E3 ligase activity of RING finger proteins that interact with Hip-2, a human ubiquitin-conjugating enzyme

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Abstract To identify proteins that interact with Huntingtin-interacting protein-2 (Hip-2), a ubiquitin-conjugating enzyme, a yeast two-hybrid screen system was used to isolate five positive clones. Sequence analyses showed that, with one exception, all Hip-2-interacting proteins contained the RING finger motifs. The interaction of Hip-2 with RNF2, one of the clones, was further confirmed through in vitro and in vivo experiments. Mutations in the RING domain of RNF2 prevented the clone from binding to Hip-2, an indication that the RING domain is the binding determinant. RNF2 showed a ubiquitin ligase (E3) activity in the presence of Hip-2, suggesting that a subset of RING finger proteins may have roles as E3s. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Huntingtin-interacting protein-2; RING motif; Ubiquitin-conjugating enzyme; Ubiquitin ligase; Yeast two-hybrid

1. Introduction

Ubiquitin-dependent proteolytic system is a major pathway involved in the eukaryotic cells for selective protein degradation. The ubiquitination pathway generally involves three classes of enzymes, including a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) [1,2]. Ubiquitin is first activated by E1 through a thiolester bond between the C-terminal glycine of ubiquitin and a cysteine residue of E1, and is then transferred into an E2 cysteine residue. E2 by itself, or in concert with E3, catalyzes the formation of an isopeptide bond between the C-terminal glycine of ubiquitin and the ε-amino group of lysine residues on the target proteins. The ubiquitinated proteins are then finally recognized and degraded by the 26S proteasome complex [3].

A large number of E2s exist; for example, in yeast, at least 10 different E2s have been identified, whereas, in *Arabidopsis thaliana*, more than 30 are present, and over 20 have been detected in mammalians [4–6]. Functions of these enzymes include roles in DNA repair, cell cycle progression, organelle biogenesis, secretion, and stress response. All known E2s contain the UBC domain, a conserved core domain of approximately 150 amino acids in size, and are classified into four groups by the presence of additional N-terminal and/or C-terminal amino acid sequences [4].

Huntingtin-interacting protein-2 (Hip-2) is homologous to a human ubiquitin-conjugating enzyme E2–25K. This protein has similarities to the UBC-1, -4, and -5 enzymes of *Saccharomyces cerevisiae*. In addition, it is highly expressed in the cortex and striatum in brain regions of Huntington's patients and interacts specifically with the N-terminus of huntingtin, implying that the cellular level of huntingtin is normally regulated in part by the ubiquitin-dependent degradation [7].

Recent studies reported that certain ubiquitin-conjugating enzymes interact with the RING finger proteins that may play roles as E3s in the ubiquitin–proteosome-dependent pathway [8–14]. In this study, we identified several RING finger proteins that interact with Hip-2 through the yeast two-hybrid system. We provide direct evidence that the RING finger domain is responsible for the interaction and is crucial for E3 activities of the proteins.

2. Materials and methods

2.1. Yeast two-hybrid screening and β-galactosidase assay

LexA yeast two-hybrid system (Clontech) was applied to identify the intracellular proteins capable of binding to Hip-2 from a human fetal brain cDNA library. A full-length Hip-2 was amplified through the polymerase chain reaction (PCR) with *Pfu* polymerase (Stratagene) and was then cloned into pLexA. The yeast two-hybrid screen was performed following the manufacturer's instructions (Clontech). Positive clones were further characterized by sequencing and were searched for possible gene homologies through the NCBI GenBank BLAST and BCM (Baylor College of Medicine) search.

Liquid β-galactosidase activity assays were performed to quantify the strength of interaction for protein pairs using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate according to the manufacturer's instructions (Clontech).

