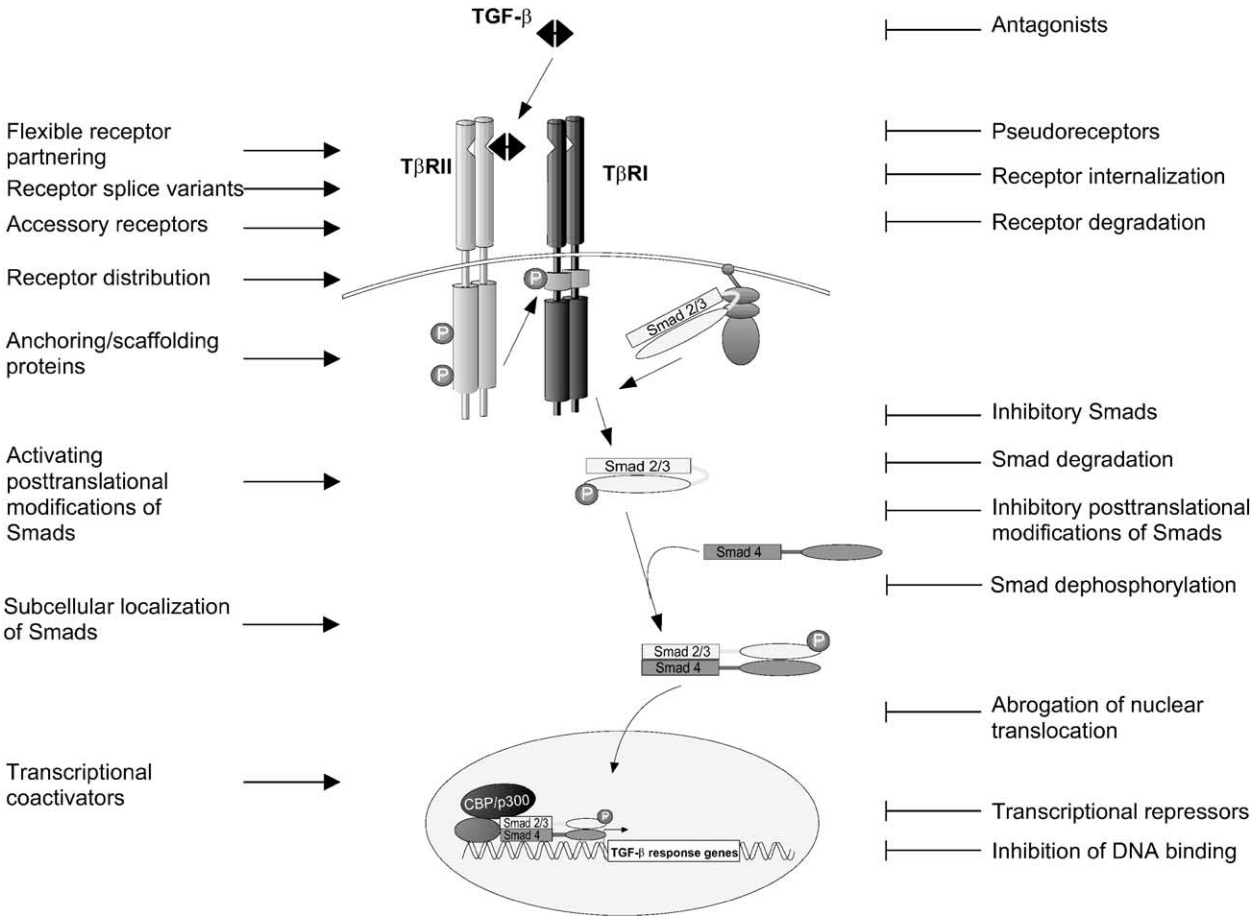

Fig. 1. Regulation of TGF-β signal transduction on various levels of the signalling cascade. The relatively simple model of signal transduction by TGF-β gets highly complex if all modulating inputs from other molecules are taken into account. From regulating the bioavailability of the ligand in the extracellular compartment to regulating gene expression in the nucleus, each step is tightly regulated. The signalling outcome is thus dependent on the sum of positive or negative influences and on the potential interacting proteins that are involved.

activation is not due to an increase of the actual kinase activity but is rather based on the creation of a binding site for Smad proteins which represent the substrates for TβRI [7]. A key determinant of TβRI–Smad interaction is represented by a region located in the kinase domain of TβRI, termed the L45-loop [8]. Within the L45-loop, four amino acids that differ in TGF-β and BMP type I receptors confer specificity for distinct Smad isoforms and thus separate TGF-β and BMP pathways [9,10].

Smad proteins can be divided into three subfamilies which will be discussed in more detail in succeeding sections: first, receptor-activated Smads (R-Smads) including BMP-activated Smads (Smad1, Smad5 and Smad8) and TGF-β-activated Smads (Smad2 and Smad3), second, the common mediator Smad4 (Co-Smad) and third, the inhibitory Smads, Smad6 and Smad7 (I-Smads) [11,12]. TβRI causes R-Smad phosphorylation at the C-terminal SSXS-motif which is conserved among all R-Smads thereby causing dissociation from the receptor and heteromeric complex formation with Smad4 [11,13,14]. Smad complexes translocate to the nucleus, assemble with specific DNA-binding co-factors and co-modulators to finally activate transcription. The choice of target genes is thereby determined by the composition of the transcriptional complex [15].

As depicted in Fig. 1, this relatively simple general signalling cascade becomes highly complex if all the potential regulatory elements are taken into account.

3. Activation of latent TGF-β

TGF-β is initially synthesized as a precursor proprotein which is cleaved during secretion. However, the mature disulfide-bonded TGF-β dimer remains non-covalently associated with the N-terminal propeptide which is referred to as latency-associated protein (LAP) and prevents receptor binding of the mature TGF-β [16,17]. In contrast to this "small latent complex", the "large latent complex" differs in the additional association of a latent TGF-β-binding protein (LTBP) which mediates deposition of the latent complex to the extracellular matrix. Release of mature TGF-β from the latent complex in vivo can be accomplished by different mechanisms such as proteolytic cleavage of

LAP by plasmin, deglycosylation of LAP or interaction with thrombospondin-1 or integrin $\alpha_v\beta_6$ (Ref. [18] and references therein). Since activation of TGF-β determines bioavailability of the ligand, it is not surprising that this process underlies tight regulation.

