Tight regulation of TGF-β (transforming growth factor-β) superfamily signalling is important for normal cellular functions and tissue homeostasis. Since TGF-β superfamily signalling pathways are activated by a short phosphorylation cascade, from receptor phosphorylation to subsequent phosphorylation and activation of downstream signal transducer R-Smads (receptor-activated Smads), reversible phosphorylation serves as a critical step to assure proper TGF-β signalling. The present article will review the current progress on the understanding of dynamic phosphorylation in TGF-β signalling and the essential role of protein phosphatases in this process.

Key words: dephosphorylation, phosphorylation, protein phosphatase, Smad, transforming growth factor-β (TGF-β) signalling.
pathways by the activation of other signalling molecules [e.g. MAPK (mitogen-activated protein kinase), Akt, Src and mTOR (mammalian target of rapamycin)] independent of Smad proteins [34,35], which amplifies the complexity of TGF-β signalling.

POST-TRANSCRIPTIONAL MODULATION OF TGF-β SUPERFAMILY SIGNALLING

As TGF-β superfamily signalling regulates a broad range of cellular responses, it is logical that the activity of TGF-β signalling must be tightly controlled. Reduction in the level of ligands, internalization and degradation of the receptors, and induction of I-Smads represent means through which TGF-β signals are modulated. Recently, it has become evident that, in addition to reversible phosphorylation (to be discussed further in the sections below), TGF-β signalling is also regulated by various types of post-transcriptional modifications such as ubiquitination, SUMOylation and acetylation [36].

Smads are regulated by the ubiquitination–proteasome pathway. Smurf (Smad ubiquitination-related factor) family members Smurf1 and Smurf2, and the related NEDD4 (neural-precursor-cell-expressed developmentally down-regulated 4)-2, which share extensive sequence similarity, have been identified as E3 ligases involved in type I receptor and R-Smad ubiquitination and degradation. Smurf1 targets Smad1 and Smad5 for ubiquitination and subsequent proteasome-mediated degradation [37]. Smurf2 is also involved in Smad1 and Smad2 ubiquitination and proteasome-mediated degradation [38–41]. Smad3, a closely-related R-Smad to Smad2, also binds to Smurf2 and NEDD4-2, yet Smad3 is not degraded by these HECT (homologous with E6-associated protein C-terminus) class E3 ligases [38–40]. Nevertheless, Smad3 is regulated by proteasomal degradation and targeted for ubiquitination via different classes of ubiquitin E3 ligase. Smad3 interacts with Rbx1 (also called ROC1 and Hrt1), a RING-containing component of the SCFβTrCP (where SCF is Skp1/cullin/F-box, and βTrCP is β-transducin repeat-containing protein) complex [also called SCFFbw1a (F-box/WD repeat-containing protein 1a)], which is partly responsible for Smad3 degradation [42]. Additionally, CHIP [C-terminus of Hsc (heat-shock cognate) 70-interacting protein] serves as a U-box-dependent E3 ligase that can directly mediate the ubiquitination and degradation of Smad3 [43] and also Smad1 [44].

Smad4 is central to canonical TGF-β superfamily signalling. Although it is a fairly stable protein, Smad4 can be targeted by polyubiquitination via various E3 ligases such as Skp2 [45], Jab1 [46] and Smurfs [47]. In addition, Smad4 is mono-ubiquitinated at Lys507 [48,49] and SUMOylated at Lys113 and Lys159 [50,51]. These modifications regulate stability and signalling strength in developmental contexts. The importance of Smad4 regulation by SUMOylation and ubiquitination events is underscored further by the interplay between phosphorylation and ubiquitin/ubiquitin-like modifications in cancer, where certain somatic mutations on Smad4 switch Smad4 from SUMOylation to ubiquitination and ultimately cause rapid degradation of the tumour suppressor [45].

Besides Smads, ubiquitination of activated TGF-β receptors is also mediated by Smurfs and related NEDD4-2, leading to the proteasome-dependent degradation of TGF-β receptors.
[40,52,53]. Smad7 plays an important role in this process [54]. Smad7 resides in the nucleus in unstimulated cells and is translocated to the plasma membrane upon ligand stimulation [55,56]. In addition to its ability to directly inhibit R-Smad phosphorylation, Smad7 recruits Smurf1 and Smurf2 to the activated TβRII complex and promotes ubiquitination and turnover of the receptors as well as Smad7 and Smurf2 themselves. In accordance, Smurf2 also mediates proteasomal degradation of the TGF-β receptors that is induced by inhibition of the molecular chaperone HSP90 (heat-shock protein 90) [57].

