Trends in Plant Science

CellPress REVIEWS

Forum

Proteolytic Proteoforms: Elusive Components of Hormonal Pathways?

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Hormonal pathways often converge on transcriptional repressors that can be degraded by the proteasome to initiate a response. We wish to draw attention to developments in a less-explored proteolytic branch called 'limited proteolysis' that, in addition to the classical proteolytic pathways, seems to regulate auxin and ethylene signaling.

Much like any biological process, proteolysis comes in different flavors. In contrast to proteasomal degradation, limited proteolysis yields protein fragments or so-called 'proteolytic proteoforms' (PPs) with potentially new interactions, localizations, and activities [1]. Here, we exclude from this definition signal peptide removal or peptide maturation to focus on unpredicted or unexpected proteolytic cleavage events. Assuming a single cleavage event in a protease substrate, two PPs are formed; the C-terminal one will have a new N terminus (neo-N terminus) while the N-terminal one will have a new C terminus (Figure 1A), which can be identified by specialized proteomic approaches collectively referred to as N-terminomics [2]. In animals, PPs affect various processes (Box 1). A recent discovery of an auxin-mediated TRANSMEMBRANE KINASE 1 (TMK1) cleavage [3], together with earlier work on the 'cleave-and-shuttle' function of ETHYLENE INSENSITIVE 2 (EIN2) [4–6], consolidates the view that PPs exert important functions in plants as well.

A TMK1 PP Fine-Tunes Auxin Signaling

While the developing seedling is pushing upwards through the soil seeking light, the apical hook protects the shoot apical meristem. Apical hook morphogenesis is controlled by the main auxin, indole-3-acetic acid (IAA), and can be divided into three sequential stages. (i) Formation stage. The hook midline is parallel to the apicobasal axis of the seedling while the hook curvature is increased. (ii) Maintenance stage. The hook midline maintains its parallel position with the hypocotyl axis as well as its curvature. (iii) Opening stage. The parallel position of the hook midline turns and the hook bends. An auxin gradient across the hook curvature is essential for proper apical hook development. The inhibition of cell elongation correlates with the auxin accumulation at the concave hook's side. The differential cell elongation between both sides of the hypocotyl's upper part promotes hook bending (Figure 1B).

In the canonical pathway, auxin promotes the recognition of the transcriptional repressors Aux/IAAs 'degron' domain II (DII) by the E3 ubiquitin ligase proteolytic complex SKP-Cullin-F-box (SCF)$^{TIR1/AFB}$, leading to their degradation (Figure 1B). While the SCF$^{TIR1/AFB}$ complex localizes in the nucleus, a newly discovered pathway in the apical hook commences at the cell membrane and involves TMK1. TMK proteins contain an intracellular kinase domain, a single transmembrane pass, and an extracellular domain with two leucine-rich repeats (LRRs) regions separated by a non-LRR region. *tmk1* loss-of-function mutants displayed disrupted apical hook development [3]. TMK1 shows a transient cytosolic and nuclear distribution at the concave side of the apical hook, specifically during the maintenance stage. The molecular mechanism of redistribution involves proteolytic cleavage of TMK1 coinciding with local auxin maxima, releasing a PP containing the intracellular kinase domain, dubbed as TMK1-C (Figure 1B).

In strict contrast to the SCF$^{TIR1/AFB}$ pathway, TMK1-C stabilizes IAA repressors [3]. The stabilization step involves the translocation of TMK1-C from the plasma membrane to the cytosol and nucleus where it interacts with and phosphorylates IAA32 and IAA34. IAA32/34 lack the DII and thus are not degraded by SCF$^{TIR1/AFB}$. A TMK1 kinase-dead mutant (K616E mutation) could not rescue the apical hook phenotype or stability of IAA32/34 in the *tmk1* mutant, highlighting the potential importance of phosphorylation in the TMK1-C pathway. In the *tmk1-2* loss-of-function mutant, IAA32/34 proteins decreased, and auxin could not induce accumulation of these proteins. However, it is unclear what determines the stability of IAA32/34, as they lack the DII degron. Perhaps these proteins contain cryptic degrons or are degraded by nonproteasomal proteolytic branches.

