Role of E2-RING Interactions in Governing RNF4-Mediated Substrate Ubiquitination

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http://dx.doi.org/10.1016/j.jmb.2016.09.018
Edited by Sachdev Sidhu

Abstract

Members of the really interesting new gene (RING) E3 ubiquitin ligase family bind to both substrate and ubiquitin-charged E2 enzyme, promoting the transfer of ubiquitin from the E2 to substrate. Either a single ubiquitin or one of the several types of polyubiquitin chains can be conjugated to substrate proteins, with different types of ubiquitin modifications signaling the distinct outcomes. E2 enzymes play a central role in governing the nature of the ubiquitin modification, although the essential features of the E2 that differentiate mono- *versus* polyubiquitinating E2 enzymes remain unclear. RNF4 is a compact RING E3 ligase that directs the ubiquitination of polySUMO chains in concert with several different E2 enzymes. RNF4 monoubiquitinates polySUMO substrates in concert with RAD6B and polyubiquitinates substrates together with UBCH5B, a promiscuous E2 that can function with a broad range of E3 ligases. We find that the divergent ubiquitination activities of RAD6B and UBCH5B are governed by differences at the RING-binding surface of the E2. By mutating the RAD6B RING-binding surface to resemble that of UBCH5B, RAD6B can be transformed into a highly active UBCH5B-like E2 that polyubiquitinates SUMO chains in concert with RNF4. The switch in RAD6B activity correlates with increased affinity of the E2 for RNF4. These results point to an important role of the affinity between an E3 and its cognate E2 in governing the multiplicity of substrate ubiquitination.

Introduction

The covalent attachment of ubiquitin (Ub) to substrates plays a central role in regulating a broad range of biological processes in eukaryotes [1,2]. Substrates can be modified with a single Ub (monoubiquitination) or with varying types of polyubiquitin chains (polyubiquitination) that are distinguished by the particular lysine through which one Ub is joined to the next [3,4]. Ub can also be conjugated to protein N termini to form an N-terminal Ub fusion [5,6] or a linear polyubiquitin chain [7]. The functional consequences of ubiquitination are determined by the distinct nature and topology of the Ub modification [3,8–10]. Ub is conjugated to substrates via the concerted action of Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub ligase (E3) enzymes [11]. An E1 Ub-activating enzyme is charged with Ub and then transfers its Ub to the active site cysteine of an E2 Ub-conjugating enzyme to yield a charged E2 ~ Ub thioester. The E3 ligase binds to both substrate and the E2 ~ Ub thioester and catalyzes the transfer of Ub to the target substrate, resulting in an isopeptide bond between the C terminus of Ub and the epsilon-amino group of the substrate lysine [3,9] or, in select cases, a peptide bond with the N-terminal alpha-amino group of the substrate [5,6,12]. In the case of really interesting new gene (RING)/U-box E3 ligases, which comprise the largest class of E3s [13], the E3 binds to both E2 ~ Ub and substrate and catalyzes the attack of the substrate lysine on the E2 ~ Ub thioester to yield the ubiquitinated substrate [14]. RING domains bind to E2 enzymes in a conserved manner, with the RING domain contacting a surface that includes the E2 N-terminal helix 1 and loops 4 and 7 [15]. In addition, the RING domain also contacts the donor ubiquitin, stabilizing a conformation of the E2 ~ Ub conjugate [16–18] that promotes a nucleophilic attack by the substrate lysine on the thioester linkage, yielding an isopeptide bond between the lysine and the Ub C terminus [19]. Most RING E3

ligases can ubiquitinate substrates in conjunction with multiple E2 enzymes. Depending upon the identity of the E2, these modifications can take on a variety of forms. In many cases, the E2 itself appears to govern the nature of the Ub modification, in terms of both the multiplicity of the modifications and, in the case of polyubiquitin chains, the linkage type [3,9,14]. While much of the core enzymology and structures of the Ub conjugation machinery are widely conserved, it is clear that individual E2–E3 pairs have evolved an array of mechanisms to generate distinct Ub modifications.

