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RING Finger Proteins: Mediators of Ubiquitin Ligase Activity

Minireview

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Until recently no specific function had been ascribed to the RING finger beyond a role in dimerization of several proteins. Over the last year and a half, however, reports from a number of laboratories studying diverse biological processes have led to the realization that RING finger proteins play critical roles in mediating the transfer of ubiquitin (Ub) both to heterologous substrates as well as to the RING finger proteins themselves. As RING fingers are found in hundreds of proteins, the number of candidate Ub protein ligases (E3s) has now increased dramatically.

Protein ubiquitination begins with the formation of a thiol-ester linkage between the C terminus of Ub and the active site cysteine (Cys) of the Ub activating enzyme (E1). Ub is then transferred to an Ub conjugating enzyme (Ubc or E2), again through a thiol-ester linkage. E3s, which are primarily responsible for providing specificity to Ub conjugation, interact with E2 and substrate, facilitating formation of isopeptide bonds between the C terminus of Ub and lysines (Lys) either on a target protein or on the last Ub of a protein-bound multi-Ub chain. Initial models for ubiquitination suggested that E3s facilitate the direct transfer of Ub from E2 to substrate. However, some E3s including E6-associated protein (E6-AP) and other HECT E3s (see below) form thiol-ester intermediates between the conserved HECT Cys and Ub as part of the process leading to the formation of multi-Ub chains on proteins. Multi-Ub chains are potent targeting signals for protein degradation in proteasomes (reviewed in Hershko and Ciechanover, 1998).

Eukaryotic genomes encode a single or at most a few E1s. Substantially more E2s exist, at least 11 in yeast and over 20 in mammals. The diversity and number of proteins that are regulated by ubiquitination predicts the existence of a large number of E3s. However, until recently relatively few E3s were known. The discovery of E6-AP as an E3 responsible for human papilloma virus E6-dependent ubiquitination of p53 led to the identification of proteins containing an ~350 amino acid region of *h*omology to the *E*6-AP *c*arboxyl *t*erminus (HECT domain), several of which have now been shown to be E3s. On the other hand, no common structural basis for known non-HECT E3s had been appreciated. However, it is now clear that all of these include a RING finger either on the same polypeptide that recognizes substrate or as a distinct polypeptide in the context of a multi-subunit E3 (Figure 1).

The first suggestion that RING fingers are associated with ubiquitination was made in 1998 by Bachmair who noted that a plant N-end rule E3 shared the RING finger motif with other proteins implicated in ubiquitination including the yeast N-end rule E3 (Ubr1p), Hrd1p/Der3p, Rad18p, and an essential anaphase promoting complex (APC) component, Apc11p. This was followed in 1999 by a series of exciting reports from the Conaway, Deshaies, Elledge, Harper, Pan, and Xiong laboratories establishing that a small noncanonical RING finger protein, Rbx1 (also referred to as ROC1 and HRT1), is an essential component of SCF (*S*kp1/*c*ullin-1/*F*-box protein) E3 complexes and that a complex containing Rbx1 and Cdc53/cullin-1 (CUL-1) is sufficient to mediate ubiquitination in vitro. Rbx1 was also determined to be an integral component of VHL-containing complexes, which have subsequently been shown to have E3 activity (reviewed in Deshaies, 1999; Tyers and Jorgensen, 2000). The activity of the yeast N-end rule E3, Ubr1, was also dependent on its RING finger (Xie and Varshavsky, 1999). c-Cbl was shown to promote the ubiquitination of activated receptor protein tyrosine kinases (RPTKs) in a RING finger–dependent manner (Waterman et al., 1999 and references therein) and subsequently determined to have the intrinsic capacity to ubiquitinate RPTKs in vitro in an SH2- and RING finger–dependent manner. Additionally, the bacterially expressed c-Cbl RING alone bound E2s and mediated ubiquitination (Joazeiro et al., 1999; Levkowitz et al., 1999; Yokouchi et al., 1999). Similarly, the E3 activity of Mdm2 toward p53 and itself has now been determined to be due to its atypical C-terminal RING (Fang et al., 2000; Honda and Yasuda, 2000). A general function for the RING finger in ubiquitination was suggested by the finding that multiple, otherwise unrelated, RING-H2 finger proteins all