The cleavage of TMK1 highlights limited proteolysis as a key step in auxin signaling; though the current model for TMK1 function in apical hook development requires some critical refinements. For example, the localization of TMK1 in the nuclei seems accurate, however, TMK1 is not consistently at the plasma membrane and at the same time, the overall signal intensity of TMK1 in the concave side is higher. Although there could certainly be a number of technical reasons that account for the reduced signal in the convex side, such as reduced antibody penetrance in this region rich in cuticular waxes, uncleaved TMK1 might represent only a small protein fraction or even TMK1 translation/stability might be induced by auxin or another signal. Furthermore, TMK1 cleavage might be induced by high levels of auxin, raising the question of whether TMK1 is cleaved in other tissues that are high in auxin levels. TMK1 cleavage may also depend on specific

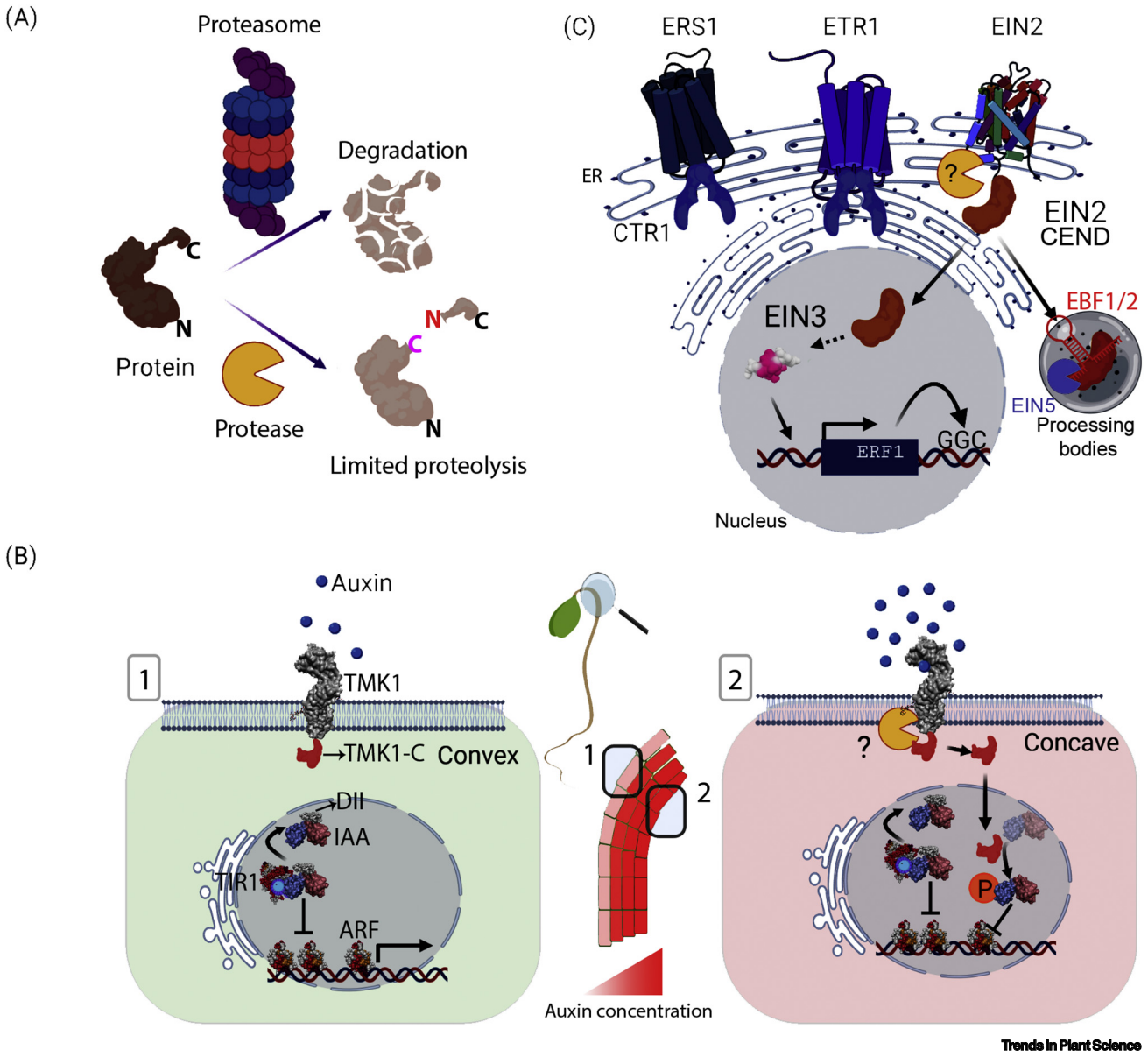

Figure 1. Proteolytic Proteoform-Mediated Phytohormone Signaling. (A) Proteasomal degradation degrades proteins down to amino acids. Limited proteolysis leads to protein partition into fragments – the proteolytic proteoforms (PPs) – some of which may exert new functions. We define PPs as N- and C-terminal. The neo-N terminus at the C-terminal proteoform is red colored, while the neo-C terminus is pink colored. (B) Upon cleavage by an unknown protease, TRANSMEMBRANE KINASE 1 (TMK1) releases a C-terminal fragment (TMK1-C) that enters the nucleus and stabilizes two non-canonical transcriptional repressors of the auxin or indole-3-acetic acid (Aux/IAA) family (IAA32 and IAA34), thereby regulating ARF (auxin response factor) transcription factors. Structure codes from Protein Data Bank (PDB) used in the illustration are 4HQ1 for TMK1, 5NQV for IAA, and 4NJ6 for ARF. (C) The plant's response to ethylene is initiated by the binding of this hormone to its cognate receptors – in *Arabidopsis*, a small family of five proteins (ETR1, ETR2, ERS1, ERS2, and EIN4) with sequence similarity to the bacterial two-component histidine kinases in the endoplasmic reticulum (ER). These receptors primarily modulate the activity of the kinase CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1). Inactivation of CTR1 results in the reduction of the phosphorylation of EIN2–CEND (ETHYLENE INSENSITIVE 2–C-terminal end of EIN2). Next, an unknown protease cleaves a fraction of the EIN2 protein pool to release the C-terminal PP that represents the CEND, which migrates to the nucleus. Downstream of EIN2, there is a rapid inhibition of growth