4. Cell surface receptors-the origin of signal transduction

4.1. Repertoire of receptors and receptor splice variants

4.1.1. Signalling receptors

In mammals, only five type II receptors and seven type I receptors have been identified for ligands belonging to the large TGF-β superfamily [19]. They all represent transmembrane receptors that contain an intracellular serine/threonine kinase domain.

The prototypic signalling receptors for TGF-β are the TGF-β type II receptor (TβRII) and the TGF-β type I receptor (TβRI). In addition, two other TGF-β binding proteins designated betaglycan (or TβRIII) [20] and endoglin [21] are frequently involved in formation of receptor complexes and function predominantly in ligand presentation. However, as shown in Table 1, the repertoire of TGF-β receptors is supplemented by other receptors or splice variants of the receptors that are also capable of transducing signals in response to TGF-β. Thus, signal diversity may be generated by different receptor combinations.

Table 1
Potential receptor complexes that transduce TGF-β signals

Ligand	Type I receptor	Type II receptor	Accessory receptor	Signal transducer	References
TGF-β1, -β3 (not -β2)	TβRI (ALK5)	TβRII	–	Smad2/3	[22,23]
TGF-β1, -β2, -β3	TβRI (ALK5)	TβRII	TβRIII (betaglycan)	Smad2/3	[24–26]
TGF-β1	TβRI-S (ALK5-S)	TβRII	–	Smad2/3	[27]
TGF-β1, -β2, -β3	TβRI (ALK5)	TβRII-B	–	Smad2/3	[28]
TGF-β1, -β2, -β3	TβRI (ALK5)	TβRII-B	TβRIII (betaglycan)	Smad2/3	[28]
TGF-β1, -β3 (not -β2)	ALK1	TβRII	Endoglin	Smad1/5	[29,30]
TGF-β1	ActRI (ALK2)	TβRII	TβRIII (betaglycan)	Smad1/5	[31,32]
?	ALK7	TβRII (?)	–	Smad2/3	[33,34]

Besides the classical TGF-β signalling receptors, TβRI and TβRII, there are additional type I receptors and receptor splice variants that can likewise contribute to formation of TGF-β responsive receptor complexes. The nature of the assembled receptor complex determines the downstream signals that are transduced either via Smad2/3 or via Smad1/5. Signals mediated by some receptor complexes require the presence of accessory receptors such as TβRIII or endoglin.

In addition to TβRI, there are other type I receptors such as ALK1 and ActRI (ALK2) that transmit signals evoked by TGF-β. Coexpression of ALK1 with TβRII and endoglin promotes efficient signalling in response to TGF-β1 and TGF-β3 but not TGF-β2 [30]. Pathological relevance is reflected by the linkage of mutations in both the endoglin and the ALK1 gene to an autosomal dominant disorder named hereditary hemorrhagic teleangiectasia (HHT) [29,35]. Moreover, ALK1 signalling plays an important role during vascular development. The two parallel signalling pathways of TGF-β1 in endothelial cells, mediated by either ALK1 or TβRI, can account for the biphasic effect of TGF-β1: ALK1 signals were found to repress expression of angiogenic factors and proteases via Smad1/5 activation, whereas TβRI phosphorylates Smad2/3 and elevates transcription of angiogenic factors and secretion of proteases [29]. Thus, the balance between ALK1 and TβRI determines the signalling outcome in endothelial cells.

ALK2 is similar to ALK1 in that it binds TGF-β following interaction with TβRII and an accessory receptor and conveys the signal via the BMP-Smads, Smad1 and Smad5, respectively [31,36]. ALK2 is postulated to promote epithelial–mesenchymal transition stimulated by TGF-β1 or TGF-β2 in murine mammary epithelial cells [32].

TβRI was found to appear in different variants arising from alternate splicing events. Agrotis et al. [27] found an in-frame deletion of 12 nucleotides adjacent to the transmembrane domain. This splice variant, referred to as ALK5-S, is also competent in TGF-β1 signalling and even depicts a tendency to be more potent than the normally processed receptor.

ALK-7 is still an orphan member of the type I receptor family which is predominantly found in the nervous system [33,37]. Nevertheless, overexpression experiments in COS cells supply evidence that ALK-7 can associate with TβRII [33] and Jörnvall et al. [34] could show that a constitutively active ALK7 mediates phosphorylation of Smad2 and Smad3, suggesting a functional role of ALK7 in TGF-β signalling.

Referring to the type II receptors, there exists a splice variant of TβRII, TβRII-B [38], which contains an insertion of 25 amino acids in the extracellular part of the receptor and shows functional differences in isoform specificity. Whereas TβRII does not bind the TGF-β2 isoform unless it is presented by TβRIII, TβRII-B is able to bind all three TGF-β isoforms irrespective of the presence of TβRIII [28]. An outstanding physiological relevance for TβRII-B expression is therefore predicted for tissues such as bone, in which TGF-β2 represents the major TGF-β isoform.

4.1.2. Accessory receptors

The major TGF-β-binding molecule on most cell types is TβRIII, also called betaglycan [20,39]. TβRIII is a transmembrane proteoglycan that is able to bind all three TGF-β isoforms via two independent binding sites in the core protein [40,41]. As mentioned above, particular importance

is assigned to TβRIII in presenting TGF-β2 to TβRII which shows only low intrinsic affinity for TGF-β2 [42]. In contrast to the facilitation of ligand access to the receptors, the soluble secreted ectodomain of TβRIII [43], which results from proteolytic processing, accounts for antagonistic effects through binding and sequestering the ligand [44]. Still puzzling is the function of the short cytoplasmic part with no discernible signalling entity. Distinctive for this domain is the high content of serines and threonines which represent suitable sites for phosphorylation [39]. Indeed, a previous report describes that phosphorylation of the cytoplasmic domain by autophosphorylated TβRII initiates the release of TβRIII from the active signalling complex, consisting of TβRI, TβRII and the bound ligand [45]. Recently, glycosaminoglycan modifications of betaglycan were proposed to account for prevention of TβRI/TβRII complex formation in renal epithelial cells [46]. Thus, depending on the cellular context, betaglycan can act as a dual modulator-either promoting or inhibiting TGF-β signal transduction.