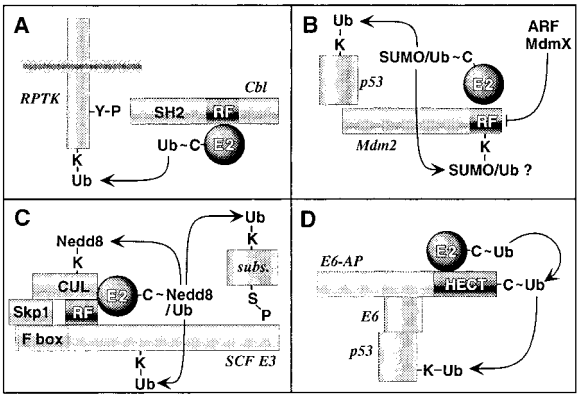

Figure 1. Variety of Action of E3s, Proteins that Recognize Specific Substrates and Mediate Ligation of the Latter to Ub- or Ub-like Proteins

(A) Ubiquitination of active RPTK by Cbl; (B) Ubiquitination of p53 by Mdm2 and modification of Mdm2 itself with SUMO-1 or Ub; (C) Multisubunit SCF-type E3: ubiquitination of substrate and of F box protein subunit; modification of cullin subunit with Nedd8; (D) Ubiquitination of p53 by the HECT-type E3 ligase E6-AP: dependence on human papilloma virus E6 protein and formation of intermediate Ub thioester with E3. Abbreviations: Y, tyrosine; K, lysine; S, serine; C, cysteine; P, phosphate group; ~, thiol-ester bond.

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Table 1. RING Finger Proteins with Demonstrated E3 Activity, E2 Binding or Involvement in Ubiquitination

Protein	Biological Role	Substrates	Comments
Hrt1/Roc1/Rbx1[a] in SCF E3	cell cycle, signaling	Sic1, IκBα, β-catenin, Cln1/2, others	component of multisubunit SCF E3 also containing Skp1, cullin, alternative F box proteins
Rbx1[a] in VCB-CUL2 E3	transcription, signaling	HIF1-α with VHL, Vav* with SOCS	component of multisubunit E3 also containing elongins B and C, CUL2, and VHL or SOCS proteins
Apc11[a]	cell cycle	Pds1, mitotic cyclins	component of multisubunit APC E3
Cbl family proteins[a]	signaling	RPTKs	negative regulator of RPTKs
Ubr1[a]	?	proteins with destabilizing N termini, Cup9, Gα	E3 ligase for the N-end rule pathway (E3α is mammalian homolog)
Mdm2[a]	apoptosis, proliferation	p53, itself	inhibited by p19ARF, MdmX
Sina[b]	transcription, signaling	Tramtrack*	fly photoreceptor development
Siah[a]	signaling	DCC*, Vav*	tumor suppressor
Prajal[a], AO7[a], TRC8[a], kf-1[a]	?	? (AO7 ubiquitinated in cells)	RING-dependent activity
NF-X1[a]	transcription	?	represses MHC-II expression
BRCA-1[a]	DNA repair, transcription	?	RING mutations in familial carcinoma
cIAPs[a], XIAP[a]	apoptosis inhibitors	themselves, caspases 3 and 7	degradation induced by apoptotic stimuli
Vmw110[b]	herpesvirus life cycle	CENP-C*, PML*, DNA-PK*	virus-encoded
Rad18[b], Rad5[b]	DNA repair	?	associate with E2s
PML[b]	?	itself SUMO-modified in vivo	binds to Ubc9 (E2 for SUMO)
Hrd1/Der3[b]	degradation from ER	HMG-CoA reductase*, others	polytopic ER membrane protein
COP1[b]	photomorphogenesis	HY5*	abundance of COP1 is regulated by light
Parkin[a], RING-IBR-RING family[b]	?	?	RING mutations associated with autosomal juvenile parkinsonism; others in the family shown to bind E2s

[a]E3 activity demonstrated; [b]activity not demonstrated; *putative substrates.

mediate RING- and E2-dependent ubiquitination of themselves in vitro. Two RING-HC proteins, the tumor suppressor, BRCA1, and a protein that mediates the proteasomal degradation of the deleted in colorectal cancer gene product, Siah-1, behaved similarly (Lorick et al., 1999). Thus, a large number of RING finger proteins are likely to mediate E2-dependent ubiquitination.

