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Species-specific variation of RPA-interacting protein (RIP) splice isoforms

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Replication Protein A (RPA) is a single stranded DNA-binding protein involved in DNA metabolic activities such as replication, repair, and recombination. RPA-Interacting Protein α (RIPα) was originally identified as a nuclear transporter of RPA in *Xenopus*. The human *RIPα* gene encodes several splice isoforms, of which hRIPα and hRIPβ are the major translation products *in vivo*. However, limited information is available about the alternative splicing of *RIPα* in eukaryotes, apart from that in humans. In this study, we examined the alternative splicing of *RIPα* in the *Drosophila, Xenopus*, and mouse system. We showed that the number of splice isoforms of *RIPα* was species-specific, and displayed a tendency to increase in higher eukaryotes. Moreover, a mouse ortholog of hRIPβ, mRIPβ2, was not SUMOylated, in contrast to hRIPβ. Based on these results, we suggest that the *RIPα* gene gains more splice isoforms and additional modifications after molecular evolution. [BMB reports 2009; 42(1): 22-27]

INTRODUCTION

Replication Protein A (RPA) is a single-stranded DNA-binding protein composed of three subunits with molecular weights of 70, 32, and 14 kDa. RPA is involved in multiple processes of eukaryotic DNA metabolism, including replication, repair, and recombination of DNA (1-3). RPA interacts tightly with single-stranded DNA (ssDNA) to facilitate its stabilization (4-6). For instance, RPA is recruited to nuclear foci to stabilize ssDNA during the repair of double-stranded DNA breaks induced by ultraviolet (UV) irradiation or ionizing radiation (7-9).

RPA is imported into the nucleus after synthesis in the cytoplasm. Jullien *et al*. (10) identified *Xenopus* RPA- Interacting Protein α (xRIPα) as a binding partner of the RPA 70 kDa subunit by yeast two-hybrid screening, and as the nuclear transporter of RPA via an *in vitro* import assay. xRIPα interacts with both RPA and importin β, and serves as an adapter molecule connecting both proteins, analogous to the linking of nuclear localization signal-containing proteins to importin β by importin α (10,11). Recently, hRIPα, the human ortholog of xRIPα, was cloned. RT-PCR (reverse transcription-polymerase chain reaction) analyses revealed that the *hRIPα* gene encodes several splice isoforms (8,12). Of the numerous isoforms of hRIPα, hRIPα and hRIPβ are the major translation products, each of which has distinct subcellular localization patterns (8). Interestingly, hRIPβ is SUMOylated, a process that determines its localization in the PML nuclear body (8,13,14). While hRIPα simply transports RPA to the nucleus, hRIPβ is likely to be post-translationally regulated for its storage in PML nuclear bodies, and subsequent release upon external stimuli, such as exposure to UV radiation (8).

RIPα is conserved among higher eukaryotes, except yeast. Msn5p is the carrier molecule of RPA in yeast (15), but does not display sequence homology with the RIPα protein family. Here, we clone RIPα cDNA from *Drosophila*, *Xenopus* and mouse, and identify their splice isoforms. *Drosophila* and *Xenopus* do not contain a homologous gene to hRIPβ. In contrast, mouse *RIPα* encodes mRIPβ2, an ortholog of hRIPβ. Unlike hRIPβ, mRIPβ2 is not SUMOylated, and amino acid sequence analyses reveal that SUMOylation sites are not conserved in mRIPβ2. The data from this study collectively indicated that higher eukaryotes contain a greater number of splice isoforms, and more elaborate mechanisms to transport RPA into the nucleus.

RESULTS

Cloning and sequencing of RIPα and its splice isoforms in Drosophila, Xenopus, and mouse

Recently, we identified seven splice isoforms of *hRIPα*, and showed that hRIPα and hRIPβ were the dominant proteins *in vivo* (8). Since hRIPβ regulates the localization of RPA in the PML nuclear body (8), we examined whether this protein was conserved in other eukaryotes, including *Drosophila, Xenopus,* and mouse. Using the nucleotide sequences of dRIPα, xRIPα and mRIPα from an expressed sequence tag (EST) database, we designed primers complementary to the 5′ and 3′ ends of each

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open reading frame to identify their respective splice isoforms. Total RNA of *Drosophila* was extracted from the whole body, while that of *Xenopus* was obtained from individual organs, such as liver, heart and ovary after dissection. Mouse and human cell lines were employed for total RNA preparation.