Endoglin, a homodimeric integral glycoprotein composed of disulfide-linked subunits is expressed at high levels on vascular endothelial cells. It shares regions of sequence similarity with TβRIII and its cytoplasmic domain likewise lacks known signalling motifs [47]. Similar to TβRII, endoglin interacts with TGF-β1 and -β3 but not with TGF-β2 [47,48]. This restrictive isoform specificity might be explained by the fact that endoglin alone is not able to bind TGF-β but requires coexpression of the type II receptor to convey ligand binding [35]. In addition, endoglin binds activin-A, BMP-2 and BMP-7 only in the presence of their respective ligand-binding receptors. Thus, unlike TβRIII, endoglin does not function in modulating ligand access to the receptors but probably contributes to recruit other proteins to the signalling complex.

In addition to TβRIII and endoglin, several glycosylphosphatidylinositol (GPI)-anchored proteins were described to serve as accessory receptors for TGF-β [22,49]. These proteins might exert TβRIII-like functions, namely, to sequester or present TGF-β [49]. Alternatively, it is conceivable that they are engaged in regulating receptor distribution since GPI-anchored proteins are known to be included in microdomains of the cell membrane, called rafts, which recruit and concentrate specific receptors and signalling molecules to facilitate signal transduction [50].

4.1.3. Pseudoreceptors

A receptor variant that cross-regulates pathways of different members of the TGF-β family is represented by the BMP and activin membrane bound inhibitor (BAMBI) [51]. BAMBI is a naturally occurring pseudoreceptor which extracellularly resembles a type I receptor but lacks the cytoplasmic kinase domain. The capacity to associate with various type I receptors prevents the formation of functional homodimeric type I receptor complexes and thus causes abrogation of BMP- as well as TGF-β- and activin-mediated signalling.

In conclusion, the repertoire of signalling receptors gives rise to multiple hetero-oligomeric receptor complexes and each of the involved receptor variants depicts a distinct expression pattern, ligand isoform specificity and ligand affinity, thus expanding signal diversity.

4.2. Distribution of receptors at the cell surface

Besides heteromeric receptor complex formation, homomeric receptor interactions as well as their spatial localization at the cell surface have strong impact on mediating and regulating intracellular signals.

At the cell surface, the overall distribution of the TGF-β receptors yields predominantly in a punctate pattern and the receptors assemble to form different oligomeric units [52,53] (Fig. 2). TβRI, TβRII and TβRIII have all been found to be present as homo-oligomers already in the absence of TGF-β [52,54–56]. In addition, heteromeric complexes consisting of TβRII and TβRIII could be detected in absence and in presence of TGF-β. However, the small amount of these complexes suggests that they represent only a minor and probably transient species [52]. Based on their inherent affinity for each other, a small but detectable proportion of TβRI/TβRII heteromeric receptor complexes exists already in unstimulated cells [57,58]. The fraction of these heteromeric complexes is significantly increased by ligand binding to TβRII which causes subsequent recruitment of TβRI. The resulting complexes were unravelled to be tetrameric, consisting of two molecules each of TβRI and TβRII, and to represent the actual signalling entity [59,60]. Homomeric receptor complexes are not sufficient to propagate TGF-β responses but are proposed to be functionally important for regulating receptor kinase activity, as reported in the case of intermolecular autophosphorylation at multiple serine residues of TβRII [56,61].

The plasma membrane of most cells is not uniform but contains microdomains called lipid rafts that constitute a dynamic assembly of cholesterol and sphingolipids. A subset of lipid rafts is represented by cell surface invaginations that contain caveolin as a marker protein and are thus referred to as caveolae. A large body of evidence has accumulated to suggest that rafts play an important role as cellular signalling centres (for review, see Refs. [50,62,63]). Recently, Razani et al. [64] could show that caveolin-1 cofractionates with TβRI, TβRII and Smad2 in caveolae-enriched membrane fractions and even directly interacts with TβRI, leading to inhibition of TGF-β-mediated Smad2 phosphorylation and subsequent downstream events. As these findings provide evidence that TGF-β receptors are associated with rafts, it will be interesting to unravel whether there are ligand-dependent mechanisms that regulate translocation of TGF-β signalling molecules in and out of lipid rafts to provide determination of signalling events.

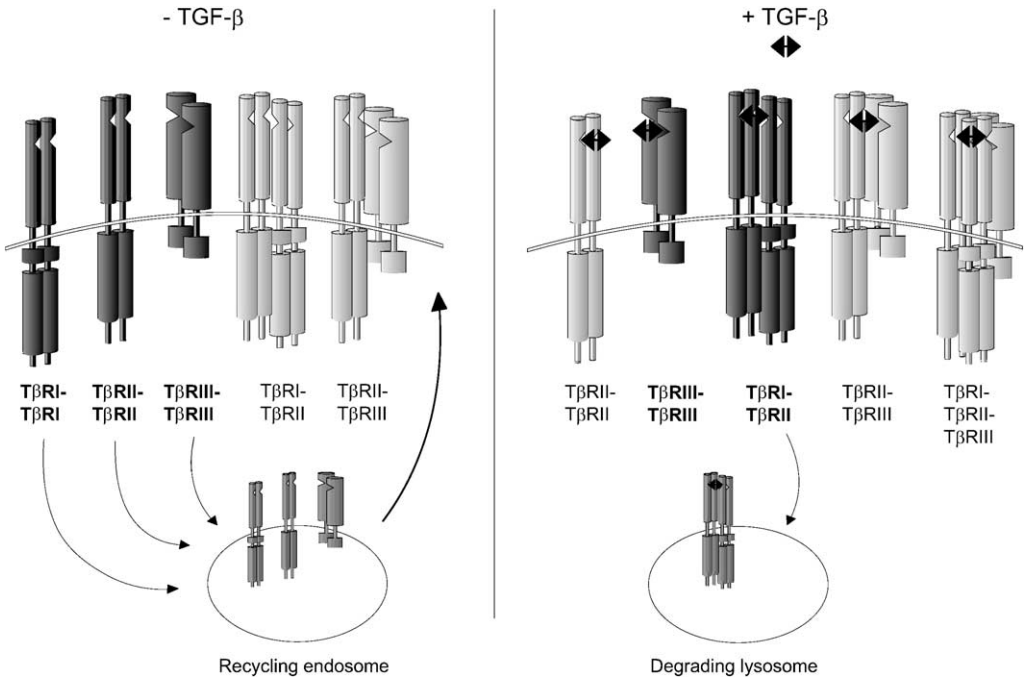

Fig. 2. Oligomeric receptor complexes and their internalization. Different oligomeric receptor complexes exist at the cell surface in absence and in presence of ligand. The bold-typed receptor names represent complexes that are abundantly present, standard-typed ones represent complexes that are present in very low amounts or are of transient nature. After ligand stimulation, both the composition of the receptor complex changes and their fate of being endocytosed and recycled or degraded.