2.2. Construction of truncate and mutant plasmids

RNF2ΔC1 (RNF_{1-93}) was generated by the digestion of a full-length RNF2 with *Eco*RI and *Spe*I, and was cloned into pB42AD. RNF2ΔC2 (RNF_{1-156}) and RNF2ΔN1 ($RNF_{180-337}$) were constructed by digesting a full-length RNF2 with *Eco*RI and *Pst*I, and with *Pst*I and *Xho*I, respectively. The resulting products were then amplified through PCR using primers, which contained *Eco*RI and *Xho*I sites on the N-terminal and the C-terminal regions, respectively. The amplified PCR products were digested with *Eco*RI and *Xho*I and inserted into pB42AD.

The constructs of C51W/C54S (cysteine to tryptophan at the amino acid 51 and cysteine to serine at the amino acid 54) and H69Y (histidine to tyrosine at the amino acid 69) mutants were generated using a Quickchange site-directed mutagenesis kit (Stratagene) and specific

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oligonucleotides. All constructs were confirmed by sequencing the mutated regions. The mutants were cloned into pB42AD and used in the yeast two-hybrid system and β-galactosidase assay.

2.3. In vitro binding assays

A full-length RNF2 was cloned into pGEX4T-1 to generate a GST–RNF2 fusion protein (Pharmacia Biotech, Uppsala, Sweden). ^{35}S-labeled Hip-2 was generated with the TNT-coupled reticulocyte lysate system following the manufacturer's instructions (Promega).

2.4. In vivo binding assays by co-precipitation experiments

For co-precipitation experiments, Hip-2 containing an N-terminal GST-epitope tag was transiently co-transfected with Xpress epitope-tagged RNF2 into COS7 cells. GST pull down assay was performed as described previously [15]. Anti-GST antibody (Ab) and anti-Xpress Ab were purchased from Amersham Pharmacia (1:3000 diluted) and Invitrogen (1:3000 diluted), respectively.

2.5. In vitro ubiquitination assay

The experiment was carried out according to a previously published report [10] with modifications. GST–RNF2 immobilized to the glutathione–Sepharose beads was combined with 20 ng of E1, 20 ng of E2, and 10 μg of ubiquitin (Sigma) in a ubiquitination buffer (50 mM Tris–HCl (pH 7.4), 2 mM ATP, 5 mM $MgCl_2$, 2 mM DTT, 1 mM creatine phosphate, 15 units of creatine phosphokinase and BL-21 cell lysates) and the mixture was incubated for 1.5 h at 30°C. The reactions were terminated by adding a stop buffer (SDS sample buffer containing β-mercaptoethanol) and processed for 10% SDS–PAGE. Western blotting experiments were performed using the anti-ubiquitin Ab.

3. Results and discussion

3.1. Hip-2 interacts with a family of RING finger proteins

To identify the proteins that interact with Hip-2, we performed a yeast two-hybrid screen. Yeasts expressing the full-length LexA–Hip-2 protein were transformed with a human brain cDNA library. Five positive clones were identified by screening approximately 2.1×10^7 Trp^+, Leu^+ auxotrophic transformants. Database searches performed at NCBI using the BLASTX program revealed that, with one exception, all Hip-2-interacting clones contained the RING finger motifs. Hip-2-interacting protein-1, termed HIPI-1, showed 97% identity to the *Homo sapiens* HSD-4 mRNA (accession number AF162680). In addition, HIPI-3 and HIPI-4 were 100% identical to the *H. sapiens* RNF2/dinG/RING1b mRNA (accession number Y10571) and RNF24 (accession number XM009564), respectively, while HIPI-8 was revealed to be a novel RING finger gene. On the other hand, HIPI-6, the one exception that did not contain the RING finger motif, was 99% identical to the *H. sapiens* HSPC035 mRNA (accession number AF078855.1).

