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Review

Regulation of TGF-β signal transduction by mono- and deubiquitylation of Smads

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ABSTRACT

Polyubiquitylation leading to proteasomal degradation is a well-established mechanism for regulating TGF-β signal transduction components such as receptors and Smads. Recently, an equally important role was suggested for monoubiquitylation of both Smad4 and receptor-associated Smads that regulates their function without protein degradation. Monoubiquitylation of Smads was discovered following the identification of deubiquitylases required for TGF-β signaling, suggesting that continuous cycles of Smad mono- and deubiquitylation are required for proper TGF-β signal transduction. Here we summarize and discuss recent work on Smad mono- and deubiquitylation.

1. Introduction

Members of the TGF-β family of proteins are pleiotropic cell signaling molecules involved in many fundamental biological processes. These include the induction of the embryonic germ layers, patterning the axes of the body plan and maintenance of homeostasis in adult tissues [1]. Mirroring these multiple roles, defects in TGF-β signaling are associated with both developmental and adult syndromes such as birth defects, tissue fibrosis and cancer [2]. Mechanisms underlying the transduction of TGF-β signals from the cell membrane to the nucleus have been extensively characterized and reviewed elsewhere [3,4]. Briefly, secreted TGF-β ligands engage in a complex with two transmembrane kinase receptors leading one member of the receptor complex to phosphorylate C-terminal serine residues of R-Smads (Receptor-associated Smad signal transducers). Phosphorylation enables R-Smads to accumulate in the nucleus where they form a DNA binding complex with their sibling protein Smad4. This Smad heteromeric complex regulates gene-expression in conjunction with promoter-specific transcription factors and cofactors. This basic scenario is shared between the TGF-β/Activin/Nodal and Dpp/BMP subfamilies of TGF-β family ligands, although each subfamily employs a different set of R-Smads (Smad2/3 or Smad1/5/8, respectively).

A growing number of studies have shown that numerous mechanisms ensure tight control over the activity of receptors and Smads, thereby regulating cellular responsiveness to TGF-β signals. Some of these strategies influence the primary phosphorylation events of the signaling cascade, such as receptor and R-Smad phosphorylation [5] while others involve a variety of post-translational modifications to pathway components. In the case of Smads these modifications include additional phosphorylation events [6,7], sumoylation [8], parpylation [9], acetylation [10] and ubiquitylation [11,12].

Ubiquitin entered our understanding of TGF-β signaling when the mammalian HECT-domain ubiquitin ligases, Smurf1 and Smurf2, were discovered as negative regulators of the pathway [13,14]. Subsequently other molecules with E3 ubiquitin ligase activity were isolated that regulate TGF-β signaling. These include additional HECT-domain family members (e.g., Nedd4L, Wwp1/Tiul1 and Aip4/Itch; 12) and RING-domain proteins such as Arkadia, Highwire and Ectodermin/Tif1-γ/Trim33 [15–17]. These molecules, except Arkadia, were isolated as inhibitors of TGF-β signaling thus assigning to ubiquitylation a predominantly negative role in the TGF-β cascade. The role of polyubiquitylation and degradation in TGF-β signaling is well known [12]. Here we discuss technical issues related to the analysis of monoubiquitylation and recent data revealing the role of mono- and deubiquitylation in the regulation of Smad activity.

2. The basics of ubiquitylation

Ubiquitylation is a regulatory mechanism that impinges on a wide variety of processes including cell-cycle checkpoints, DNA damage responses and signal transduction pathways [e.g., [18]].

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Ubiquitylation is the covalent attachment of a ubiquitin polypeptide to a target protein via the sequential action of three enzymes: a ubiquitin activating enzyme (E1), a conjugating enzyme (E2) that carries the activated ubiquitin and transfers it to the target protein and a ubiquitin ligase (E3) that binds both the target and the E2 to promote efficient ubiquitin transfer.

Compared to other post-translational modifications, ubiquitylation is a particularly diverse process [19]. Ubiquitin can be linked to a target protein as a monomer on a single lysine (monoubiquitylation), as a monomer to multiple lysines (oligoubiquitylation), or several ubiquitin molecules can be added serially to the same lysine forming a polyubiquitin chain. Furthermore, polyubiquitin chains can assume different geometries according to which of the seven internal lysines of ubiquitin (K48, K63, K29, etc.) is used for polymerization. As a result ubiquitylation can participate in a variety of regulatory mechanisms. For example: (1) as a trigger for proteasomal degradation (K48-linked polyubiquitylation), (2) as a scaffold for protein–protein interactions (K63-linked polyubiquitylation in the NF-κB pathway), (3) or as a tag for vesicle sorting during endocytosis (EGF receptor signaling). In addition, ubiquitylation can be reversed. Cells have at their disposal over 100 different deubiquitylating enzymes that can remove ubiquitin from modified proteins, thus resetting the system or enabling cells to switch from one type of ubiquitylation to another [20].