4.3. Receptor turnover

TGF-β receptor expression at the cell membrane can also be controlled by mechanisms such as internalization and down-regulation. Ligand binding to the TGF-β receptors triggers not only signalling but also initiates internalization of both ligand and receptors [65]. In absence of TGF-β, the receptors are constantly internalized and recycled back to the membrane. Ligand binding causes heteromeric TβRI/TβRII receptor complexes to get internalized and rapidly targeted to a degradative pathway, whereas signalling incompetent homomeric type I or type II receptor complexes are not down-regulated but rather recycled [66,67] (Fig. 2). Prerequisite for optimal down-regulation is the interaction between TβRI and TβRII [68]. However, TβRII transphosphorylation activity was shown to be necessary to divert heteromeric complexes to degradative pathways in fibroblasts but not in epithelial cells [67,69].

The precise mechanism of TGF-β receptor internalization is still controversially discussed. While Anders et al. [70] propose clathrin-coated vesicles to mediate endocytosis, others suggest from experiments using various inhibitors of endocytosis that internalization is mediated by either non-coated vesicles or caveolae [65].

Furthermore, it is conceivable that more than one mechanism causes TGF-β receptor internalization. In conclusion, endocytic activities are dependent on the composition of the receptor complex as well as the cell type [66,67,70].

4.4. Receptor-interacting proteins

Various receptor-interacting proteins have been identified that function to regulate TGF-β signalling by completely different mechanisms (Fig. 3).

The basal receptor activity is kept under tight control by the immunophilin FKBP12 [71]. The inherent tendency of TβRI and TβRII to associate with each other allows complex formation and TβRI activation already in absence of ligand. By binding to the unphosphorylated GS-box of TβRI [72,73], FKBP12 stabilizes a conformation of TβRI that is incapable of getting transphosphorylated by TβRII, thus preventing ligand-independent signalling [74,75]. Ligand binding to TβRII, however, induces conformational changes that lead to displacement of FKBP12 and subsequent TβRI activation by TβRII [7,74]. Furthermore, binding of FKBP12 to TβRI was proposed to be involved in negative regulation of receptor internalization, mediated by favouring a conformation that is not recognized by the endocytic machinery [76].

Several receptor-interacting proteins exert important functions in subcellular localization of Smad proteins. Among these, there are the Smad anchor for receptor activation (SARA) [53], disabled-2 (Dab2) [77] and the TβRI-associated protein-1 (TRAP-1) [78]. SARA contains a FYVE domain which mediates binding to membrane phospholipids and consequently association with the membrane. The adjacent Smad binding domain allows interaction with

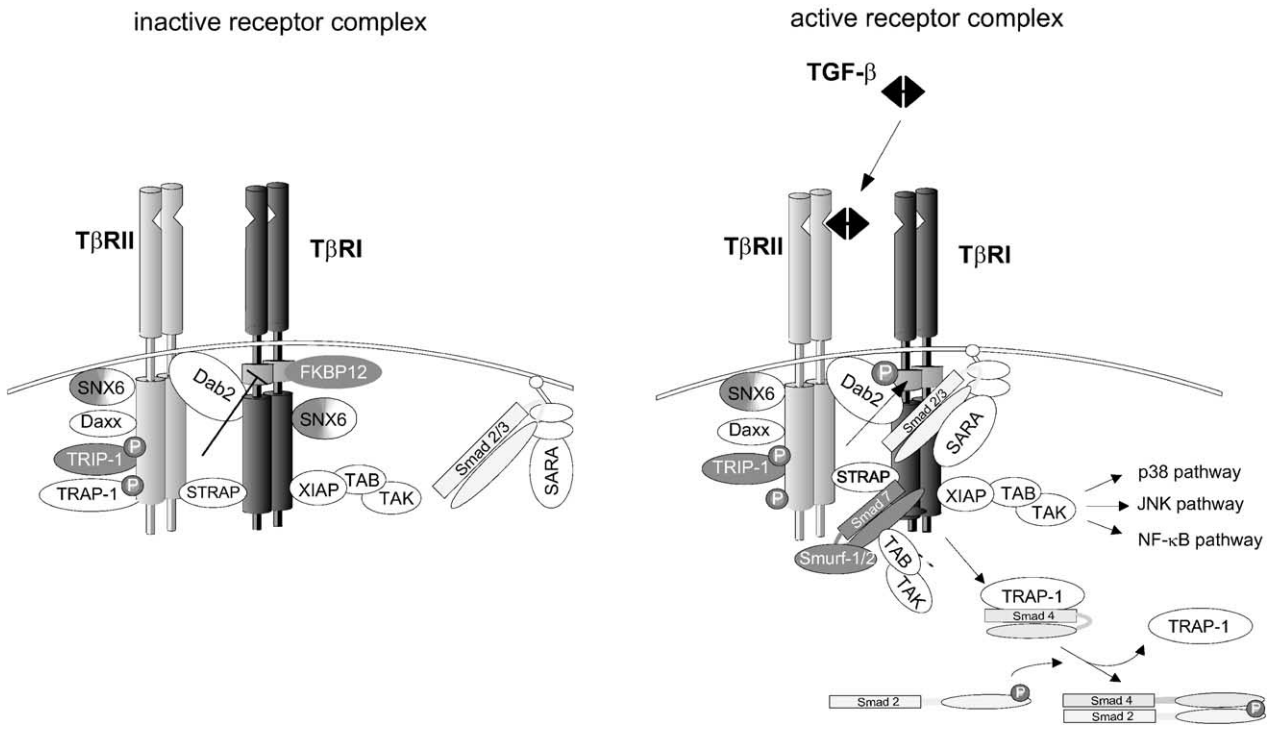

Fig. 3. Receptor-interacting proteins. A number of proteins that positively or negatively influence TGF-β-induced signals were shown to interact with the cytoplasmic domain of the TGF-β receptors. Some of them (TRIP-1, Dab-2, XIAP, STRAP, SNX6 and Daxx) bind the receptors regardless of the kinase activity of the receptors, whereas others associate specifically with the activated receptor complex (SARA, Smad7, Smurf1/2) or are released from the receptor complex following ligand stimulation (TRAP-1, FKBP-12, SNX6). Antagonizing regulators are depicted in grey; synergizing regulators are shown in white. Proteins whose function is not yet known are demonstrated as half grey and half white molecules.