One of the most fundamental distinctions between Ub signals is substrate monoubiquitination *versus* polyubiquitination. With the exception of the E2, UBE2W, which represents a special case because it only ubiquitinates the flexible N-termini of substrates [5,6,20], most examples of monoubiquitination studied to date involve a role for the E3 ligase in either suppressing intrinsic polyubiquitination activity of the E2 [21] or directing the E2 to a preferred target lysine in the substrate [22,23]. The E3 ligase APC/C is capable of driving both the direct multi-monoubiquitination of substrate in conjunction with UBE2C and the extension of polyubiquitin chains on substrate with UBE2S, by interacting with each E2 using two distinct binding architectures [24]. UBCH5C contains a non-covalent ubiquitin-binding site on its "backside", distal from the active site, which mediates polyubiquitination as assayed in E3 autoubiquitination assays [25]. However, the Bmi1–RING1 E3 complex targets UBCH5C to monoubiquitinate histone H2A–Lys119 [26–28] by juxtaposing the UBCH5C ~ Ub thioester and the target lysine [22,23]. The E2, RAD6B, can synthesize polyubiquitin chains on its own *in vitro* [21,29] but monoubiquitinates PCNA together with the E3, RAD18 [30,31]. RAD18 suppresses intrinsic RAD6B polyubiquitination activity through a special domain called the R6BD, which binds to the RAD6B backside and suppresses its polyubiquitination activity [21]. Yeast Rad6 also has intrinsic polyubiquitinating activity [32] that is mediated in part through backside interactions [33]. Rad6 monoubiquitinates histone H2B–Lys123 in conjunction with the E3 ligase, Bre1 [32]. Although Bre1, like human RAD18, contains a special domain that contacts the Rad6 backside, the Bre1 RING domain alone is sufficient to direct the monoubiquitination of nucleosomal H2B [34], potentially through substrate targeting as recently observed in the structure of Bmi1–RING1/UBCH5C bound to a nucleosome [23]. While backside interactions and direct targeting of the E2 to its substrate are clearly important in governing mono- *versus* polyubiquitination, there is evidence that the molecular interface between the E2 and the RING domain may also govern substrate ubiquitination [35]. Indeed, a screen for mutants of the U-box E3, UBE4B, which enhanced the auto-polyubiquitination of UBE4B in conjunction with the E2, UBCH5C, yielded a subclass of activating point mutations in UBE4B that increased its affinity for the E2 [36]. The activating mutations in UBE4B, while increasing the rate at which polyubiquitin accumulated, did not impact the multiplicity of autoubiquitination. Interestingly, whether the E2 enzyme, UBCM2 (UBE2E3), mono- or polyubiquitinates a RING E3 partner in autoubiquitination assays correlates with the ability of glutathione *S*-transferase (GST)-tagged UBCM2 to interact with the E3 in a pull-down assay: GST–UBCM2 pulls down AO7T, which is polyubiquitinated in conjunction with UBCM2, but not BD/BC, which is monoubiquitinated in conjunction with UBCM2 [37]. Since the E3 is also a substrate in these reactions, it is not possible to separate the potential contributions of substrate *versus* E3 affinity to the observed differences in ubiquitination.

While a number of studies have helped elucidate the principles of target amino group specificity, UBL selectivity, and Ub linkage specificity [3,4,9,10,14,19,20,23,24,33,34,37–41], the role of the canonical E2-RING interface in governing substrate ubiquitination is less well-understood. We therefore investigated how the differences in E2–E3 RING interactions affect substrate ubiquitination. In order to separate the role of interactions between the RING domain and the E2 *versus* the interactions between the charged E2 and the substrate, we utilized a system in which we could monitor the ubiquitination of a substrate other than the E3 ligase itself. RING finger protein 4 (RNF4) is a compact 190-residue E3 ligase that belongs to the SUMO-targeted Ub ligase subfamily and directs the ubiquitination of polySUMO chains [42,43]. RNF4 contains a C-terminal RING domain that binds the E2 and N-terminal SUMO-interacting motifs that bind to the polySUMO substrate [44–46]. RNF4 monoubiquitinates polySUMO substrates in concert with RAD6B and robustly polyubiquitinates the substrate together with UBCH5B, a promiscuous E2 that can function with a broad range of E3 ligases [46–49]. We find that the ubiquitinating activities of RAD6B and UBCH5B in concert with RNF4 are governed by interactions between the E2 and the RNF4 RING domain. By reengineering the RAD6B RING-binding surface to resemble that of UBCH5B, we transformed RAD6B into a UBCH5B-like E2 that polyubiquitinates polySUMO in the presence of RNF4. The switch from weak monoubiquitinating activity to robust polyubiquitinating activity correlates with increased affinity of the E2 for RNF4. Our results shed new light on the characteristics of E2-RING interactions that govern the activity and nature of substrate ubiquitination.

Results

Mono- *versus* polyubiquitination of polySUMO by RNF4 is E2-dependent

To ask how differences in E2–E3 interactions affect RNF4 substrate ubiquitination, we monitored