that takes place within minutes of exposure to the hormone. There are also many other, and possibly slower, changes induced by this hormone, including transcript level alterations that seem independent of the key transcriptional regulators EIN3 and EIN3/EIN3-LIKE 1 (EIL1). The fast growth inhibition response remains a mystery but may involve the CEND translocation to processing bodies (P-bodies). In P-bodies, CEND halts translation of EIN3-BINDING F-BOX (EBF)1/2 and potentially translation of more proteins. The CEND upregulates expression of ETHYLENE RESPONSIVE FACTOR 1 (ERF1) by activating EIN3. ERF1 can interact with the GCC box in the promoter of target genes and activate downstream ethylene responses. Again, in this P-body-related pathway, cleavage of EIN2 seems important for the release of CEND. The structures of ERS1, ETR1, and EIN2 are hypothetical. Structure code from PDB used in the illustration for EIN3 is 1WIJ. Abbreviations: DII, domain II; TIR1, TRANSPORT INHIBITOR RESPONSE 1. This figure was created using BioRender (https://biorender.com/).

Box 1. Examples of PPs with Altered Functions, Stability, and Localization Following Proteolytic Cleavage

(i) A C-terminal PP of MET tyrosine kinase, a transmembrane receptor of HGF/SF (a hepatocyte growth factor/scatter factor), is produced by proteolysis in the absence of HGF/SF [12]. When binding HGF/SF, the receptor activates signaling pathways that mediate cell survival, migration, and differentiation. In the absence of HGF/SF, the receptor acquires an 'opposite' activity through the formation of a death-inducing C-terminal cytosolic PP(s) that functions in the cytosol and/or the nucleus.

(ii) ETK/BMX tyrosine-protein kinase is engaged in phosphatidylinositol 3-kinase (PI3-kinase) pathway and plays a pivotal role in interleukin 6 (IL-6) signaling. The C-terminal PP of the ETK/BMX tyrosine-protein kinase that is produced by proteases induces cell death [13].

(iii) The gasdermin PP produced by proteolysis is involved in the execution of cell death. Upon cleavage, gasdermin releases an N-terminal PP with plasma membrane pore-forming abilities [14]. The cleavage removes the inhibitory C-terminal PP of gasdermin, allowing the N-terminal PP to assemble into pores. The segregation of N- and C-terminal PPs, especially in the presence of membranes, suggests that at least one of these newly formed PPs undergoes conformational rearrangements that favor dissociation, as opposed to just a simple covalent decoupling upon proteolytic cleavage.

proteases induced by auxin. Furthermore, the fate and function (if any) of the N-terminal PP is elusive.