The RING Finger: Structure and Function

RING fingers have been defined by the consensus sequence CX2CX(9–39)CX(1–3)HX(2–3)C/HX2CX(4–48)CX2C with the Cys and His representing zinc binding residues. RING fingers are subcategorized into RING-HC and RING-H2 depending of whether a Cys or His occupies the fifth coordination site, respectively. Structures of RING-HC fingers show two interleaved zinc binding sites. This is in contrast to the tandem arrangement of zinc binding sites characteristic of zinc fingers (reviewed in Freemont, 2000).

There is substantial evidence for interactions between RING fingers and E2s. This is now supported by the crystallization of the c-Cbl SH2 and RING domains together with a tyrosine-phosphorylated peptide and an E2, UbcH7 (Zheng et al., 2000). UbcH7 binds to the RING domain of c-Cbl through contacts between a groove within the RING domain of c-Cbl and two loops in the E2 fold of UbcH7. These loops also provide the site of interaction between UbcH7 and E6-AP. The interactions between UbcH7 and the c-Cbl RING are largely due to van der Waals interactions involving hydrophobic residues in UbcH7 and the c-Cbl RING. Interacting residues in c-Cbl include a tryptophan (Trp) found in a number of active RING proteins, mutation of which abrogates E2 interaction and E3 activity (Joazeiro et al., 1999). Notably, at least one RING-E2 pair (Rad18p and Rad6p/Ubc2p) have corresponding residues that are polar and therefore likely interact by hydrogen bonding. Thus, the nature of the residues in the site of E2-RING interactions may play a significant role in determining RING-E2 pairs.

While HECT E3s form a thiol-ester with Ub, there is little evidence for such intermediates involving RING E3s. It is likely that RING fingers mediate ubiquitination by facilitating the direct transfer of Ub from E2s to target Lys. This is consistent with the original model of ubiquitination based on biochemical studies on what is now known to be a RING finger E3, E3α. How the RING facilitates ubiquitination remains to be established. There is evidence that the role of the RING finger is not merely to recruit E2s to the vicinity of proteins to be ubiquitinated. For example, Ubr1p binds its cognate E2, Rad6p, predominantly via regions outside the RING, yet mutations in this region only weakly reduce E3 activity, while a RING mutation did not affect binding to E2 or substrate, but abolished ubiquitination (Xie and Varshavsky, 1999). One possibility is that the RING serves as an allosteric activator of E2. However, UbcH7 shows no change in conformation when bound to the c-Cbl RING. Whether an allosteric modification of the complex of E2 with Ub occurs on binding to the RING awaits determination.

RING Finger Proteins Regulate Diverse Cellular Processes: Tip of the Iceberg?

RING finger-containing E3s play pivotal roles in diverse cellular processes and are implicated in contributing to disease (Table 1). A role for RING finger proteins in the cell cycle has been clearly established. Mitotic cyclins

are targeted for degradation by ubiquitination mediated by the APC (or cyclosome), which includes a small RING finger protein, Apc11p. The yeast APC consists of at least 12 essential subunits, including the cullin-family member, Apc2p. As with other RING fingers proteins, Apc11p mediates its own ubiquitination in vitro but interestingly also directly and specifically ubiquitinates the APC substrates cyclin B and securin (Leverson et al., 2000; Gmachl et al., 2000; reviewed in Deshaies, 1999; Tyers and Jorgensen, 2000). The cyclin-dependent kinase inhibitor Sic1p, G1 cyclins, other cell cycle regulators, and proteins involved in transcriptional regulation are targets for the Rbx1-containing SCF E3s (Figure 1). Together with the cullin Cdc53p/CUL-1, Rbx1 forms a core that binds to and activates the E2 Cdc34p. Skp1p binds to this core and mediates recruitment of various F-box-containing proteins, which confer substrate specificity to this family of E3s.

The RING finger protein Rbx1 is also a component of the VCB-CUL2 E3 complex, which includes the von Hippel Lindau (VHL) tumor suppressor protein, elongins C and B and CUL2. VHL mutations that prevent assembly of this E3 are associated with the malignancies of VHL disease, perhaps due to the stabilization of proteins such as hypoxia inducing factor (HIF) 1α. VHL can be replaced in this complex by SOCS box-containing proteins, which, analogous to F-box proteins, presumably confer substrate specificity (reviewed in Deshaies, 1999; Tyers and Jorgensen, 2000).

