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Regulation of Catalytic Activities of HECT Ubiquitin Ligases

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Abstract

Studies in yeast and mammalian cells over the past decade have shown that HECT domain ubiquitin ligases (HECT E3 enzymes) are involved in diverse physiological pathways. Many substrates of specific HECT E3s have been identified, as well as many adaptor proteins that aid in defining substrate specificity or intracellular localization of HECT E3s. Here we review some recently discovered mechanisms for regulation of the catalytic activities of HECT E3s, including regulation at the level of E2 recruitment, phosphorylation-dependent relief of inhibitory intramolecular interactions, and regulation by association with a deubiquitinating enzyme.

Introduction

Conjugation of ubiquitin to target proteins involves three groups of enzymes, the E1, E2, and E3 enzymes, which function cooperatively in a cascade of ubiquitin transfer reactions [1]. The E3 enzymes, or ubiquitin ligases, interact with both an upstream E2 enzyme and specific target proteins, facilitating protein ubiquitination. RING type E3s, which include both monomeric proteins (*e.g.*, Mdm2, Cbl) and multimeric protein complexes (*e.g.*, cullin-based E3s, the APC), function primarily as scaffolds, orienting the E2~ubiquitin thioester complex and target protein for ubiquitin transfer. HECT domain E3s are unique among the several classes of E3s in that ubiquitin is transferred from the E2 to an active-site cysteine within the HECT domain, forming an E3~ubiquitin thioester complex [2]. Ubiquitin is then transferred to target proteins that are bound to the substrate recognition determinants of the E3.

The HECT domain, itself, is an approximately 350 amino acid domain that is always found at the C-terminus of the ligase, and structural information is available for three HECT domains and one HECT domain – E2 complex [3-5]. Briefly, the HECT domain consists of a larger N lobe that contains the E2 binding site, and a smaller C lobe that contains the active-site cysteine. The lobes are connected by a short flexible linker, and conformational flexibility about this linker appears to be critical for juxtaposing the active site cysteines of the E2 and E3 in order to facilitate the transthioesterification reaction. Most aspects of isopeptide bond catalysis are uncharacterized, particularly with respect to how polyubiquitin chains are formed, although one key requirement for ubiquitin transfer from the active-site cysteine to the target protein is a phenylalanine residue located, in most HECT E3s, four amino acids from the end of the protein [6]. This may function to properly orient the ubiquitin molecule that is tethered to the active-site cysteine.

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The smallest HECT E3s are approximately 90 kD and the largest are over 500 kD. The region upstream of the HECT domain contains determinants of substrate specificity, intracellular localization, and in some cases, as discussed below, regulation. E6AP was the first HECT E3 identified, and this protein mediates the ubiquitination of p53 in cells that express the human papillomavirus (HPV) E6 oncoprotein. This appears to be an unregulated reaction in that cells that express E6 constitutively ubiquitinate p53, causing it to be constantly degraded. There are approximately 50 HECT E3s in humans and five in *S. cerevisiae*. Only a few of these have been characterized in detail, although the best characterized subgroup of HECT E3s, with respect to both substrate identification and regulation, are the C2-WW-HECT E3s [7]. These proteins contain an N-terminal C2 phospholipid binding domain and multiple (from two to four) WW domains in the central portion of the protein that mediate enzyme-substrate interactions. Yeast Rsp5 belong to this group, as well as seven human proteins, including Nedd4, Smurf1, Smurf2, and Itch. The C2-WW-HECT E3s have been more amenable to analysis than other HECT E3s, in part because of genetic tools in yeast and the fact that it has been relatively simple to isolate proteins (substrates) that bind specifically to WW domains. The regulation of substrate recognition by C2-WW-HECT E3s, as well as the physiologic functions of these enzymes, has been reviewed recently [7]. We will focus here on mechanisms of regulation of the catalytic activity of HECT E3s.

Regulation at the level of E2 recruitment

The TGF- β signaling pathway has multiple effects on growth and differentiation processes. The pathway is regulated at many different levels to achieve balanced and temporal responses, including down-regulation of the TGF- β receptor, itself [8]. Smurf1 and Smurf2 are C2-WW-HECT E3s that mediate ubiquitination and degradation of the TGF- β receptor, and these proteins are regulated at several points by Smad7, an inhibitory Smad (I-Smad). Smad7 consists of an N-terminal domain (NTD), a central region with a PY motif that binds to the WW domains of Smurf1 and Smurf2, and an MH2 domain, which engages the TGF- β receptor. Smad7 therefore recruits Smurf1 and Smurf2 to the receptor, where they then mediate receptor downregulation. In addition to mediating enzyme-substrate interactions, Smad7 also activates Smurf2 by enhancing its ability to interact with the activating E2 enzyme, UbcH7 [4]. This study found that while the PY motif of Smad7 binds to the WW domains of Smurf2, the NTD of Smad7 binds to both the HECT domain and UbcH7 and facilitates the interaction between the E2 and E3 enzymes (Figure 1). At the same time, the crystal structure of Smurf2 HECT domain revealed that, while being overall similar to other HECT domain structures (E6AP and WWP1), the predicted E2 binding surface of Smurf2 lacked certain key hydrophobic residues that were critical for E2 interaction in the E6AP-UbcH7 structure (corresponding to E6AP residues I656 and F691) [4]. The corresponding residues in Smurf2 are hydrophilic amino acids (His547 and Tyr581). This suggested that Smurf2 might have an inherently low affinity for its E2 enzyme, and that Smad7 might activate Smurf2 by aiding in the recruitment of UbcH7. Indeed, when the key residues in the E2 binding pocket of the Smurf2 were replaced with the hydrophobic residues found in E6AP, Smurf2 became constitutively active.