Smad2 and Smad3 and the C-terminal part directly associates with the activated TβRI [53]. The cooperative binding of these proteins enables R-Smad phosphorylation by TβRI, which is followed by dissociation of the R-Smad from SARA and formation of a heteromeric complex with Smad4. By masking the nuclear localization signal of Smad2, SARA functions also in retaining non-activated Smad2 in the cytoplasm [79]. Interestingly, SARA is required for Smad2-mediated signalling but is dispensable for Smad3-mediated signals [80]. The crystal structure of SARA binding to Smad2 was recently determined by Wu et al. [81]. Similar to the SARA protein, the member of the disabled gene family, Dab2, facilitates TGF-β signalling by bridging the receptor complex to the Smad proteins. Dab2 interacts with both TβRI and TβRII in absence and in presence of TGF-β. The protein contains a C-terminal proline-rich domain (PRD) and an N-terminal phosphotyrosine binding site (PTB) that is likely to allow membrane association and binding to the MH2 domain of Smad2 and Smad3 in a ligand-dependent manner [77]. In contrast to SARA and Dab-2, TRAP-1 recruits the Co-Smad, Smad4, into the vicinity of the receptor complex. In absence of ligand, TRAP-1 interacts via multiple binding sites with TβRII. Ligand-induced changes in receptor conformation result in release of TRAP-1 from the complex. Transient interaction of TRAP-1 and Smad4 serves to attract the Co-Smad into the proximity of the receptor complex, thus facilitating heteromeric complex formation between activated R-Smads and Smad4 [82].

Negative regulation of TGF-β signalling is achieved by proteins such as the inhibitory Smad7 [83,84], serine/threonine kinase receptor-associated protein (STRAP) [85] or the Smad ubiquitination regulatory factors (Smurf), Smurf1 [86] and Smurf2 [87]. Smad7 belongs to the subfamily of inhibitory Smads which are highly divergent from R-Smads referring the N-terminal MH1 domain but contain conserved MH2 domains. Since inhibitory Smads lack the SSXS-motif, they do not serve as a substrate for TβRI. Smad7 stably interacts with the activated TβRI, thereby competing with the R-Smads for receptor association. As a consequence, phosphorylation of R-Smads and the succeeding downstream events are efficiently blocked by Smad7. Being an immediate early response gene for TGF-β, Smad7 triggers an efficient negative feedback [88,89]. Recently, it was demonstrated that physical interaction between the N-terminal domain of Smad7 and its MH2 domain enhances the inhibitory activity of Smad7, most likely by promoting receptor association [90]. In synergism with Smad7, the WD domain containing protein STRAP inhibits TGF-β-mediated signals [85]. STRAP was described to interact with both TβRI and TβRII, and assists in stabilizing the ternary complex consisting of Smad7, TβRI and STRAP. Moreover, STRAP is proposed to be involved in recruiting Smad7 to the TGF-β receptors [91].

E3 ubiquitin ligases of the HECT family, Smurf1 and Smurf2, are bridged to the receptor by Smad7. They possess two WW domains that mediate protein–protein interactions with the PPXY sequence (PY-motif) in the linker region of I-Smads as well as R-Smads. Smurf1 and Smurf2 associate with Smad7 in the nucleus and both proteins get recruited to the TGF-β receptor complex following ligand-induced upregulation of Smad7 expression [87,92]. Induction of ubiquitination of Smad7 leads to subsequent proteasomal and lysosomal degradation of the complex containing Smad7 and the receptor proteins.

Based on their capacity to interact additionally with R-Smads, Smurf proteins can also modulate TGF-β signals by association with and degradation of R-Smads. Degradation of R-Smads, in particular Smad1, by Smurf1 or Smurf2 occurs regardless of receptor activation, indicating that Smurf proteins play an important role in controlling their steady-state levels [86,93]. Phosphorylated Smad2 was described to associate preferably with Smurf2 which targets the ligand-activated nuclear Smad2 to proteasomal degradation [94]. Thus, Smurf proteins can specifically and selectively direct R-Smads for either cytoplasmic or nuclear degradation.

An indirect interaction with the TβRI receptor can be assumed for TGF-β-activated kinase-binding protein 1 (TAB1) [95]. Although TAB interaction with components of the TGF-β/BMP cascade has been investigated predominantly in BMP signalling, it is likely that this association likewise occurs in response to TGF-β. TAB1 is an upstream signalling molecule of TGF-β-activated kinase (TAK1) [96] which in turn triggers multiple other pathways such as the p38 MAPK and the c-Jun N-terminal kinase (JNK) cascade [97,98]. Thus, the linkage of the TGF-β receptors to TAB1 and TAK1 represents a point of convergence with several other substantial signalling pathways. TAB1 was reported to associate with Smad7, thereby causing inhibition of TAK-induced p38 activation and BMP-2-induced neurite outgrowth of PC12 cells [99]. In addition, TAB1 can bind to the X-linked inhibitor of apoptosis (XIAP) [100] which serves as a cofactor in TGF-β signalling. XIAP contains a C-terminal RING finger domain and three N-terminal baculovirus IAP repeats (BIR), whereby the latter represent the binding site for TβRI. Although XIAP serves as a bridging molecule between the receptors and TAB1, cooperative effects of XIAP and TGF-β are not necessarily mediated by TAB1/TAK1-dependent processes. The exact mechanism of how XIAP cooperates with TGF-β is not yet fully understood but activation of TGF-β responsive genes by XIAP was shown to be dependent on Smad4, whereas the anti-apoptotic effects are elicited independent of Smad4 [101].