RING finger E3s can influence the balance between proliferation and apoptosis. Mdm2 binds p53 through its N terminus and ubiquitinates p53 and itself, targeting both to proteasomes. Notably, Mdm2 binds the p53-like molecule p73 in a similar fashion to p53, but does not target p73 for proteasomal degradation. Additionally, Mdm2 bearing a heterologous RING finger mediates its own ubiquitination in vitro and targets itself for degradation in vivo but does not ubiquitinate p53 in vitro or target it to proteasomes in cells (Fang et al., 2000 and references therein). Together these observations suggest RING specificity in the ubiquitination of heterologous substrates. The activity of RING finger proteins can also contribute to apoptosis. The observation that proteasome inhibitors block apoptosis led to the determination that the RING finger-containing Inhibitors of Apoptosis (IAPs) are degraded in a proteasome-dependent manner in response to apoptotic stimuli, and that they undergo RING-dependent auto-ubiquitination in vitro and in cells. The stability of RING finger-deficient forms correlates with enhanced survival. Thus, in response to apoptotic stimuli the E3 activity of IAPs leads to their auto-ubiquitination, degradation, and progression toward cell death (Yang et al., 2000).

Ubiquitination plays important roles in regulating levels of plasma membrane proteins both through proteasomal degradation and by facilitating endocytosis and lysosomal/vacuolar targeting of transmembrane proteins. The means by which Ub influences protein trafficking in the endocytic pathway remains obscure, but multi-Ub chains may not be required. RPTKs are ubiquitinated in response to activating stimuli and degraded primarily in lysosomes. A molecular basis for RPTK ubiquitination has now been provided with the determination that proteins of the Cbl family target activated RPTKs for ubiquitination in a RING-dependent manner. Cbl-dependent ubiquitination correlates with trafficking to lysosomes and the termination of signaling. These observations provide a molecular explanation for the failure to terminate signaling in cells expressing an oncogenic form of c-Cbl that lacks a complete RING finger (Joazeiro et al., 1999; Levkowitz et al., 1999; Yokouchi et al., 1999).

RING finger E3s also play key roles in the secretory pathway. Hrd1p is a yeast endoplasmic reticulum (ER) membrane RING finger protein that functions in quality control in the ER through its RING finger-dependent role in proteasomal degradation of abnormal ER proteins. Hrd1p also mediates the ubiquitination of the resident ER membrane protein HMGCoA reductase. Other RING finger E3s similarly facilitate disposal of membrane proteins from the ER. βTrCP, an F-box SCF component that targets β-catenin and IκBα for ubiquitination, also recognizes phosphorylated HIV-encoded Vpu in the ER membrane, thereby targeting Vpu-bound CD4 for degradation. This results in an increase in the amount of HIV Env available for virus production. Thus, Vpu uses an SCF E3 to target a cellular protein for degradation and contributes to the pathogenesis of AIDS (reviewed in Deshaies, 1999). In addition to proteins that use cellular E3s, viral genomes encode RING finger proteins that may be E3s themselves. Among these is the Herpes simplex virus-1 (HSV-1) protein, Vmw110/ICP0. Although E3 activity has not yet been demonstrated for Vmw110, this protein targets specific host proteins for proteasomal degradation in a RING finger dependent manner (Everett et al., 1999) (Table 1).

The tumor suppressor BRCA1 provides an example where loss of RING finger function is potentially associated with dysregulated growth and malignancy. BRCA1 mediates its own ubiquitination in vitro, and although in vivo activity is yet to be demonstrated, mutations in its N-terminal RING finger are associated with familial breast and ovarian cancer. The BRCA1 RING finger also mediates interaction with another RING finger protein, BARD1, and with a deubiquitinating enzyme. It remains to be determined whether E3 activity is critical for BRCA1's role in DNA repair. In yeast, post-replicative DNA repair involves two RING finger proteins, Rad18p and Rad5p, which physically associate through regions outside of their RINGs. Rad18p binds Rad6p while Rad5p binds Ubc13p, which in turn physically and functionally interacts with a non-canonical E2-like protein, Mms2p. Epistasis analyses implicate these proteins in a common DNA repair pathway. Thus, this E2-RING complex in yeast and their mammalian analogs/homologs may target specific proteins for ubiquitination at sites of DNA damage (Ulrich and Jentsch, 2000).