Comparisons of the amino acid sequences among HIPIs revealed no significant homologies in the structures except that all proteins contained the RING domain sequences (Fig. 1). RNF2, HIPI-8, and HSD-4 belong to the RING-HC (C_3HC_4)-type and, interestingly, have 12 amino acid sequences between Cys2 and Cys3 (Fig. 1). On the other hand, RNF24 belongs to the RING-H2 ($C_3H_2C_3$)-type and has 14 amino acid residues between Cys2 and Cys3. One common characteristics of HIPIs that contain the RING finger domains, as compared to other RING proteins, is that a phenylalanine (F) residue exists in front of the Cys4 position. Recently, ubcM4, a ubiquitin-conjugating enzyme, was revealed to interact with the RING finger proteins, termed UIPs (ubcM4-interacting proteins) [8]. Though all UIPs belong to the RING-HC (C_3HC_4)-type, they possess more amino acid residues between Cys5 and Cy6 than HIPIs.

3.2. Interaction of RNF2 in vitro and in vivo with Hip-2

In order to confirm the interaction between Hip-2 and RNF2 (HIPI-3), in vitro binding assays were performed using GST fusion proteins. ^{35}S-Radiolabeled Hip-2s were generated using the rabbit reticulocyte lysates and assessed for their capability to form a stable complex with the GST–RNF2 protein or GST alone, a negative control. Hip-2 was shown to efficiently bind to GST–RNF2, but not to GST alone (Fig. 2A).

To further verify whether RNF2 interacts with Hip-2 in mammalian cells, in vivo binding assays were performed. COS-7 cells were harvested 48 h after co-transfection with pcDNA3.1/Xpress-RNF2 and pcDNA3.0/GST–Hip-2. The transfected cell extracts were mixed with glutathione–Sepharose beads, and proteins that were bound to the GST–Hip-2 coupled with the beads were then probed with an anti-Xpress Ab to detect the RNF2 protein. Results revealed that RNF2 proteins were bound to the GST–Hip-2 fusion proteins (Fig. 2B)., while no interaction was observed between RNF2s and GST proteins (data not shown). These results suggest that RNF2 binds to Hip-2 under in vivo conditions and, furthermore, confirm the interaction detected through the yeast two-hybrid assays.

3.3. The RING finger domain in RNF2 is essential for the recognition of Hip-2

To define the protein region responsible for the interaction between RNF2 and Hip-2, deleted versions of RNF2 were expressed as LexA–BD fusion proteins in yeasts. *LacZ* reporter gene was activated in cells coexpressing LexA–Hip-2 and pB42AD full-length RNF2, -RNF2ΔC1 or -RNF2ΔC2 (Fig. 3A,B). However, no detectable β-galactosidase activity was observed in cells expressing the protein pair LexA–Hip-2 and pB42AD–RNF2 C-terminal stretch (aa 180–337). These results indicate that the N-terminal region of RNF2, including the RING finger domain, is essential for the observed protein–protein interaction.

To further verify the role of the RING domain as the Hip-2-

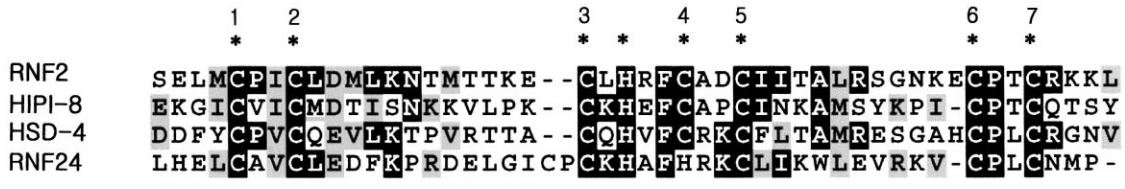

Fig. 1. RING finger motifs of Hip-2-interacting proteins. A comparison of the ring finger structure of HIPIs is shown. Cysteine residues are numbered. The conserved cysteine and histidine residues of the RING finger motif are marked with asterisks. White letters on the dark background and dark letters on the gray background indicate residues that are conserved in at least 80 and 50–80% of the aligned sequences, respectively.