The TGF-β receptor-interacting protein (TRIP-1) belongs to the WD40-repeat-containing proteins and interacts with and gets phosphorylated by TβRII. By receptor-dependent as well as receptor-independent mechanisms, TRIP-1 specifically impedes the TGF-β-induced gene expression from the PAI-1 promoter but does not repress TGF-β-triggered growth inhibition [102].

Additional receptor-interacting proteins, the functions and signalling mechanisms of which are not yet fully clear, are the dimeric 14-3-3 protein, the sorting nexin 6 (SNX6) and the Daxx protein. The 14-3-3 protein was first found in the parasitic trematode *Schistosoma mansoni*. It was shown to interact with TβRI and to act as a positive regulator of TGF-β-mediated growth arrest, probably through binding to Cdc25 [103]. SNX6 belongs to the family of sorting nexin proteins that interact with each other as well as with tyrosine kinase receptors and each of them can bind to specific receptors of the TGF-β family. SNX6 binds to TβRII and to the inactive TβRI. Functional relevance of sorting nexin proteins is proposed to reside in intracellular trafficking of receptors [104]. Daxx was originally identified as a Fas-receptor-binding protein that triggers JNK activation and apoptosis [105]. Recently, it was demonstrated that Daxx can interact with the constitutively active TβRII receptor and function as an intermediary between both the Fas and the TGF-β receptors on the one hand and the apoptotic machinery on the other. Furthermore, expression of a dominant-negative Daxx mutant impedes TGF-β-induced JNK activation as well as TGF-β-stimulated apoptosis [106].

Collectively, a number of receptor-associated proteins are engaged in precisely modulating TGF-β signalling, either positively by controlling subcellular localization of signalling components and allowing crosstalk with other pathways, or negatively by preventing receptor activation or triggering degradation of signalling molecules.

5. Smads-the signal transducers

5.1. Structure and function of Smad proteins

Smad proteins are so far the only known substrates for the TGF-β type I receptor that elicit signalling function in response to TGF-β. Although other receptor-interacting proteins can synergize with TGF-β signalling or mediate crosstalk, it is unique to the Smads that they transmit the signal directly from the receptors to the nuclear transcriptional machinery.

In vertebrates, eight members of the Smad family have been identified which can be further classified based on their structural and functional properties: (i) receptor-activated Smads [R-Smads: Smad1, Smad5, Smad8 (BMP activated); Smad2, Smad3 (TGF-β activated)] that become phosphorylated by type I receptors, (ii) the common mediator Smad (Co-Smad: Smad4) which, as an obligate partner, oligomerizes with activated R-Smads and (iii) the inhibitory Smads (I-Smads: Smad6 and Smad7) which antagonize TGF-β or BMP signals by competing with R-Smads for type I receptor interaction (reviewed in Refs. [2,11,107]).

The overall structure of R-Smads and Co-Smads comprises the highly conserved N-terminal Mad homology 1 (MH1) and the C-terminal Mad homology 2 (MH2) domains which form globular structures and are linked by a divergent proline-rich region of variable length. I-Smads likewise contain the conserved MH2 domain but show very little similarity to other Smads in their N-terminal part (reviewed in Refs. [12,108]).

Lacking an intrinsic enzymatic activity, the Smad proteins exert their effects exclusively by protein–protein and protein–DNA interactions which are mediated by distinct functional regions within the proteins (Fig. 4).

5.1.1. The MH1 domain

The MH1 domain was first characterized to mediate autoinhibition by physically interacting with the MH2 domain, thereby impeding its effector function in the absence of ligand [109]. Furthermore, the MH1 domain accounts for the ability to bind directly to DNA. Determination of the crystal structure of a Smad3 MH1 domain bound to DNA revealed that a conserved β-hairpin loop is responsible for binding to the major groove of the DNA helix [110]. An exception is represented by Smad2, which lacks DNA-binding capacity due to an insertion encoded by exon3 [111]. However, in cells that removed exon3 from the *smad2* gene by alternative splicing, DNA binding of Smad2 is enabled [112]. Protein–protein interactions with transcription factors such as ATF-2 [113], c-Jun [114,115], SP1 [116] or TFE3 [117] are also mediated by the MH1 domain.

Based on a basic nuclear localization signal (NLS)-like motif present in the MH1 domain, an intrinsic nuclear import activity is inherent to R-Smads. It is hypothesized for Smad3 that, by virtue of the intra-molecular interaction of MH1 and MH2, the NLS-like motif is buried in the basal state. Phosphorylation of Smad3 causes conformational changes that expose the NLS-like motif, allowing interaction with importin-β1 and recognition by the nuclear import machinery [118,119]. In addition, Smad2 was shown to be retained in the cytoplasm by interaction with the anchor protein SARA. Ligand-induced release of SARA unmasks the NLS-like motif and leads to Smad2 nuclear translocation by a mechanism that is independent of importin-β1 but requires a specific region in the MH2 domain which elicits constitutive nuclear import function [79]. Although Smad4 contains only an incomplete NLS-like motif in its MH1 domain, it has the ability to constitutively enter the nucleus in absence of ligand. The cytoplasmic localization of Smad4 is observed under unstimulated conditions and is based on active nuclear export mediated by the leucin-rich nuclear export signal (NES) that resides in the Smad4 linker region. Ligand-dependent nuclear entry of Smad4 presupposes inactivation of the NES which is achieved by hetero-oligomerization with R-Smads [120,121].