RT-PCR analysis of *dRIPα* disclosed a single transcript, which was cloned and nucleotide sequences determined. This transcript was reported to encode the full-length dRIPα protein (Fig. 1A, panel i). No other splice isoforms were detected in *Drosophila*. RT-PCR of *xRIPα* resulted in the amplification of a single dominant transcript representing the full-length protein (Fig. 1A, panel ii). However, repeated RT-PCR of the *xRIPα* gene resulted in the amplification of a novel splice isoform designated *xRIPδ* (Fig. 1B, panel ii). The PCR product of xRIPδ was similar to that of xRIPα, and thus difficult to distinguish from the major xRIPα product (Fig. 1B, panel ii). The peptide sequence of xRIPδ was noted to be homologous to that of hRIPδ (Data not shown). xRIPδ is postulated to be a minor transcript of the *xRIPα* gene, since the levels of the xRIPδ transcript were hardly detectable by RT-PCR.

In an attempt to clone mouse orthologs of hRIPα, RT-PCR was performed on RNA extracted from NIH3T3 and B16F10 cells. The mRIPα gene yielded various splice isoforms, which were cloned into the pCR2.1 TOPO vector to determine their nucleotide sequences (Fig. 1A, panel iii). Repeated experiments disclosed more distinctive bands in humans, compared to mouse (Fig. 1A, panels iii and iv). Six different splice isoforms, denoted mRIPα, mRIPβ1, mRIPβ2, mRIPδ1, mRIPδ2, and mRIPε, were cloned (Fig. 1B, panel iii), based on their homology to the hRIPα isoforms (Fig. 2B). Each splice isoform was derived from a unique combination of exon 1 through to

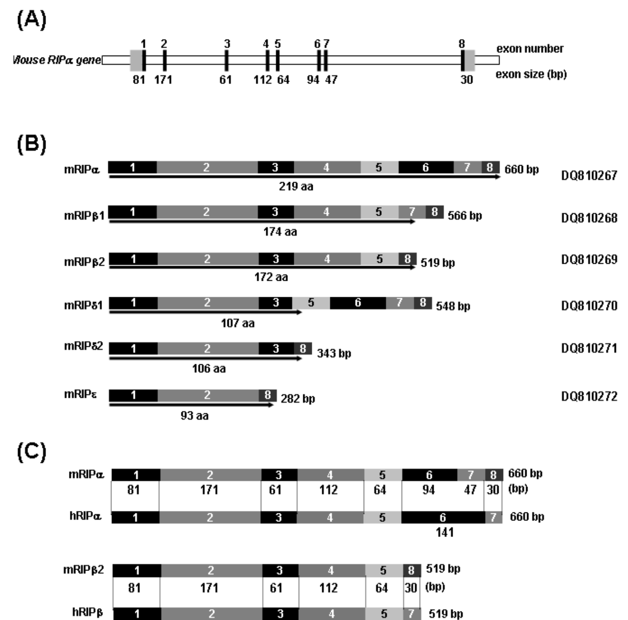

Fig. 2. *mRIPα* gene structure and alternative splicing. (A) Schematic diagram of *mRIPα*. Coding exons are shaded in black boxes, and their sizes are specified. (B) Structures of the alternative splicing products of *mRIPα*. The exon combinations are specified, and protein products are depicted as arrows under each isoform. GenBank accession numbers are specified. (C) mRIPα is composed of 8 exons, while hRIPα comprises 7 exons. A combination of exons 6 and 7 of mRIPα is equivalent to the single exon 6 of hRIPα. The splicing pattern of mRIPβ2 was identical to that of hRIPβ.