Interestingly, a recent study determined the affinity of the E2-E3 interaction for E6AP and UbcH7 and found it to be quite low, approximately 6 μ M [9]. In addition, alteration of residues at the E2-E3 interface that increased the binding affinity decreased the ability of the E2 to be charged with ubiquitin by E1. This is consistent with structural studies that have indicated that the surface of the E2 that interacts with the E3 is likely to significantly overlap with the surface that interacts with the E1 enzyme [3,10,11]. Together, these results suggest that polyubiquitination catalyzed by HECT E3s (as well as RING E3s) requires multiple rounds of E2-E3 binding and release, and that relatively low E2-E3 affinities are therefore likely to facilitate the reaction. Therefore, in the Smurf2 case, one would presume that Smad7 is increasing the affinity for UbcH7 to be in the range seen with wild-type E6AP (as opposed to

promoting very tight binding), and that in the absence of Smad7 the affinity for UbcH7 is indeed very low. It will be important to confirm this by determining the affinity of Smurf2 for UbcH7, with and without Smad7. It has so far not been reported whether Smurf1 is also regulated at the level of E2 recruitment.

Phosphorylation-dependent relief of inhibitor interactions

Itch is a HECT E3 involved in activation of T helper 2 (Th2) cells [12] through ubiquitination of JunB [13]. Phosphorylation has been proposed to activate the catalytic activity of Itch by relieving repression due to intramolecular interactions between the WW domain region and the HECT domain [14]. Itch is activated by phosphorylation by the JNK1 serine/threonine kinase at three residues (S199, T222, S232) that lie within a proline-rich region (PRR) upstream of the WW domains. JNK1 is recruited to Itch by a D domain-like sequence within the N-lobe of the HECT domain. D domains consist of a group of basic residues followed by hydrophobic residues, and these have been shown to recruit MAP kinases [15]. Phosphorylation was proposed to trigger a conformational change that disrupts inhibitory intra-molecular interactions between the central WW domain region (containing the PRR and four WW domains) and the HECT domain, switching the E3 to a mode capable of ubiquitinating target proteins (Figure 1C). It was not determined whether the PRR, itself, or one of the four WW domains mediates the interaction with the HECT domain, nor was the recognition site on the HECT domain mapped.

While these intramolecular interactions inhibited Itch in an *in vitro* autoubiquitination assay, it is not clear which step in the enzymatic cycle of E3 is actually blocked. That is, the interactions might block the enzyme by preventing E2 binding, by inhibiting the transthiolation reaction between the E2 and E3, or inhibiting isopeptide bond catalysis by the E3. The conservation of the putative D domain sequence of Itch within other C2-WW-HECT E3s raises the possibility that this mode of regulation might be common to other HECT E3s. While phosphorylation events have been proposed to regulate substrate targeting by other HECT E3s [7], this study provides a mechanism for linking a kinase signaling pathway to the enhancement of the catalytic activity of a HECT E3.

Modulation by an associated DUB

Deubiquitinating enzymes (DUBs) are ubiquitin-specific proteases that can reverse target protein ubiquitination and play an important role in several aspects of the ubiquitin-proteasome pathway [16]. Several recent studies have found that some ubiquitin ligases are physically associated with DUBs and directly reverse the reactions catalyzed by these ligases. There are several examples of DUBs reversing autoubiquitination of the ligase, itself. This has been seen with human Nrdp1 ligase and the USP8 DUB [17], the herpes simplex virus ICP0 ligase and USP7 [18], the TRAF2/TRAF6 ligases and the CYLD DUB [19,20]. The ligases in these cases are all RING domain E3s.

The only HECT E3 that has so far been shown to associate with a DUB is Rsp5, a yeast C2-WW-HECT E3 [21]. Rsp5, the only essential HECT E3 in *S. cerevisiae*, associates with Ubp2, one of the 16 ubiquitin-specific proteases (UBPs) in yeast. This interaction is mediated by a third protein, Rup1, whose only distinguishing feature is a UBA ubiquitin binding domain. Rup1 and Ubp2 do not influence the stability of Rsp5, and indeed there is no evidence that Rsp5 is regulated at the level of autoubiquitination. Rather, genetic and biochemical evidence indicate that Ubp2 reverses ubiquitination of Rsp5 substrates (Figure 1D). One line of genetic evidence is that the phenotypes of the *rsp5-1* hypomorphic mutation can be partially rescued by either *ubp2Δ* or *rup1Δ* mutations, suggesting that low Rsp5 activity can be compensated by loss of the antagonizing DUB activity. Ubp2 reverses Rsp5 substrate ubiquitination *in vitro*, as well, and these experiments revealed that both Rsp5 and Ubp2 have a distinct biochemical preference

for synthesizing and disassembling, respectively, K63-linked polyubiquitin chains. In addition to the role of Rup1 in mediating association of Rsp5 and Ubp2, it is tempting to speculate that the UBA domain of Rup1 is required for binding the polyubiquitin chains formed on substrates and presenting these to Ubp2.