5.1.2. The linker

The linker which connects MH1 and MH2 domains is less conserved between the different Smad classes but has been well conserved through evolution and contains several important regulatory peptide motifs. There are multiple consensus phosphorylation sites for mitogen-activated protein kinases (MAPK) [122] which, upon phosphorylation, cause sequestration of R-Smads in the cytoplasm by impeding nuclear translocation [122,123]. Additionally, a proline–tyrosine (PY) motif present in R-Smads and I-Smads enables recognition by the WW domains of the E3 ubiquitin ligases, Smurf1 and Smurf2 [87,92]. The Smad4 linker is equipped with the aforementioned NES in the N-terminal

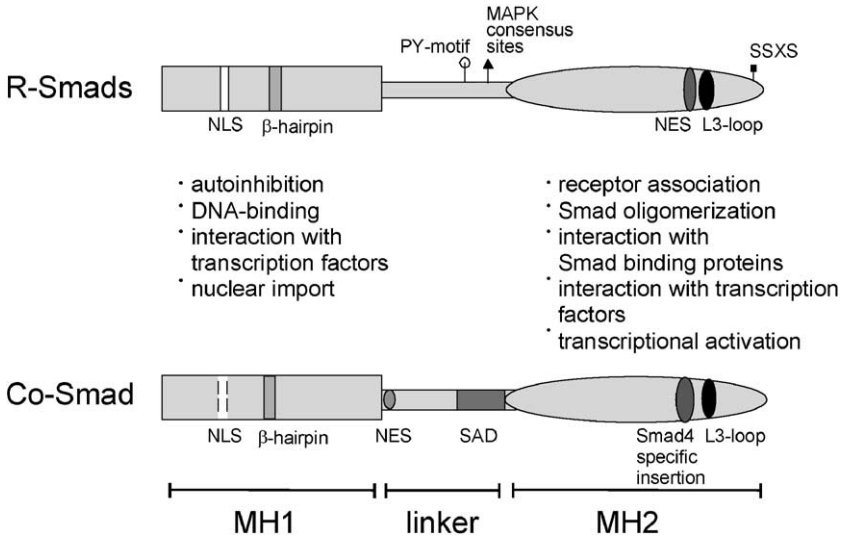

Fig. 4. Functional domains of R-Smads and Co-Smads. The overall structure of Smad proteins comprises the conserved MH1 and MH2 domains and the intervening linker region. Each part exerts specific functions that are conferred by specific structural motifs in the proteins. The functional differences between R-Smads and Co-Smads are thus reflected in the presence or absence of distinct protein modules.

part and with the Smad activation domain (SAD) that is essential for its transactivation activity [124].

5.1.3. The MH2 domain

The MH2 domain is known to mediate diverse protein–protein interactions, to exert effector function and, in case of the R-Smads, to provide receptor phosphorylation sites. In addition, an NES residing in the MH2 domain of R-Smads suggests a function of the MH2 domain in cytoplasmic targeting. Resembling Smad4, Smad1 was described as undergoing constant nucleoplasmic shuttling conferred by the presence of both functional NLS and NES. The four hydrophobic amino acids that are critical for conveying nuclear export are conserved among all R-Smads, implying that they also trigger nuclear export of TGF-β-specific R-Smads. However, despite the conserved sequence of the motif, structural and functional investigations rather hint to an executed activity of the NES only in BMP- but not TGF-β-specific R-Smads [125].

Protein–protein interactions mediated by the MH2 domain cover homomeric as well as heteromeric complex formation. The stoichiometry is still a matter of controversy for both the homomeric [126,127] and the heteromeric Smad complexes. The heteromeric R-Smad/Smad4 complexes that assemble upon signal activation have been suggested to be hexameric [128], trimeric [126,129–132] or dimeric [127,133]. Recent publications suggest that for Smad1 [130] and Smad2 [134], these R-Smads exist as monomers in the basal state. Upon ligand-induced phosphorylation, they assemble to form a homotrimer which is stabilized by the subunit interface and by specific contacts between the phosphorylated tail and the L3 loop in the MH2 domain of the neighbouring monomer [131,135]. However, formation of heterotrimers is energetically favoured over the homotrimer formation.

Moreover, the MH2 domain is involved in mediating Smad-receptor association. The specificity for distinct Smad isoforms is thereby determined by the L3 loop in the R-Smads and the L45 loop in the type I receptor [9,10]. The crystal structure of the Smad2 MH2 domain in complex with the Smad-binding domain (SBD) of SARA indicated that a basic patch adjacent to the L3 loop is likely to serve as a docking site for the receptor [81]. The activated type I receptor triggers phosphorylation of R-Smads and initiates the downstream cascade. In the nucleus, a multitude of co-activators, co-repressors and DNA-binding partners can assemble with Smads to regulate expression of distinct target genes.

As there are several recent reviews that deal nicely and extensively with the cohort of cytoplasmic and nuclear Smad-interacting proteins [15,108,136–139], this aspect of TGF-β signalling will not be subject of the present review. However, these proteins have an extremely high impact on determining the signalling outcome and on the complexity of TGF-β/Smad signalling as they represent modulators as well as points of convergence between different pathways.

5.2. Phosphorylation of Smad proteins

TGF-β-induced activation of TβRI is followed by transient interaction between TβRI and R-Smads which become phosphorylated at the last two serine residues within the C-terminal SSXS-motif [140]. Consequences of this phosphorylation event are the release of Smad autoinhibition [11,109], the release of the R-Smad from cytoplasmic retention proteins such as SARA [79], microtubuli [141] or potentially filamin [142], as well as from the type I receptor. At the same time, the affinity for Smad4 is increased [131,140,143].

Several lines of evidence reveal that Smad proteins are not solely phosphorylated by TβRI but additionally rank as substrates for a growing number of cytoplasmic kinases (Fig. 5).

Members of the MAP kinase family are frequently involved in TGF-β/Smad signalling and several of them were shown to represent kinases for Smad proteins. Smad1 was found to be a substrate for the Erk subfamily of MAP kinases which becomes activated following binding of hepatocyte growth factor (HGF) or epidermal growth factor (EGF) to tyrosine kinase receptors [123]. Phosphorylation of four Erk consensus sites (PXSP motifs) located in the linker region of Smad1 results in prevention of nuclear accumulation of Smad1. Although the Erk consensus sites are not completely conserved in Smad2 and Smad3, EGF likewise induces linker phosphorylation in these R-Smads leading to their cytoplasmic retention. It is proposed that instead of the Erk sites, serine/proline motifs can equally function as targets for phosphorylation [122] (Fig. 5). Oncogenic Ras was determined to initiate the same response but to produce stronger effects compared to EGF. Efficient abrogation of TGF-β signalling can account for the loss of TGF-β-induced growth inhibition in tumours harbouring oncogenic mutations in Ras [122]. Deviating results, however, provide evidence that HGF and EGF mediate TGF-β-independent C-terminal Smad2 phosphorylation by a kinase downstream of MEK-1 [144]. The opposing effects of TGF-β and HGF/EGF demonstrate that Smad activity can be balanced depending on whether signals are emanating from receptor serine/threonine kinases (RSKs) or from receptor tyrosine kinases (RTKs).