The finding that mutations in Parkin are associated with Juvenile familial Parkinson's disease establishes an association of RING finger protein function with a genetic neurodegenerative disorder. Parkin contains an N-terminal Ub-like domain with its C-terminal half including two atypical RING finger sequences. Parkin associates with UbcH7 in a RING finger-dependent manner. When expressed in, and purified from, a neuroblastoma cell line, but not fibroblasts, Parkin co-purifies with ubiquitinated species and mediates in vitro ubiquitination. The factors that allow for ubiquitination in specific lysates, and the nature of the ubiquitinated species now become of great interest (Shimura et al., 2000).

Regulation of RING Finger E3s

There are several examples where phosphorylation plays regulatory roles in E3 function. The F box component of SCF E3s selectively binds phosphorylated substrates, and phosphorylation of Mdm2 inhibits p53 binding. Phosphorylation of c-Cbl is neither required for it to

mediate ubiquitination, nor to bind the EGF-R. However, mutation of a tyrosine (Tyr) in the vicinity of the c-Cbl RING abolishes EGF-R ubiquitination. It has been suggested that phosphorylation at this site may promote a conformational change required for ubiquitination in the context of the full molecule (Levkowitz et al., 1999). However, this tyrosine is not readily available to phosphorylation according to the c-Cbl SH2-RING structure (Zheng et al., 2000).

For Mdm2, protein–protein interactions clearly affect E3 activity. Binding of ARF by Mdm2 exposes a nucleolar localization signal in the Mdm2 RING, leading to its compartmental segregation from p53. ARF also inhibits Mdm2's intrinsic E3 activity, perhaps through a conformational change in the RING finger, and as a result both p53 and Mdm2 are stabilized. Mdm2's activity is also inhibited by dimerization with its close relative MdmX through their RING fingers. A number of other RING finger proteins form hetero- or homodimers including BRCA1 and BARD1, c-Cbl, and Siah. The significance of these interactions to the regulation of ubiquitination is unknown.

Modification with the Ub-like molecules Nedd8 and SUMO-1 can protect proteins from ubiquitination and degradation, as well as regulate localization, activity, and protein–protein interactions. Modification of the cullin components of VCB-CUL2 and SCF E3s with Nedd8, by a Nedd8-specific E2 and Rbx1, has been reported and its significance demonstrated with the finding that ubiquitination of IκBα occurs preferentially through association with βTrCP SCF complexes containing Nedd8-modified CUL-1 (Read et al., 2000 and references therein). IκBα is also a substrate for modification by SUMO-1, which has been reported to prevent its ubiquitination, thereby stabilizing the protein. As with Nedd8 and Ub, a specific E1 and E2 for SUMO-1 have been identified (references in Buschmann et al., 2000).

Mdm2 mediates its own modification with SUMO-1, apparently on a Lys within the Mdm2 RING. This Lys has also been suggested to be a major site for Mdm2 ubiquitination. Importantly, SUMO-1 modification of Mdm2 inhibits its auto-ubiquitination while enhancing its capacity to ubiquitinate p53 (Buschmann et al., 2000). The RING finger proteins PML and Siah-1 also interact with the E2 for SUMO-1 (Ubc9). Modification of proteins with Ub-like molecules as a means of modulating E3 activity will likely be a recurrent regulatory theme.

Implications and Future Directions

Ubiquitination is a highly regulated and common cellular process with E3s as central players. RING finger proteins represent the largest class of E3s to date. Whether RING fingers are primarily modules that mediate ubiquitination and other protein modifications or if they have other discrete functions should soon become apparent. Mammalian genomes encode hundreds of RING finger proteins. Extrapolating from in vitro studies, we predict that many of these have the capacity to interact with E2s and to potentially mediate ubiquitination. It remains to be determined how many of these will be bona fide E3s for heterologous substrates, targets for ubiquitination, or both. The number of potential E3s is further enhanced by the combinatorial association of certain RING finger proteins with other proteins that provide docking sites for substrates.

The field of intracellular protein degradation now leaves the era where mediators of substrate-specific ubiquitination were scarce and enters a new and exciting phase where databases provide us with a large number of candidate E3s awaiting characterization. Each has the potential to help us understand how cells orchestrate the complexities of rapidly regulating protein levels and activity.

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