Smurf-mediated ubiquitination of Smad7 is antagonized by acetylation. Smad7 is acetylated in the nucleus as a result of the interaction with the transcriptional co-activator p300 [58]. This acetylation blocks ubiquitination on the same residues, resulting in a higher concentration of Smad7 available to bind to receptors and block receptor–R-Smad binding. Conversely, Smad7 can be deacetylated by HDAC1 (histone deacetylase 1), which decreases the stability of Smad7 by enhancing its ubiquitination [59]. Thus the activity of Smad7 is regulated by a balance among acetylation, deacetylation and ubiquitination.

**PROTEIN PHOSPHATASES**

Although protein kinases phosphorylate and, in most cases, induce the activities of their substrates, protein phosphatases reverse the actions of protein kinases and thus inactivate the substrates. This reversible phosphorylation provides the balance for the proper functioning of signalling molecules. Sequence analysis predicts that there are ~150 putative protein phosphatases in the human genome, which can be classified into three groups: 38 PTP (protein tyrosine phosphatase) family members, ~40 PS/PT (protein serine/threonine phosphatase) members and ~68 DUSPs (dual-specific serine/threonine phosphatases) [60,61]. Furthermore, the PS/TP family is classified into three subfamilies by their structurally distinctness: PPM (metal ion-dependent protein phosphatase), PPP (phosphoprotein phosphatase) and FCP/SCP (farnesylcysteine phosphatase)-containing component of CTBD (C-terminal domain) phosphatase/small CTBD (C-terminal domain) phosphatase). The PPM subfamily consists of PPM1A/PP (protein phosphatase) 2Ca, PPM1B/PP2Cβ and other PP2C-domain-containing proteins [60,62]. Members of this subfamily are monomeric phosphatases with a single subunit, the catalytic subunit. The PPP subfamily mainly includes PP1, PP2A, PP4, PP5 and PP6. Among them, PP1 is ubiquitously expressed and highly conserved. Members of this subfamily often contain multiple subunits, including the catalytic subunit and regulatory/targeting subunits, that guide the catalytic subunit to the right place at the right time. The founding member of the FCP/SCP subfamily is represented by FCP1, which is a phosphatase that dephosphorylates the C-terminal domain of RNA polymerase II [63].

**PHOSPHATASES AND TGF-β RECEPTOR ACTIVITY**

The phosphorylation cascade from receptor to Smad proteins plays an important role in the activation of TGF-β signalling. Dephosphorylation of receptors and Smad proteins contributes to the duration and intensity of TGF-β signalling. An increasing number of protein phosphatases have been reported to regulate TGF-β signalling at both the receptor and Smad levels.

At the receptor level, several studies have reported the involvement of PPP family phosphatases in modulating the receptor activity. Members of the PPP family contain the catalytic subunit and a highly diverse array of regulatory and/or targeting subunits, and therefore represent hundreds of PS/TPs. For example, PP1 has at least 16 regulatory subunits, each of which may play a unique role in targeting PP1c (catalytic subunit of PP1) to its specific substrates in a tempo-spatial manner. A previous study in Drosophila melanogaster identified PP1c as a negative regulator of Dpp (Decapentaplegic) signalling. PP1c binds to SARA (Smad anchor for receptor activation) through a specific motif and is targeted to the Dpp receptor complexes [64]. Disruption of this PP1–SARA interaction leads to hyperphosphorylation of the Dpp type I receptor. Although there is no direct evidence supporting dephosphorylation of the type I receptor by PP1, SARA-recruited PP1c can reduce the phosphorylation level of the type I receptor and negatively regulate Dpp signalling [64]. Thus SARA is a novel targeting subunit that recruits PP1c to the type I receptor.