Nuclear accumulation of Smad2 was demonstrated to be affected by Ca^{2+}-calmodulin-dependent protein kinase II (Cam kinase II) which triggers phosphorylation of several serine residues (Ser110, Ser240 and Ser260) within the Smad protein [145] (Fig. 5). The exact mechanism that results in inhibition of Smad signalling is not yet known but is likely to be complex due to the distinct sites that might convey signal regulation by different means. Recently, phosphorylation of Ser240 in Smad2 by Cam kinase II

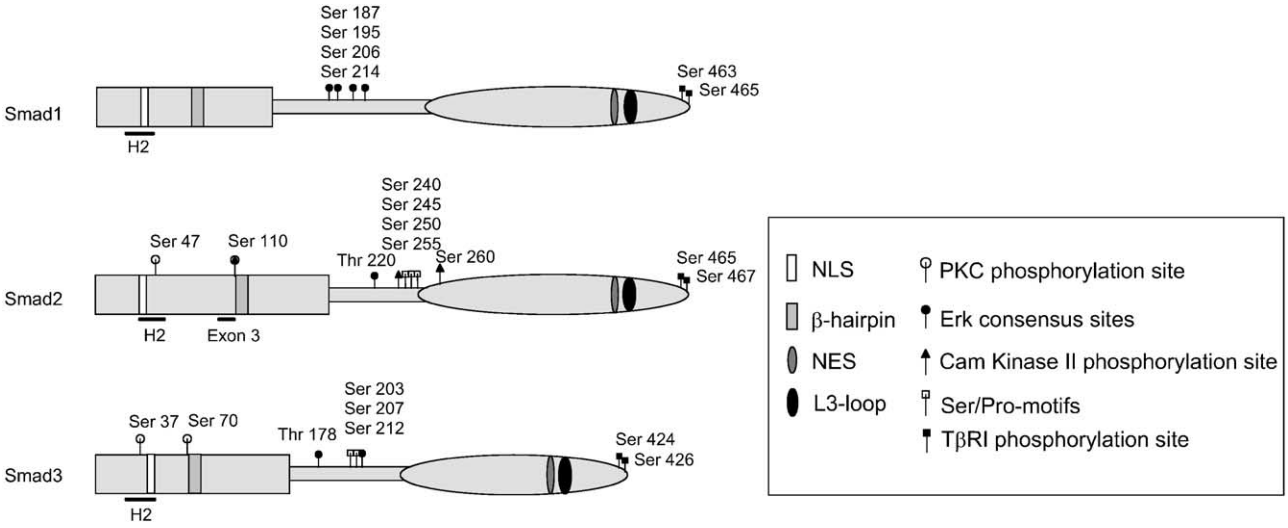

Fig. 5. Phosphorylation sites in R-Smad proteins. R-Smad proteins get phosphorylated by the activated TβRI at the C-terminal serine residues. Other sites of phosphorylation are targeted by different kinases such as protein kinase C (PKC) or Erk/MAP kinases, demonstrating that regulation of Smad activity by posttranslational modifications can be achieved by multiple proteins.

was suggested to be the mechanism by which decorin elicits inhibition of TGF-β-induced fibrotic responses [146].

Protein kinase C which is activated by TGF-β can provide a negative feedback by phosphorylating specific serine residues in the MH1 domain of Smad3 [147] (Fig. 5). The phosphorylation sites reside on the surface that is facing the DNA and therefore preclude DNA-binding of Smad3. Although corresponding sites get phosphorylated in Smad2, the inhibitory effect is specific for Smad3 because Smad2 is not able to directly bind to DNA in any case.

Further cytoplasmic kinases which mediate Smad phosphorylation outside the C-terminal site that is used by TβRI are the c-Jun N-terminal kinase (JNK) [148] and the MAP kinase kinase kinase (MEKK-1) [149]. In both cases, the exact position of the target residues are not yet identified. TGF-β-stimulated JNK activation was shown to be biphasic: a rapid Smad-independent JNK activation is followed by sustained Smad-dependent JNK activity. The initial JNK activation leads to Smad3 phosphorylation at sites other than the SSXS motif, facilitating subsequent C-terminal phosphorylation by TβRI. The downstream signals emanating from activated Smad3 synergize in turn with JNK signalling by augmenting AP-1 activity. Referring to the MEKK-1 kinase, it was reported that constitutively active MEKK-1 can mediate Smad2-dependent transcriptional responses in endothelial cells. The activating Smad2 phosphorylation induced by MEKK-1 does not occur on the C-terminal serine residues but is likely to target amino acids residing in the linker region or in the MH2 domain.

In conclusion, there is an increasing number of cellular kinases besides TβRI that can account for modulation of Smad activity via phosphorylation of distinct amino acid residues. Additionally, the interdependent relationship of JNK and Smad signalling demonstrates that phosphorylation events can be critical to maintain the balance between different signalling pathways.

5.3. Termination of Smad activity

Several proteins have already been mentioned that negatively regulate TGF-β signalling by controlling Smad activity: first, inhibitory Smad proteins rapidly induced by TGF-β block phosphorylation of R-Smads; second, various Smad binding proteins and transcriptional co-repressors inhibit expression of Smad-dependent target genes; third, the Smurf proteins that target R-Smad for degradation. However, evidences for the most obvious mechanism of Smad inactivation, namely, dephosphorylation of R-Smads are just beginning to emerge. Smad2 dephosphorylation after prolonged TGF-β signalling could be observed in HaCaT and in NIH 3T3 cells. Dephosphorylated Smad2 dissociates from Smad4 and both proteins are subsequently accumulating back in the cytoplasm [121].

Elucidation of phosphatases and mechanisms responsible for Smad dephosphorylation turns out to be a challenging milestone for future research.

6. Concluding remarks

From an immense number of TGF-β-related signalling studies, it became evident that the Smad pathway plays a central role for many cellular responses such as control of proliferation or differentiation. However, the pleiotropic effect of TGF-β is established by the interplay of the Smad pathway with Smad-independent pathways (e.g. MAPK or Jak/STAT) in a specific cellular context.

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