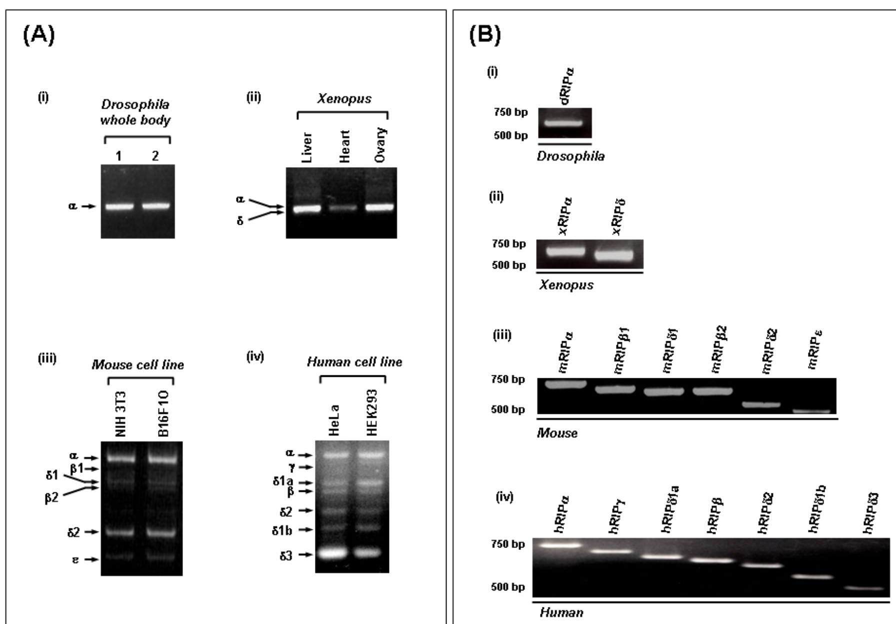

Fig. 1. Alternative splicing of *RIPα* in *Drosophila*, *Xenopus*, mouse, and human. (A) RT-PCR analysis of *RIPα*. Total RNA was extracted from the whole body (*Drosophila*, panel i), organs (*Xenopus*, panel ii), and cell lines (mouse and human, panels iii and iv). The mRNA transcripts were analyzed by RT-PCR. (B) Splice isoforms were cloned and fully sequenced. The numbers of splice isoforms were found to be variable among *Drosophila* (one), *Xenopus* (two), mouse (six), and human (seven or more).

exon 8. Notably, exons 1, 2 and 8 were conserved in all splice isoforms (Fig. 2A and B).

mRIPβ2, the mouse ortholog of hRIPβ

The *mRIPα* gene is composed of 8 exons, while *hRIPα* is composed of 7 exons (Fig. 2C). However, further analyses revealed that the association of exon 6 (94 bp) and exon 7 (47 bp) of mRIPα were equivalent to exon 6 (141 bp) of hRIPα (Fig. 2C). All six splice isoforms potentially encoded polypeptides. Alignment of the polypeptides revealed the presence of conserved N-terminal sequences (residues 1 to 84) (Data not shown). Interestingly, *mRIPα* encoded the unique splice isoform, mRIPε, which was found to be shorter than the other isoforms (Fig. 2B).

Based on an alignment of mRIPα and its splice isoforms, we concluded that mRIPβ2 was the mouse ortholog of hRIPβ. Both mRIPβ2 and hRIPβ contained 519 nucleotides, and potentially encoded for 172 amino acids (Fig. 2C). The splicing pattern of mRIPβ2 was almost identical to that of hRIPβ, except that exon 8 was used instead of exon 7 in human (Fig. 2C). Alternative splicing of the *mRIPα* gene produced mRIPβ1, another mouse homolog of hRIPβ. However, the C-terminal sequences (residues 163 to 174) of mRIPβ1 were not found to be homologous to those of hRIPβ and mRIPβ2. These results indicated that mRIPβ2 was the mouse ortholog of hRIPβ.

mRIPα and mRIPβ2, the major translation products

mRIPα and its splice isoforms were subcloned into the pcDNA3 vector and expressed in rabbit reticulocytes in the presence of [^{35}S] methionine *in vitro*. Each splice isoform produced the expected size of polypeptide *in vitro*, as observed by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis; Fig. 3A). To confirm the expression of the genes *in vivo*, HEK293 cells were transiently transfected with plasmids encoding mRIPα and its splice isoforms. Cell lysates were subjected to immunoblotting with the anti-FLAG antibody. While mRIPα and mRIPβ2 were readily detected, the levels of the other isoforms were low. This pointed toward mRIPα and mRIPβ2 as the major splice isoforms (Fig. 3B, left panel). To confirm this hypothesis, we generated an anti-mRIPα antibody in rabbits, and analyzed the endogenous expression of mRIPα and mRIPβ2 in B16F10 cells (Fig. 3B, right panel). Immunoblot analysis revealed that the upper band represented mRIPα, while the lower band corresponded to mRIPβ2 (Fig. 3B). Repeated experiments showed that the upper band was comparatively more abundant (Fig. 3B). These