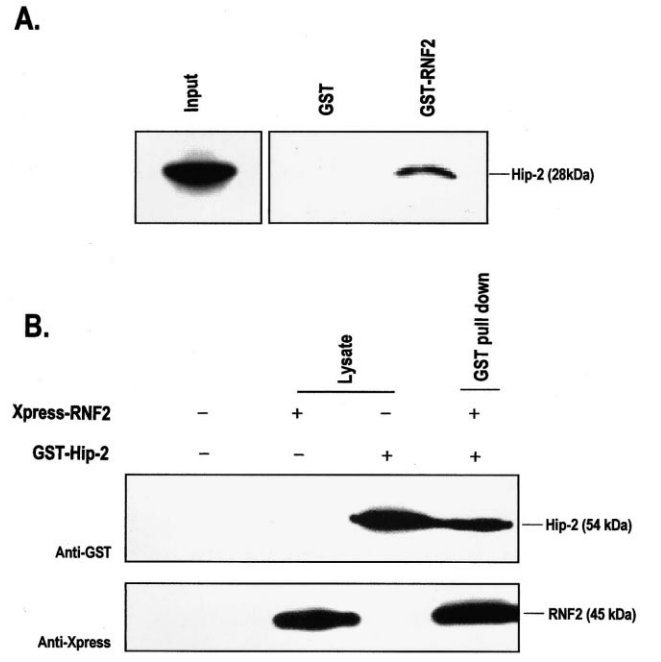

Fig. 2. Hip-2 interacts with RNF2 in vitro and in vivo. A: ^{35}S-radiolabeled Hip-2s were generated using rabbit reticulocyte lysates and assessed for their capability to form a stable complex with GST–RNF2 protein or GST alone. B: Co-precipitation of the RNF2 protein with Hip-2. GST-tagged-Hip-2 and Xpress-tagged-RNF2 were transiently transfected into COS-7 cells. After 48 h, the cells were harvested, and Western blotting experiments were performed. Detections were made using an anti-Xpress Ab (1:3000), and the blot was stripped to reprobe with an anti-GST Ab (1:3000).

interacting region, we generated the following two missense mutations in the domain, which disrupt the RING structure: C51W/C54S (cysteine to tryptophan at the amino acid 51 and cysteine to serine at the amino acid 54) and H69Y (histidine to tyrosine at the amino acid 69). Yeast two-hybrid and β-galactosidase assays revealed a very weak β-galactosidase activity in the double mutant C51W/C54S, and no activity in the single mutant H69Y (Fig. 3A,B). In control experiments performed to verify our results, coexpression of pB42AD–RNF2 and LexABD-atropine-1 (DRPLA gene) as a negative control showed no β-galactosidase activity (data not shown). Taken together, these data indicate that the RING finger domain in RNF2 has a critical role in the recognition of Hip-2 ubiquitin-conjugating enzyme.

3.4. RNF2 plays a role as an E2-dependent ubiquitin ligase

Recently, several proteins containing the RING finger domain revealed to be E3 ubiquitin ligases (reviewed in [16–19]). Therefore, we performed in vitro ubiquitination assays to assess whether RNF2 has an E2-dependent E3 activity. A ubiquitination pattern was observed in the presence of E1, Hip-2, and RNF2 (lane 5 in Fig. 4A), whereas none was detected in the absence of RNF2 (lane 4 in Fig. 4A), which suggests that RNF2 functions as E3. A mixture of E1, ubcH5b, and AO7, as a positive control, generated high molecular weight-smeared bands indicative of polyubiquitination (lane 2 in Fig. 4A). Similar results were obtained using ^{32}P-labeled ubiquitin (data not shown).

To verify that the RING finger domain is important for the E3 activity, we used the deleted or mutant RNF2 proteins instead of the wild-type in the ubiquitination assays. Ubiquitinated proteins were only detected in the presence of the wild-type RNF2 (lane 2 in Fig. 4B), indicating that RNF2 has an E3 activity in a RING-dependent manner and exhibits a RING-type E3 ligase activity through the interaction with Hip-2 in an E2-dependent manner.