Further studies suggest that the PP1 protein also plays a role in regulating TβRII in various mammalian cell types. Shi et al. [65] found that, upon TGF-β stimulation, Smad7 interacts with GADD (growth-arrest and DNA-damage-inducible protein) 34, a regulatory/targeting subunit of the human PP1 holoenzyme. The formation of the Smad7–GADD34 complex recruits PP1c to dephosphorylate TβRII in Mv1Lu mink lung epithelial cells, a process enhanced by SARA as in the case in Drosophila. The Smad7–GADD–PP1c complex significantly inhibits TGF-β-mediated cellular responses, indicating that the formation of PP1 holoenzyme mediated by Smad7 functions as a negative feedback signal in the TGF-β signalling pathway (Figure 2) [65]. Consistent with this finding, ectopic expression of Smad7 and PP1α (PP1c isoform α) potently inhibit TGF-β/ALK1-induced Smad1/5 phosphorylation in ECs (endothelial cells), whereas knockdown of either Smad7 or PP1α enhances Smad1/5 phosphorylation [33]. It is interesting to note that Smad7 and PP1α gene expression is up-regulated by TGF-β treatment in ECs through a non-canonical TGF-β/ALK1 pathway. These studies suggest that Smad7 may recruit PP1 to dephosphorylate type I receptors and consequently control receptor-induced downstream signalling events.

In addition to PP1, PP2A is another well-known protein phosphatase that associates with TGF-β receptors [66–68]. PP2A is also a member of the PPP family and this remarkably conserved phosphatase accounts for the majority of PS/TP activity in most cells. PP2A functions as heterotrimERIC enzyme, which consists of a catalytic C subunit, a structural/scaffolding A subunit and a regulatory B-type subunit. The B-type subunits function as
targeting factors, and hence confer specificity, diversity and regulation of the PP2A holoenzyme. The interaction between TGF-β receptors and PP2A subunits was first reported in 1998 [68], and a recent study [66] has suggested an opposite effect of two PP2A B-type regulatory subunits, Bα and Bδ, in the TGF-β/activin/Nodal signalling pathway (Figure 2). In the latter study, Batut et al. [66] noticed that, in Xenopus embryos, the phenotype of Bα knockdown or Bδ overexpression is highly similar to those with loss of Nodal signalling. Further studies suggest that Bα enhances TGF-β/Activin/Nodal signalling by stabilizing the basal level of the type I receptor, whereas Bδ negatively modulates these pathways by restricting receptor activity [66]. Unexpectedly, there is no solid evidence supporting the role of PP2A in direct dephosphorylation of either the TGF-β receptors or Smad2. However, PP2A may dephosphorylate Smad3, but not Smad2, under hypoxic conditions, suggesting a complicated regulatory function of the PP2A phosphatase in the regulation of TGF-β signalling [69].

Despite these nuclear-localized phosphatases that function to facilitate R-Smad export from the nucleus to cytoplasm, a DUSP family phosphatase, MTMR4 (myotubularin-related protein 4), also negatively regulates TGF-β signalling by interacting with and dephosphorylating activated R-Smads in the early embryo, and then blocking R-Smad nuclear translocation [76].

Similar to Smad2/3, Smad1/5/8 is also regulated through reversible phosphorylation. Duan et al. [77] reported that PPM1A dephosphorylates the C-terminal SXS motif of phosphorylated Smad1/5/8, suggesting that PPM1A is a pan-Smad phosphatase. Are there any phosphatases that specifically target the TGF-β or BMP branch? It has been reported that SCP1/2/3 and PDP (pyruvate dehydrogenase phosphatase) were initially identified to dephosphorylate the SXS motif of Smad1 in Xenopus and Drosophila respectively [78,79]. Furthermore, knockdown of SCP1/2 [78,79] or PDP [78,79] appears to increase the level of Smad1 phosphorylation in mammalian cells.

PHOSPHATASES THAT TARGET THE SXS MOTIF OF R-SMADS

Phosphorylation of R-Smad proteins in the SXS motif by type I receptors is a central event during TGF-β signal transduction activation. SXS phosphorylation triggers a cascade of intracellular responses from R-Smad–Co-Smad complex formation in the cytoplasm to the transcriptional control in the nucleus (Figure 1). Since signal transduction pathways are regulated by the dynamic interplay between protein kinases and phosphatases, it is reasonable to postulate that the SXS motif must be dephosphorylated by phosphatases to ensure a proper balance of Smad signalling. Experimental evidence has shown that R-Smads shuttle between the nucleus and cytoplasm. Although persistent accumulation of the R-Smad–Smad4 complex in the nucleus requires continuous TGF-β receptor activity, nuclear export of R-Smads has been shown to depend on their dephosphorylation and dissociation of Smad complexes [70–73]. This further suggests the existence of nuclear Smad phosphatases that target the SXS motif to terminate TGF-β/Smad signalling.