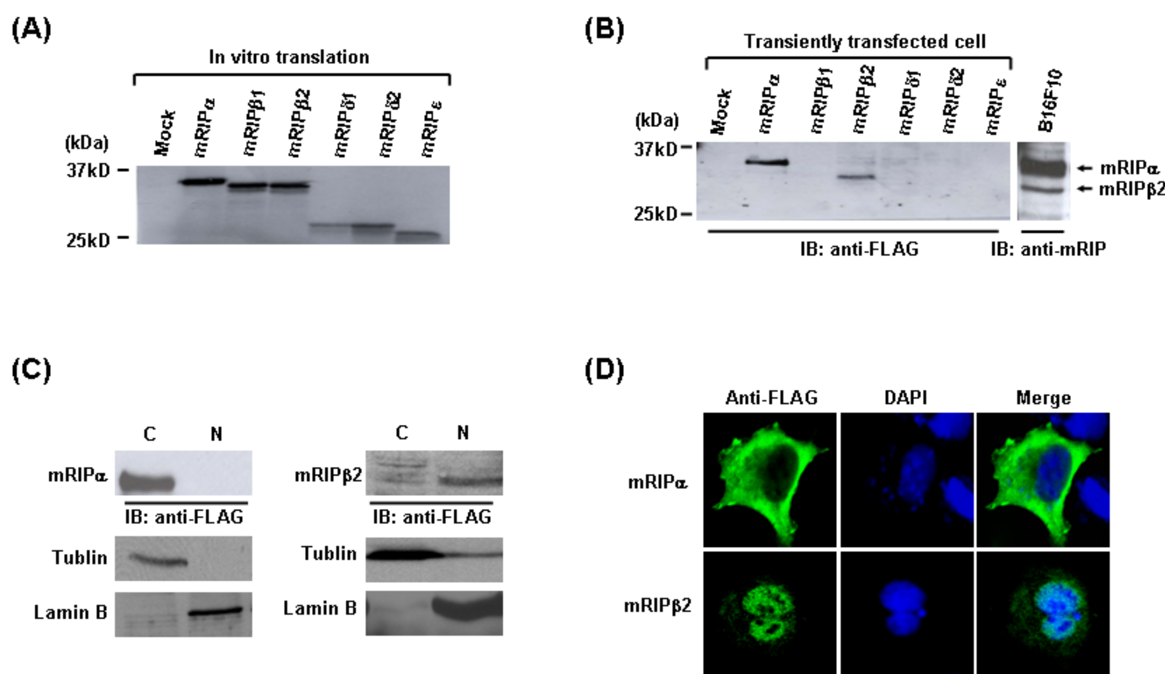

Fig. 3. Expression analysis of mRIPα and its splice isoforms. (A) *In vitro* translation of mRIPα and its splice isoforms. mRIPα and its splice isoforms were translated in rabbit reticulocytes in the presence of [^{35}S] methionine *in vitro*, and analyzed by SDS-PAGE and autoradiography. (B) Transient expression of mRIPα and its splice isoforms. HEK293 cells were transfected with plasmids encoding mRIP*α* and its splice isoforms. Equal amounts of protein were analyzed by immunoblotting with anti-FLAG antibody. B16F10 cell lysates were probed with anti-mRIP*α* antibody to analyze endogenous mRIP*α* and mRIPβ2 expression. (C) mRIP*α* mainly localized to the cytoplasm, while mRIPβ2 localized to the nucleus. Subcellular fractionation (C) and confocal microscopy (D) were used to identify the subcellular localization of mRI*α* and mRIPβ2. Tubulin and laminin B were used as markers for cytoplasmic and nuclear fractions, respectively.

results indicated that mRIPα and mRIPβ2 were the major translation products in the mouse system *in vivo*, corresponding to hRIPα and hRIPβ in humans (8).