3.5. RNF2 can be paired with closely related ubc4 and ubcH5b in the ubiquitination pathway

Although E2s have a common biological feature of ubiquitin conjugation, all E2s appear to possess their respective cognate E3s. We investigated whether RNF2 has a specific interaction with other E2s. ubc4, ubcH5b, ubcH6, and ubcH9 proteins, which were expressed in *Escherichia coli* and purified using a glutathione affinity column, were assayed in the presence of mammalian E1, ubiquitin, and an ATP-regenerating system. High molecular-ubiquitinated bands were observed only in the presence of the Hip-2-related proteins, ubc4 and ubcH5b (Fig. 4C), but not in the unrelated proteins, ubcH6 and ubcH9, indicating that RING finger proteins functioning as E3s interact with specific E2s. These results imply that RING proteins may contribute to the substrate specificity in the degradation of proteins through a ubiquitin-dependent pathway.

The diversity and the number of proteins that are regulated by the ubiquitination foretell the existence of a large number of E3s. However, to date, relatively few E3s have been found. In this study, we identified several RING finger proteins that

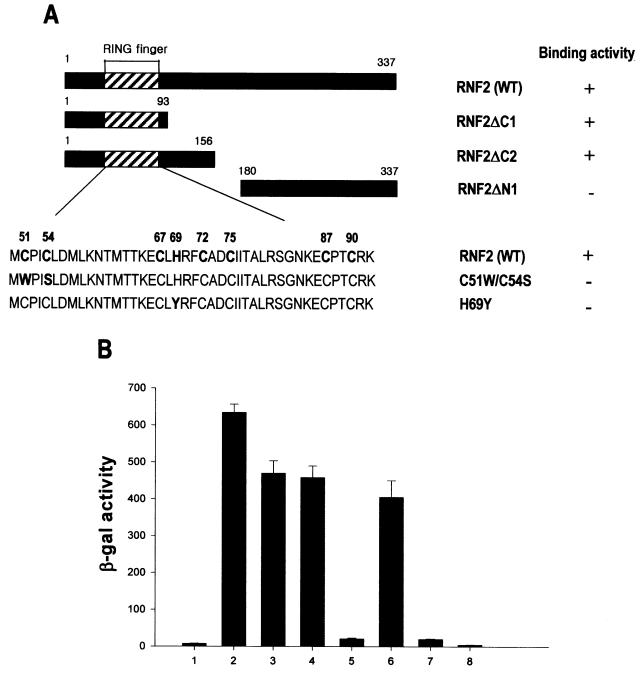

Fig. 3. RING domain of RNF2 is necessary for the interaction with Hip-2. A: Schematic representation of wild-type RNF2 and various mutant constructs. The interaction with Hip-2 was determined using the yeast two-hybrid assays (right). The RING finger domain is indicated with striped bars. B: The strength of interaction with Hip-2 was measured using the β-galactosidase assays. Lane 1, BD/empty-AD/TAg; lane 2, BD/p53-AD/TAg; lane 3, BD/Hip-2–AD/RNF2ΔC1; lane 4, BD/Hip-2–AD/RNF2ΔC2; lane 5, BD/Hip-2–AD/RNF2ΔN1; lane 6, BD/Hip-2–AD/RNF2 (wt); lane 7, BD/Hip-2–AD/RNF2 (C51W/C54S); lane 8, BD/Hip-2–AD/RNF2 (H69Y).