While work to this point indicates that Rup1 and Ubp2 are important players in modulating Rsp5 activity, the basis for determining whether a substrate is ultimately polyubiquitinated or deubiquitinated is still unknown. It is also possible that Rup1/Ubp2 serve to generate monoubiquitinated target proteins by partial disassembly of polyubiquitin chains. This is an attractive hypothesis because Rsp5 is a very processive enzyme *in vitro*, yet it catalyzes monoubiquitination of some substrates *in vivo* [22,23]. Finally, Rup1 and Ubp2 appear to cooperate with, rather than antagonize, Rsp5 function in promoting sorting of membrane cargo proteins into the multivesicular body (MVB) pathway [24]. This might be consistent with the model that Ubp2 promotes monoubiquitination of certain targets, or alternatively, that a “futile cycle” of ubiquitination and deubiquitination is involved in the sorting process.

Other mechanisms

ARF is a tumor suppressor that has been shown to stabilize p53 by inhibiting both the Mdm2 RING ubiquitin ligase and the Mule/ARF-BP1 HECT ubiquitin ligase [25,26]. ARF inhibits Mdm2 by blocking its ability to interact with p53. While it is not entirely clear how ARF inhibits Mule/ARF-BP1, it appears to do so by inhibiting the catalytic activity rather than substrate binding activity. Consistent with this, the region of Mule/ARF-BP1 required for ARF binding includes the HECT domain, suggesting that it may inhibit either E2 binding or any of the downstream ubiquitin transfer events.

Finally, E4 activities have been proposed to be factors that extend shorter polyubiquitin chains formed by E3s, and these have been suggested to modulate Rsp5-catalyzed events *in vivo*. Bul1 and Bul2 are Rsp5-associated proteins that have been shown to promote polyubiquitination of the Gap1 amino acid permease at the golgi membrane [22]. Gap1 ubiquitination is regulated by nitrogen levels, with Gap1 being monoubiquitinated under low nitrogen conditions, in which case it is sorted to the plasma membrane. Under high nitrogen conditions Gap1 is polyubiquitinated, which directs it to the vacuole for degradation. Bul1 and Bul2 promote the polyubiquitination and trafficking of Gap1 to the vacuole, suggesting that they may be acting as E4 proteins. While conceptually attractive, there has so far not been an *in vitro* demonstration of E4 activity of Bul1 or Bul2, and as noted above, Rsp5 is already an extremely processive enzyme *in vitro* in the absence of any factors other than E1 and E2 enzymes.

Conclusions

The clearest examples of regulation of HECT E3 catalytic activities, as well as regulation of substrate recognition, have come from study of the C2-WW domain subclass of HECT E3s. In humans, these represent only seven of approximately 50 HECT E3s, so additionally insights and surprises are surely in store. Probing the control and regulation of these proteins is also likely to lead to insights into aspects of the general mechanism of these enzymes, particularly with respect to the transthiolation and ubiquitination/polyubiquitination steps of the reaction. An understanding of enzyme mechanism, in turn, is critical for intervening or enhancing HECT E3 function in disease-related pathways, including cervical cancer and Angelman syndrome (E6AP), TGF- β signaling pathways (Smurf1 and Smurf2), Liddle's syndrome (Nedd4), and p53 control (Mule/ARF-BP1).

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Figure 1.

Modes of regulating or modulating the catalytic activities of C2-WW-HECT E3s. A. Some C2-WW-HECT E3s, such as Rsp5, appear to be constitutively active and can be charged with ubiquitin in the presence of only ATP, E1 enzyme (not shown), and an appropriate E2 enzyme. The arrow indicates ubiquitin transfer (a transthiolation reaction) from the E2 active-site cysteine to the E3 active-site cysteine. B. Smad7 activates Smurf2 by recruitment of the E2 enzyme, Ubch7. The PY motif of Smad7 interacts with the WW domain region of Smurf2, while the NTD interacts with both the HECT domain and the E2. These interactions increase the affinity of Smurf2 for Ubch7. C. Itch is a C2-WW-HECT E3 that adopts a "closed" inactive conformation due to intramolecular interactions between the WW-PRR region and the HECT domain. In response to signaling events, JNK1 is recruited by a D domain within the HECT domain and then phosphorylates multiple sites within the PRR region. This relieves the inhibitory interactions, leading to activation of the E3. D. While Rsp5 appears to be constitutively active, its activity is modulated following ubiquitination by Rup1- and Ubp2-

dependent deubiquitination. Rup1 and Ubp2 (a ubiquitin-specific cysteine protease) are stably associated with Rsp5, but do not inhibit substrate association.