We then examined the localization pattern of mRIPα and mRIPβ2 within the cell, by subcellular fractionation of transfected cells. Immunoblotting with anti-FLAG antibody disclosed that mRIPα predominantly localized to the cytoplasm, while mRIPβ2 was present in the nucleus (Fig. 3C). Confocal microscopy was applied to confirm these findings. HEK293 cells were transfected with plasmids encoding mRIPα and mRIPβ2 and stained with the anti-FLAG antibody. Notably, while the majority of mRIPα was localized in the cytoplasm, mRIPβ2 mainly translocated to the nucleus, confirming the results of the subcellular fractionation analyses (Fig. 3D).

In view of the finding that SUMOylation of hRIPβ is essential for its function (8), we examined whether mRIPβ2 was SUMOylated *in vivo*. hRIPβ contains the SUMOylation consensus sequence ($IK^{103}QE/\Psi KxE$). Moreover, three other lysines (K^{114}, K^{121} and K^{142}) at the C-terminus are involved in SUMOylation (8,16). However, this SUMOylation consensus sequence is not conserved in mRIPβ2, and two out of three lysines in the C-terminus of hRIPβ are not conserved (Fig. 4A), suggesting that mRIPβ2 is not likely to be SUMOylated.

HEK293 cells were transiently transfected with plasmids encoding mRIPα and mRIPβ2, while hRIPβ was employed as a positive control. Since SUMO isopeptidase is capable of readily cleaving the isopeptide bond between SUMO and the target protein, direct boiling of the cell lysates was employed to inactivate the enzyme (17). SUMOylation of hRIPβ was readily detected by immunoblotting, while mRIPβ2 was not SUMOylated (Fig. 4B and C). To confirm these results, we examined the co-localization of mRIPβ2 with SUMO-1. Previous analyses show that SUMOylation of hRIPβ was directly linked to co-localization with SUMO-1 (8). As expected, hRIPβ co-localized completely with SUMO-1, while mRIPβ2 did not (Fig. 4D). Based on these results, we concluded that unlike hRIPβ, mRIPβ2 was not SUMOylated.

DISCUSSION

In the present study, we examined the splice isoforms of dRIPα, xRIPα and mRIPα, and demonstrated that the numbers of splice isoform were species-specific. Notably, dRIPα did not have a splice isoform, while xRIPα contained one splice isoform, xRIPδ. Because the levels of expression of xRIPδ were hardly detectable by RT-PCR analysis, xRIPδ was predicted to be a minor transcript. So it is reasonable to suppose that dRIPα and xRIPα are the major transcripts in *Drosophila* and *Xenopus*. In the mouse system, we cloned six splice isoforms of *mRIPα*, including mRIPβ2. Similar to hRIPα and hRIPβ, mRIPα and mRIPβ2 were readily detected by transient transfection, suggestive of major translation products.

The number of splice isoforms possibly increases as the species evolves. Interestingly, yeast does not contain RIPα, *Drosophila* has one isoform, *Xenopus* has two, mouse has six, and humans have seven or more (8,12,15). It is reasonable to assume that the number of RIPα splice isoforms expands to cope with the complexity of DNA metabolism in higher eukaryotes.

RPA is essential in DNA metabolism, and is involved in DNA replication, repair and recombination. Therefore, the regulation of RPA transport and storage is likely to be one of the

