

Protein family review

Supplementary Text

The RBR protein family

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Running Title: RBR proteins

Keywords: RBR / IBR / E3 ligases / Parkin / PAUL / ARIADNE

Abbreviations: see list attached

Vienna
September 2006

Alternative splicing of RBR proteins

There is yet another level of complexity: Genes of RBR proteins are dispersed throughout the genome. Typically, they contain several exons. Splicing isoforms of RBR-domain containing proteins are widespread. Mladek *et al.* [1] have studied the Ariadne gene family in *Arabidopsis*. The 16 AtARI genes are distributed on all five chromosomes at 10 loci. Despite the conserved sequence, they possess distinct gene structures. The number of exons varies between one (AtARI3/CAD52885.1; here and below, the sequence entries of the resulting proteins are listed) and 15 (AtARI5/CAD52887.1, AtARI7/CAD52889.1, and AtARI8/CAD52890.1). The AtARI genes are differentially expressed during plant development and in an organ-specific manner. An alternative splicing event has been experimentally detected for AtARI15 (CAD52897.1). For the human TRIAD3 gene, at least 5 splicing variants have been reported [2]. The two isoforms XAP3 and XAP4 (human Q9BYM8) differ in their N-terminal region.

At least for some RBR proteins, alternative splicing appears a mechanism for the control of the subcellular localization and function of the parental RBR protein. For the rat transcription factor RBCK1 (AAC72243.1), another isoform of XAP3, the RBR domain plays a crucial role for its transcriptional activity [3]. RBCK2 (BAA33957.1) has been identified as an alternative splice variant of RBCK1, which lacks the C-terminal part of RBCK1 including the RBR region [3]. Yoshimoto *et al.* [4] could show that RBCK2 represses the transcriptional activity of RBCK1 by tethering it within the cytoplasm. A similar alternative splice variant lacking the RBR region has been reported recently for Parkin [5].

Function and Localization

Several human RBR proteins have a function in neurodegenerative and infectious diseases although the molecular mechanisms are not fully understood. Parkin protects dopaminergic neurons from the consequences of mitochondrial damage [6,7] and from harmful levels of aggregation-prone proteins by ubiquitin-mediated proteasomal degradation and/or subcellular tethering [8,9]. In addition, population specific variants of the regulatory region of the Parkin gene act as risk factors for the susceptibility to infections with intracellular pathogens such as *Mycobacterium leprae* and *Salmonella typhi* and *Salmonella paratyphi* [10-12].

The first evidence that RBR proteins exhibit E3 activity was published for the human HHARI in 1999 (Suppl. Tables 3, 4 and Figure 5) [13]. E3 activity associated with the IBR and the C-RING was also demonstrated for Parkin [14-16], which catalyzes multiple mono-ubiquitination [17]. As typical E3s, RBR proteins interact with E2s. For most RBR-proteins analyzed so far, the N-RING is essential and functions as recruiting region for specific E2s and substrates (Suppl. Tables 3, 4 and Figure 5). One exception is Parkin where the IBR and the C-RING are responsible for the interaction with E2s, the proteasomal subunit alpha-4, microtubules, SIM2, synphilin-1 and LRRK2. The latter has the tendency to aggregate and enhance the E3 activity of Parkin but is not directly ubiquitinated [18]. Other exceptions are Dorfin and the human Parc protein where interactions have been assigned to either the C- or N-terminal non-RBR part, respectively (Suppl. Tables 3, 4 and Figure 5).

Thirteen Parkin substrates and 31 interactors have been described so far (Suppl. Tables 3, 4 and Figure 5). The ubiquitination of synphilin-1 is promoted by Parkin. This modification is impaired in all familial-linked mutations of Parkin [19]. Both p38/JTV-1 and FBP-1 localize to the Parkin interactors and chaperones Hsp70 and Hdj-2 and accumulate in Parkin knock out mice and in patients with Parkinson's and Lewy Body disease [20,21]. Dorfin ubiquitinates superoxide dismutase-1 (SOD1) [22] and the Parkin target synphilin-1 [23]. Dorfin reduces the accumulation of mutant SOD1 in mitochondria by enhancing its degradation in the cytosol [24]. The interaction of Parkin through its IBR and N-RING with two regulators of synaptic vesicle dynamics, the septin GTPases, CDCrel-1 and Septin5_v2, promotes their proteasome dependent degradation [14,25,26].

That Parkin and Dorfin are involved in the ERAD pathway is not only supported by their interaction with ER associated E2s, UBC6 and UBC7 [27]. Parkin also promotes the degradation of un- or mis-folded forms of transmembrane proteins such as Pael-R [28], Synaptotagmin XI [29] and the dopamine transporter (DAT) [30] preventing their accumulation in the ER. Furthermore, Dorfin interacts with the intracellular C-terminus of CaR and with the AAA-ATPase VCP, a proposed component of ERAD [31].

In addition to its single-molecule E3 ligase activity, Parkin was identified as part of the SCF-like E3 complex that includes the F-box/WD repeat protein hSel-10 and Cullin-1. HSel-10 also

interacts with cyclin E and Parkin deficiency elevates cyclin E levels causing apoptosis of neuronal cells [28]. Furthermore, Parkin weakly interacts with Rpn10, the regulatory subunit of the 26S proteasome [32]. The *C. elegans* Parkin homolog PDR-1 is an interactor of the proteasomal subunit PRT-2 [33].