In the study by Lin et al. [74], a library of human serine/threonine phosphatases were screened for their activity towards dephosphorylating Smad2/3. Experimentally, the screen is rather simple by first co-expressing Smad2/3 (the substrate) and a phosphatase (the enzyme) in the same cell and then looking for those phosphatases that reduce or abolish the phosphorylation of Smad2/3 that is induced by a constitutively active mutant of TβRI. Out of these 39 phosphatases, only a nuclear-localized protein phosphatase, PPM1A/PP2Cα, reduces the level of Smad2/3 phosphorylation induced by the activated TGF-β receptor [74]. Furthermore, RNAi (RNA interference)-mediated knockdown of endogenous PPM1A expression increases the strength and duration of Smad2/3 SXS phosphorylation in the nucleus. A series of biochemical experiments pinpoint further PPM1A as a direct phosphatase towards Smad2/3 dephosphorylation, rather than inactivation of the upstream type I receptor [74]. PPM1A can directly interact with phospho-Smad2/3, but not unphosphorylated Smad2/3, and can dephosphorylate recombinant phospho-Smad2/3 proteins in vitro [74]. Importantly, PPM1A facilitates the interaction of dephosphorylated Smad2/3 with RanBP3 (Ran-binding protein 3), a nuclear export factor [75]. As a result, PPM1A-mediated dephosphorylation of Smad2/3 promotes nuclear export of Smad2/3 and shuts off TGF-β-induced anti-proliferative and transcriptional responses (Figure 3). The Smad-antagonizing activity of PPM1A is also observed during Nodal-dependent early embryogenesis in zebrafish [74].

REVERSIBLE PHOSPHORYLATION AT THE LINKER REGION OF R-SMADS

Besides phosphorylation at the C-terminal SXS motif of Smads by TβRIs that represents the key event in Smad activation, phosphorylation at additional sites also regulates the activity of Smads. In contrast with the conserved N-terminal MH1 domain and C-terminal MH2 domain, sequences in the linker region between the MH1 and MH2 domains in R-Smad proteins are very divergent. The linker region contains multiple sites of phosphorylation by a number of intracellular protein kinases that are activated by signals, such as mitogenic growth factors, and therefore the linker integrates the activation of these protein kinases into the fine-tuning of Smad activation.

Proline-directed protein kinases, such as MAPKs and CDKs (cyclin-dependent kinases), are major groups of protein kinases that exhibit preference for specific serine/threonine residues in the linker region [80,81]. Studies have demonstrated that phosphorylation in the linker region of R-Smads plays both positive and negative roles in TGF-β signalling. Phosphorylation of Smad3 by CDK2/4 inhibits its transcriptional activity, reduces the anti-proliferative action of TGF-β, and serves as a novel means by which CDK2 promotes aberrant cell-cycle progression and confers cancer cell resistance to the growth-inhibitory effects of TGF-β [81]. Although CDK-mediated R-Smad phosphorylation appears to play negative roles in TGF-β signalling, MAPK-mediated phosphorylation has a dual role in Smad2/3 regulation. For example, ERK (extracellular-signal-regulated kinase)-dependent Smad2 phosphorylation at Thr245/247 and Ser255 and Thr265 as well as Smad3 phosphorylation at Ser204/Ser206 and Thr210 play an inhibitory role on Smad2/3 transcriptional activity [82]. In addition to CDKs and ERK, a number of intracellular protein kinases have been found to phosphorylate the linker region of R-Smads. p38, Rho kinase and JNK (c-Jun N-terminal kinase) phosphorylate Smad2/3 at multiple sites and enhance their transcriptional activity [83–87]. Other kinases, e.g. CK1δ/ε, CaMKII (Ca2+/calmodulin-dependent protein kinase II), PKC (protein kinase C), GRK2 (G-protein-coupled-receptor kinase 2) and MEKK1 (MAPK/ERK kinase kinase 1), can also target R-Smads and regulate Smad-dependent transcriptional responses [88,89]. Like Smad2/3 in the TGF-β/activin pathway, Smad1 in the BMP pathway can also be phosphorylated in its linker region by protein kinases. For example, Ser198, Ser195, Ser206 and Ser214 sites in the linker region of Smad1 are phosphorylated by ERK [90,91], whereas Ser190 and
Figure 3 Regulation of R-Smad nucleocytoplasmic cycling by reversible phosphorylation

R-Smads are phosphorylated by type I receptor kinases and are then translocated into the nucleus. In the nucleus, Smads are dephosphorylated by phosphatases such as PPM1A, leading to their export back to the cytoplasm.