An EIN2 PP Regulates Ethylene Signaling

Ethylene modulates plant growth and development throughout the plant life cycle. The pathway starts by ethylene binding to endoplasmic reticulum (ER)-localized receptors that form complexes with the kinase CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) and the integral membrane protein EIN2 which relays the ethylene signal to downstream effectors (Figure 1C). EIN2 activates the transcription factors EIN3/EIN3-LIKE 1 (EIL1), which in turn activate ETHYLENE RESPONSIVE FACTOR 1 (ERF1) transcription.

How does the ER-localized integral membrane EIN2 protein activate EIN3/EIL1 in the nucleus? The answer seems to lay in the C-terminal end of EIN2 (CEND). The CEND exerts three functions: (i) EIN2 turnover regulation, (ii) shuttling in the nucleus, and (iii) retention in cytoplasmic granules, so-called processing bodies (P-bodies). In the absence of ethylene, two F-box proteins, EIN2-TARGETING PROTEIN (ETP)1 and ETP2, interact with CEND, mediating EIN2 degradation by the proteasome, and thereby negatively regulating ethylene signal transduction [7]. In the presence of ethylene, EIN2 undergoes limited proteolysis and CEND, which contains a nuclear localization signal (NLS), translocates to the nucleus and stabilizes the EIN3/EIL1 through a yet unknown mechanism [4–6]. CEND might outcompete EIN3/EIL1 recognition by two F-box proteins, EIN3-BINDING F-BOX (EBF)1 and EBF2, which promote EIN3/EIL1 degradation. In the absence of ethylene, CTR1-mediated phosphorylation at the CEND represses EIN2 activity. Mutations that mimic EIN2 dephosphorylation, or inactivate CTR1, show constitutive cleavage and nuclear localization of CEND and EIN3-dependent activation of ethylene responses.

Furthermore, upon ethylene treatment, the CEND can also localize in P-bodies, through interaction with the EBF1 3′-UTR (untranslated region). This interaction quickly represses EBF1/2 translation [8,9], leading to a rapid EBF1/2 protein pool depletion by the proteasome. Meanwhile, a CEND pool translocates into the nucleus to stabilize EIN3/EIL1. This dynamic model should be more elegantly detailed in the future to demonstrate what determines the balance between the intact/cleaved CEND and the full composition of ethylene-induced P-bodies.

Outlook

The breadth of biological processes steered by hormones begs the question of how this regulatory plasticity is brought about by using similar signaling components. PPs, such as CEND and TMK1-C, may provide the required regulatory plasticity. An important layer of regulation, the smoking gun for CEND and TMK1-C – the responsible protease(s) – is missing. PPs can arise from very different mechanisms, including alternative splicing and alternative transcription initiation [10]. Ideally, in the case of PPs, the protease is identified, the cleavage site determined, and mutation of either protease or cleavage site leads to a defective signaling pathway or failure to revert a phenotype, as exemplified by developments in the field of peptide signaling [11]. The identification of proteases will allow us to genetically or chemically control the pathways by designing specialized drugs when identified proteases fall into functionally redundant families. Still, CEND and TMK1-C PPs do not represent altered functions compared to their progenitors but rather an expansion of functionalities and localizations. In plants, we are yet to identify PPs with altered functions compared to their progenitors. Nevertheless, we believe that the PPs CEND and TMK1-C represent just the tip of the iceberg. Further research aimed at identifying similar mechanisms in other physiological or pathological contexts will aid in revealing new modes of protein function regulation as well as providing evidence for novel protein activities, moonlighting functions, and additional localizations. Uncovering this layer of information systematically, for example by terminomics, will be a cornerstone in our attempts to reveal so far unrecognized key steps in plant signaling.

Acknowledgments

This work was supported by grants to P.N.M. from the VR Research Council (grant number 21679000), FORMAS Research Council (grant number 22924-000), the Carl Tryggers Foundation (grant numbers 15:336 and 17:317), and start-up grants from IMBB-FORTH; and to S.S. by the Research Foundation – Flanders (grant number FWO14/PDO/166). We apologize for failing to cite relevant research due to space constraints.

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https://doi.org/10.1016/j.tplants.2020.01.002

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