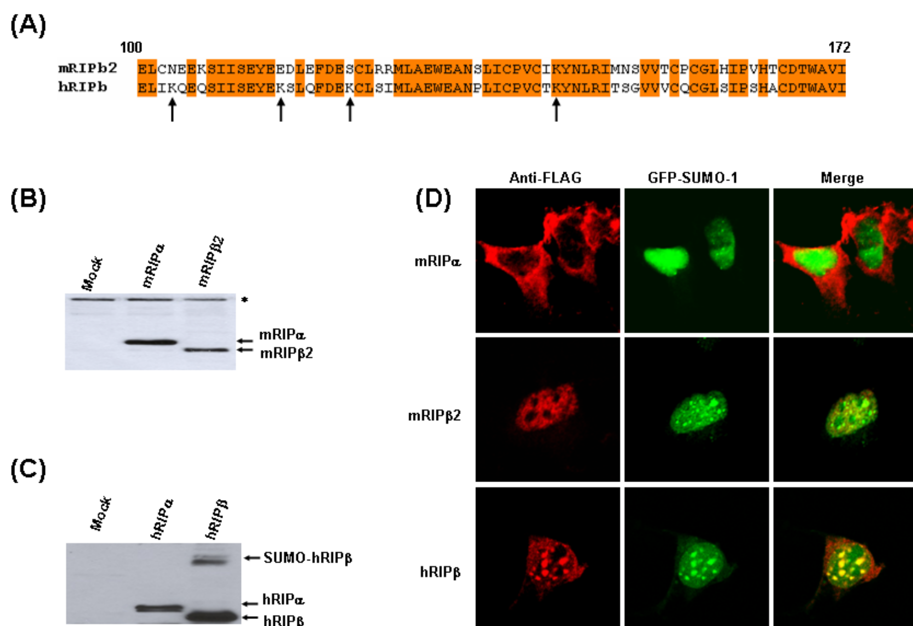

Fig. 4. mRIPβ2 is not SUMOylated. (A) SUMOylation sites of hRIPβ are poorly conserved in mRIPβ2. C-terminal sequences (residues 100 to 172) of mRIPβ2 and hRIPβ were aligned, and each lysine in hRIPβ sequences is indicated with an arrow. (B) mRIPβ2 is not SUMOylated *in vivo*. HEK293 cells were transfected with plasmids encoding mRIPα and mRIPβ2, and cell lysates analyzed with the anti-FLAG antibody. The star represents non-specific bands. (C) hRIPα and hRIPβ are transiently expressed. SUMOylation of hRIPβ was used as a positive control. (D) mRIPβ2 is not co-localized with SUMO-1. HEK293 cells were transfected with plasmids encoding mRIPα, mRIPβ2 or hRIPβ in the presence of GFP-SUMO-1. hRIPβ was employed as a positive control.

main factors affecting DNA metabolism. Previous reports demonstrate that hRIPα simply transports RPA into the nucleus, while hRIPβ regulates the storage of RPA in the PML nuclear body and releases the protein upon external insult (8). To determine whether a similar mechanism exists in other eukaryotes, we searched for RIPβ orthologs in *Drosophila, Xenopus* and mouse.

Drosophila and *Xenopus* do not contain a splice isoform homologous to hRIPβ. On the other hand, protein alignment analyses showed that mRIPβ2 corresponded to the mouse ortholog of hRIPβ, similar to mRIPα, which is the mouse ortholog of hRIPα. The two proteins contained a similar number of amino acids and identical splicing patterns. We further tested whether mRIPβ2 was SUMOylated. Since SUMOylation of hRIPβ is known to be essential for RPA deposition and release in the PML nuclear body (8), it was likely that mRIPβ2 did not possess a mechanism to regulate RPA storage in the PML nuclear body. The function of mRIPβ2 is analogous to that of the hRIPβ 4KM mutant, which is involved in transporting RPA to the nucleus, and is not SUMOylated (8). However, low levels of RPA co-localized with hRIPβ 4KM, compared to hRIPβ, implying that SUMOylation was required for the efficient co-localization of these proteins. Accordingly, we speculated that hRIPβ had a more robust mechanism to regulate RPA, compared to mRIPβ2. Our results suggested that the RPA storage systems of higher eukaryotes required additional splice isoforms and modifications, which contributed to efficient DNA metabolism, such as rapid and accurate DNA repair and DNA recombination under various conditions.

In this study, we examined the alternative splicing patterns of *RIPα* in *Drosophila*, *Xenopus* and mouse, and showed that mRIPβ2 was the mouse ortholog of hRIPβ. Moreover, mRIPβ2 was not SUMOylated, indicating that SUMOylation of RIPβ is unique to the human system. Thus, we propose that RIPα gains more splice isoforms and additional modifications to cope with the complexity of DNA metabolism in higher eukaryotes. Further studies are required to identify the physiological roles of the additional splice isoforms, mRIPβ1 and mRIPδ.

MATERIALS AND METHODS

Cell culture and transfection

HEK293 cells were grown in EME medium supplemented with 10% fetal bovine serum. NIH3T3, HeLa and B16F10 cells were grown in DMEM medium supplemented with 10% fetal bovine serum. HEK293 transfection was performed using FuGENE6 (Roche, Germany), according to the manufacturer's instructions.