Ser210 sites are phosphorylated by GSK-3β (glycogen synthase kinase-3β) [92, 93]. Thus the linker region is a critical regulatory platform in TGF-β superfamily signalling.

Phosphorylation in the linker regions serves an important function in regulating the activity, stability and transport of R-Smads. Linker phosphorylation of Smad2/3 by TGF-β facilitates the binding of NEDD4L to Smad2/3 and consequently results in Smad2/3 polyubiquitination and degradation [41]. Similarly, Smurf1 binding to the phosphorylated linker promotes Smad1 ubiquitination; Smurf1 also causes cytoplasmic retention of Smad1 through blocking the Smad1–Nup214 interaction [92].

With the increase in evidence indicating the importance of the linker phosphorylation in TGF-β signalling, protein phosphatases have been identified to reversely control this dynamic process. Specifically, phosphatases SCP1/2/3 dephosphorylate specific residues in the linker, but not in the SXS motif of Smad2/3 [94, 95]. The activity of SCPs antagonizes the mitogen-mediated inhibition of TGF-β transcriptional responses. Interestingly, for Smad1, SCPs not only mediate dephosphorylation in its linker region, but also dephosphorylate its SXS motif [78, 95]. However, SCPs do not dephosphorylate all phospho-residues in the linker and MH1 domain. It is of great interest to identify additional phosphatases that selectively dephosphorylate individual phospho-residues in these regions.

CONCLUSIONS AND PERSPECTIVES

Accumulating evidence illustrates the important role of post-translational modification in regulating components of the TGF-β signalling pathway and, ultimately, the critical cellular responses induced by TGF-β ligands. Recent advances emphasize the importance of how reversible phosphorylation and dephosphorylation controls the physiological consequences in response to developmental or environmental cues. Notably, a few phosphatases have been identified that both negatively and positively regulate TGF-β superfamily signalling.

Despite the progress, the precise role of most of the phosphatases remains undisclosed, largely due to the complexity and pleiotropy of both the TGF-β signalling and phosphatases. For example, the molecular mechanism of PPP-mediated regulation of receptor activity is still elusive. What are the direct substrates of PP1 or PP2A during receptor regulation? How and in which subcellular compartments are the individual subunits or the holoenzyme recruited to the receptor? Even for nuclear phosphatases such as PPM1A, do they dephosphorylate the Smad activator complex on chromatin and dissociate the complex or only dephosphorylate phospho-R-Smads after Smad/DNA dissociation? The answers to these questions are important in addressing the functions and mechanism of how phosphatases fine-tune the TGF-β signalling strength and duration during embryogenesis and other developmental processes.

Because the TGF-β superfamily is evolutionarily conserved, more exhaustive genetic screens in multiple species (e.g. *Drosophila, Caenorhabditis elegans* and RNAi screens in mammalian cells) are anticipated to search for additional protein phosphatases involved in the regulation of TGF-β signalling. Several features for these new phosphatases include: (i) they either directly dephosphorylate the receptors or Smads; (ii) they act either for the entire TGF-β superfamily or in a pathway-restricted
manner; (iii) functionally, they may play either positive or negative roles, depending on the targeted residues of the receptors or Smads; and (iv) they may also function to regulate Smad nuclear entry, act to stabilize/dissociate the Smad transcription complex or to modulate Smad nuclear export. Identification and characterization of new phosphatases will not only help us to draw a clearer view for the complex regulation of TGF-β signalling, but also lead to a more complete understanding of signalling cross-talk and integration between the TGF-β signalling pathway and the diverse signalling pathways in cells.

Considering critical roles of TGF-β signalling in tumorigenesis, deregulation of phosphatases in the TGF-β pathway may be an important requirement for tumour progression. Since both PP2A complexes and PPM1A are implicated in tumorigenesis, it is plausible that these phosphatases exert their functions in tumour development through regulation of the TGF-β signalling. Further investigations are needed to elucidate the pathological roles of TGF-β receptor/Smad phosphatases in cancer and other human diseases (e.g. vascular diseases and autoimmune diseases) where TGF-β signalling is a critical player.

FUNDING

The work in the laboratories of the authors are supported by China’s Fundamental Research Funds for National/Central Universities, and the National Institutes of Health [grant numbers R01AR053591, R01GM063773 and R01CA108454].

REFERENCES

Funds for National/Central Universities, and the National Institutes of Health [grant numbers R01AR053591, R01GM063773 and R01CA108454].
Regulation of TGF-β signalling by protein phosphatases