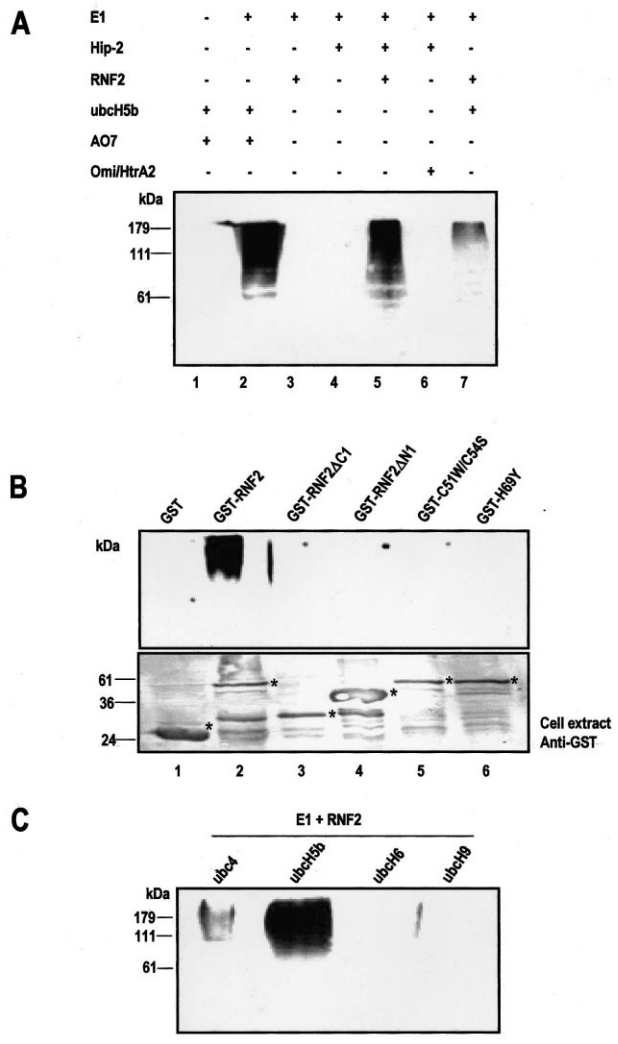

Fig. 4. RNF2 is involved in the E2-dependent ubiquitination in vitro. A: GST–RNF2 fusion protein was incubated with or without E1 and E2, and ubiquitination patterns were investigated using a ubiquitin Ab. The ubcH5b and AO7 are positive controls, and Omi/HtrA2 is a negative control. B: GST-fused mutants were evaluated for the ubiquitination activity (upper panel). In the lower panel, anti-GST antibodies were used to confirm the expression of the fusion proteins, which are indicated with asterisks. C: Glutathione bead-immobilized E2s were incubated with mammalian E1 and RNF2. The experiments were performed as described in Section 2. RNF2 exhibits ubiquitin ligase activity in the presence of ubc4 and ubcH5b, but not with ubcH6 and ubcH9.

interact with an E2 enzyme, Hip-2 (hE2–25K), and found evidence that RNF2 has a role as an E3 ligase, which suggest that other proteins identified through the yeast two-hybrid screen may also function as E3 ligases. We generated various truncates and missense mutations in the RING finger domain of RNF2 protein. Both the yeast two-hybrid assays and β-galactosidase experiments showed that mutant forms of the RING domain lost their ability to bind to Hip-2 (Fig. 3). In addition, the mutants did not act as E3s in the ubiquitination assays (Fig. 4). Thus, the RING finger domain of RNF2 is not only required for interacting with Hip-2 but also for acting as an E3 ligase. These studies would expand the pool of E3 ligases and provide information of specificity on the ubiquitination-related proteins for determining the ubiquitination target substrates.

RNF2/dinG/RING1B is a homolog of the human RING1 protein. RING1A and RING1B were recently shown to interact with other mammalian PcG proteins and were identified as new members of the Pc group family [20–23]. Ring1A has been known to behave as a transcriptional repressor, whereas the function of RNF2/Ring1B protein has not yet been revealed. Our study demonstrates that RNF2 has a role as an E3 ligase, providing an insight into the new function of the RNF2/dinG/RING1B protein.

Abnormal ubiquitinations have been found at increased levels in neuropathological diseases including Alzheimer's disease and Parkinson's disease, suggesting that the ubiquitination is common pathological pathway among neurodegenerative disorders. Thus, an abnormal ubiquitination pathway may be related to HD in which Hip-2 and RNF2 may have a role as important ubiquitination components in HD. These studies may lead to novel diagnostic and therapeutic approaches to HD.

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