RNA isolation and RT-PCR

Total RNA from *Xenopus* was extracted from organs, including brain, heart, liver, kidney, ovary and muscle, using a tissue homogenizer and TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA of *Drosophila* was isolated from the whole body using TRIzol reagent, and NIH3T3 cells were employed to prepare mouse RNA. The entire coding regions of the splice isoforms of dRIP, xRIP, and mRIP were amplified by RT-PCR using the specific primers (18). dRIP forward: ATGAACTCCAC TCAAAGCCCGG; dRIP reverse: CTAGACGGAGGAGAAGTA GTCGCAG; xRIP forward: ATGGAGGCTGAAAGGAGGCAT C; xRIP reverse: CTATAGGATGACAACCATTGCATC; mRIP forward: ATGGCAGAGTCCTCGGGGTCTCC; mRIP reverse: CTAGAGGATCACAGCCCAGGTG. Amplified cDNA fragments were cloned into the pCR2.1 TOPO vector (Invitrogen). Each cloned cDNA was completely sequenced. Clones for mRIP splice isoforms were PCR-amplified and cleaved with *EcoRI* and *XhoI* restriction enzymes and subcloned into pcDNA3 (Invitrogen), pME18S (FLAG-tagged SRα promoter plasmid) or pGEX4T-1 (Amersham-Pharmacia Biotech, Uppsala, Sweden). Clones for the hRIP splice isoforms are described in a previous report (8) and the plasmid encoding GFP-SUMO-1 (pEGFP-C1/SUMO-1) was kindly provided by J. Choe (KAIST, Korea).

In vitro translation of mRIPα splice isoforms

One microgram of pcDNA3/mRIPα, pcDNA3/mRIPβ1, pcDNA3 /mRIPβ2, pcDNA3/mRIPδ1, pcDNA3/mRIPδ2 or pcDNA3/hRIPε was incubated with the TNT-coupled transcription and translation system (Promega, Madison, WI, USA), and ^{35}S labeled proteins were synthesized *in vitro*. Labeled proteins were analyzed by SDS-PAGE and autoradiography.

Antibody production and immunoblotting

The GST-mRIPα fusion protein was used as a source antigen for anti-mRIP antibody production. The fusion protein was prepared according to a previous report (19), and used to immunize rabbits. Antibodies for tubulin and lamin B were purchased from Santa Cruz (Santa Cruz, CA, USA). Immunoblot detection was performed with the primary antibody (1:1000 or 1:2000 dilution) and an enhanced chemiluminescence system (ECL; Amersham, Chicago, IL, USA). To detect SUMOylated protein, cells were washed with PBS (phosphate buffered saline) and directly lysed with SDS-loading buffer (100 mM Tris-HCl [pH 6.8], 20% glycerol, 4% SDS, 0.001% bromophenol blue) supplemented with 20 μM N-ethylmaleimide (Sigma, St. Louis, MO, USA). Finally, cell lysates were boiled for 5 min, centrifuged for 10 min at 13000 rpm, and analyzed by SDS-PAGE.

Immunofluorescence and confocal microscopy

Cells were grown on sterilized glass coverslips, fixed with 4% paraformaldehyde, and blocked with 0.1% BSA (bovine serum albumin) in PBS. Following this, the cells were stained with primary antibody (1:500 dilution) in PBS, and allowed to react with Alexa 488-conjugated or Alexa 568-conjugated secondary antibody (1:5000 dilution) (Vector, Burlingame, CA, USA). The slides were rinsed three times with PBS and mounted in mounting media (Vector). Images were captured with a

Bio-Rad confocal microscope (Bio-Rad laboratories, Hercules, CA, USA).

Subcellular fractionation

Cells were harvested with 500 μl CLB buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) with a protease inhibitor cocktail. Cells were allowed to swell for 5 min, and homogenized 50 times using a Dounce homogenizer (Wheaton, Millville, IL, USA). Lysates were centrifuged for 10 min at 7500 rpm at 4°C, and the cytoplasmic fraction was transferred to a clean tube. The nuclear pellet was washed once with CLB buffer before lysis in the presence of SDS-loading buffer.

Acknowledgements

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