Monoubiquitylation received particular attention in the last decade when a number of observations in fields as disparate as DNA repair, histone regulation, membrane receptor trafficking and regulation of tumor suppressors (such as PTEN, FOXO and p53) pointed to a key role for monoubiquitylation as a general modulator of protein function, rather than as a dedicated signal for protein destruction [21,22]. In this respect, monoubiquitylation is comparable to protein phosphorylation: it can regulate protein activity and subcellular localization, it can form or conceal protein–protein interaction surfaces, it can be used for regulation in a time- and space-dependent manner without the need of regulating total protein levels and it can be rapidly reversed by the activity of deubiquitylases. Moreover, given the size of ubiquitin (76 amino acids), monoubiquitylation could in principle enable interactions based on the newly resulting tridimensional structure of the targeted protein.

3. Technical challenges in the analysis of ubiquitylation

When studying a regulatory mechanism based on ubiquitylation, there are three key experimental issues to be considered: (1) what is the relevant ubiquitylation pattern of the target – mono-, oligo- or polyubiquitylation; (2) does ubiquitylation regulate degradation of the target or is it regulative; and (3) in the regulative situation - to what extent can the biological function of the ligase (or the deubiquitylase) be explained by regulation of the proposed target?

For the first point (relevant ubiquitylation pattern of the target), it is clear that studying the endogenous ubiquitylation pattern of a protein is challenging. In rare instances, the pattern is so obvious that the mono- or polyubiquitylated isoforms can be readily detected by a simple western blot, as is the case for Hif1α or Fancd2 [23]. Generally this is not observed, even for a paradigmatic example of ubiquitin-dependent degradation such as p53. At least in part, this is because polyubiquitylated proteins are rapidly degraded and because many deubiquitylases are thought to be aspecifically activated upon cell lysis. Thus, in most cases it is necessary to immunoprecipitate the protein from cell extracts and identify double positive higher molecular weight bands with both anti-ubiquitin and an antibody to the target protein (or its epitope tag). Even then interpretation of the results may be complicated by the presence of other coprecipitating ubiquitylated proteins and by the fact that available antibodies detecting endogenous ubiquitin are often not very sensitive, such that low levels of ubiquitylation or monoubiquitylation can be easily missed. The use of overexpressed epitope-tagged ubiquitin constructs can solve the last problem, but they introduce an extra variable. Alternatively raising antibodies specific to ubiquitylated proteins would circumvent the need for immunoprecipitation. However this approach is very difficult with only two or three antibodies available. Thus it is often hard to assess, in a quantitative fashion, the degree of ubiquitylation of a protein.

Once the ubiquitylation pattern has been defined, the next question is where this modification is occurring on the target protein (i.e. on which lysine). Bioinformatics studies of evolutionary conservation can provide clues to the identity of targeted lysines based on the idea that evolution will act to conserve important regulatory interactions [24]. Alternatively direct mapping of ubiquitylation sites can be addressed either by systematic lysine mutation [25,26] or by mass-spectrometry [27]. The first approach can be complicated by the effect of amino acid mutations that are independent from ubiquitylation (e.g. modification of protein structure). The second approach is more direct but is limited to proteolytic peptides that can be detected, so that often there are lysines that cannot be queried. In the end, mutation of the relevant residue(s) should render the protein insensitive, both biochemically and functionally, to ubiquitylation.

For the second point (ubiquitylation regulates degradation of the target or is regulative), this too can be challenging. For a few proteins such as Hif1α, p53 and β-catenin it is self-apparent because inhibition of the proteasome greatly enhances detection of the polyubiquitylated protein and readily stabilizes its steady-state levels. In general it is more difficult to establish which is the relevant process. For example, degradation may be visible only in pulse-chase assays. Alternatively, polyubiquitylation and degradation may be visible only upon overexpression of the E3 ligase. Here, in the absence of supporting loss-of-function evidence E3 overexpression can be misleading as it can mask regulative ubiquitylation. Lastly, polyubiquitylation does not automatically lead to degradation. For example in the NF-κB pathway, K63-linked polyubiquitylation acts as a "scaffold" to enable signal transduction [19]. Also, in the TGF-β pathway K63-linked polyubiquitylation promoted by TRAF6 plays a key role in the regulation of non-Smad TGF-β receptor signaling [28–30].

For the third point (the biological function of an E3 ligase or a deubiquitylase is dependent upon its target protein), it is essential to identify the appropriate enzyme/target pair. In the ubiquitylation reaction, target specificity is thought to be primarily determined at the level of E3 ligase/target interaction [31]. However, E3 specificity is not absolute as one ligase can have multiple targets. This also applies to deubiquitylases where the potential for multiple interactions is even higher as the human genome encodes for over 100 deubiquitylases [23]. To determine the functional enzyme/target pair one relies on both biochemical and genetic evidence. For biochemical evidence one should observe a requirement of the E3 ligase for ubiquitylation. For genetic evidence, phenotypes due to loss of the E3 ligase should also depend on the target and phenotypes due to loss of the target should dominate those due to loss of the E3 ligase in double mutants (i.e. the target should be epistatic to the ligase). An example of strong genetic data supporting an enzyme/target pair is that of Mdm2 and p53: mouse knockouts for the p53 ligase Mdm2 die during embryogenesis and are fully rescued by the concomitant knockout of p53 [32].