The interaction of HHARI with the proteins 4EHP and SIM2 (which is also a substrate of Parkin) sheds light on the HHARI function [34,35]. Apparently, ubiquitination of 4EHP alters its binding efficiency to the cap of mRNAs, thereby regulating the translational machinery. It is not excluded that 4EHP ubiquitination may be a signal for compartmentalization of specific mRNA populations. The fly and mouse homologues of SIM2 are involved in brain development. SIM2 is poly-ubiquitinated at multiple lysines within the PAS1-PAS2 region (residues 141- 289) but the impact of this modification on transcriptional activity is unknown.

The Ariadne member Parc acts as negative regulator of and interacts with the tumor suppressor p53 via its N-terminus [36]. As a sensor of DNA damage and other stresses, p53 must enter the nucleus to deter cell cycle progression and to induce apoptosis. Although Parc was unable to ubiquitinate p53 *in vivo* and *in vitro*, its overexpression sequestered p53 into the cytoplasm whereas depletion led to its nuclear relocalization. [36,37]. In the cytoplasm, Parc-bound p53 might be ubiquitinated by additional factors, such as CHIP, a co-chaperone with E4 function [38,39]. The N-terminal region of cullins such as Parc dictates binding to bridging and to substrate specificity proteins. p53 binding to Parc may block these interactions, inactivating a Parc-based, SCF-like E3 ubiquitin ligase. Under stress-free conditions, an active Parc-based complex could form in isolation from nuclear p53. Upon exposure to genotoxic stresses, the stabilization of p53 might allow the cytoplasmic concentration of p53 to rise, acting as an inhibitor of the cytoplasmic Parc complexes [40].

RBR proteins are found in various cellular compartments depending on the characteristics of the additional domains (Figure 4) and the function of their interaction partners. TRIAD1 and HHARI from the Ariadne subfamily are detected predominantly in the nucleus [35,41]. Parkin and PAUL are cytoplasmic [42]. The ARA54-GFP construct is constitutively localized throughout the cytoplasm and the nucleus [43].

RBR-proteins can be recruited by their interaction partners to a particular compartment. The example of the nuclear translocation of RBCK1s from the cytoplasm depending on interaction with its RBR-domain deficient splicing variant RBCK2 was discussed in detail above [4]. RBCK1's transcriptional activity is mediated through the N-RING and IBR domain [44] and can be modulated by the antagonistic action of two other RBCK1 interacting proteins, the positive regulator CBP and its negative regulator PML [45]. For the human XAP3 member, HOIL-1, interaction with hepatitis B virus X protein enhances the ability to activate X-responsive promoters [46]. HOIL-1 ubiquitinates SOCS6 and the oxidized form of the modulator of iron metabolism IRP2 [47,48]. HOIL-1 expression stabilizes SOCS6 by delaying proteasome-mediated protein degradation either by preventing the binding of another protein responsible for SOCS6 degradation or by altering the association to the proteasomal machinery [48].

PAUL redistributes depending on co-expression with its substrate MuSK. The cytoplasmic domain of MuSK is important for the co-localization to MuSK-positive membrane-associated patches [42]. A membrane association of Dorfin, IBRDC1 and RNF144 cannot be excluded in context with their transmembrane helix-like hydrophobic segments (see above). RNF144 was initially identified as transcriptional target p53. Since the abundance of the inhibitor of cyclin-dependent kinase, p21^{WAF1} correlates with p53RFP expression, it is suggested that p53 induces p53RFP which targets p21^{WAF1} for degradation driving cells into caspase-independent apoptosis [38,49]. Thus, p53RFP might switch cells from p53-mediated growth arrest to apoptosis [38].

For the two human splicing variants of TRIAD3, ZIN and Triad3A, distinct functions have been assigned. ZIN co-localizes with its substrate RIP in the cytoplasm and regulates apoptosis by repressing RIP induced NF- κ B activation [50]. It translocates to the nucleus by co-transfection with the Vif protein of HIV1. Vif is important for viral particle assembly and the stability of the reverse transcription complex [51,52]. Thus, ZIN is an attractive candidate to interfere with HIV replication. The "full length" variant, Triad3A, has an 377 residue extended N-terminus and promotes ubiquitination of the Toll-like receptors, TLR4 and TLR9 [2]. This led to the hypothesis that Triad3A controls the intensity and duration of pro-inflammatory responses mediated by Toll signaling.

The nuclear-cytoplasmic shuttling is a frequent phenomenon for RBR proteins and is further supported by the protein-interaction map of *Drosophila* [53]. In this survey, 30, 2, 8, 10, 9 and a single putative interaction partners were identified for ARI-2, ARI-1a, ARI-1b, RNF144, Parkin and PAUL, respectively (Suppl. Table 4). These include cytoplasmic proteins as the ribosomal protein S3, the TNF-receptor associated factor TRAF3, the E3 ligases deltex and Hakai, that promote endocytosis of Notch's and cadherin, respectively but also β -tubulin. Interestingly, Parkin is anchored to and able to regulate the turnover rate of microtubules and tubulin α - β -heterodimers by enhancing ubiquitination [29,54,55]. The large protein-protein interaction survey includes also nuclear proteins such as the fly homolog of the homeobox HOX11 protein clawless, the HLH4C transcription factor, the DNA-binding and repressor of Dpp signaling, brinker [56,57] or the evolutionary conserved protein dup that is essential for DNA replication and co-localizes with the origin recognition complex in the nucleus [58]. Furthermore, for the most promiscuous ARI-2 protein, an interaction with a classical shuttling nuclear transport receptor, karyopherin 3, has been detected (Suppl. Table 4) [53].

That Parkin regulates nucleocytoplasmic protein transport and transcription is supported by its interactor and substrate, RanBP2, a component of the nuclear pore complex that associates to the nuclear membrane [59]. RanBP2 belongs to a family SUMO E3 ligases and sumoylates HDAC4.

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