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The linker connecting the tandem ubiquitin binding domains of RAP80 is critical for lysine 63-linked polyubiquitin-dependent binding activity

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The tandem ubiquitin-interacting motif (UIM) domain located at the N-terminus of Receptor Associated Protein 80 (RAP80) plays a crucial role in ionizing radiation (IR)-induced DNA damage response. RAP80 translocates to sites of IR-induced DNA damage through interaction of its UIM domain with ubiquitinated H2A and Lys63-linked polyubiquitin chains. The exact mechanism, however, through which RAP80 associates with Lys63-linked polyubiquitin chains is not clear. Here, we show by *in vitro* GST-pull down assays that modifying the linker region between the tandem ubiquitin binding domains of RAP80 changes the binding affinity for Lys63-linked polyubiquitin chains and affects translocation to sites of DNA breaks. Based on these findings, we suggest that the length of the linker region between the tandem ubiquitin binding domains of RAP80 may be a key factor in the binding of RAP80 with Lys63-linked polyubiquitin chains as well as in the translocation of RAP80 to DNA break sites. [BMB reports 2009; 42(11): 764-768]

INTRODUCTION

DNA damage caused by external and internal factors such as ultraviolet radiation (UV), ionizing radiation (IR), numerous chemicals, reactive oxygen species, replication errors and mitotic errors induces cell cycle checkpoints that promote genome stability through DNA lesion repair and apoptosis (1-3). The proteins involved in DNA damage-induced cell cycle checkpoint signaling are divided into three groups (3). The first group consists of so-called DNA damage sensors, which detect the original DNA damage signal and initiate the DNA damaged signaling pathway. Ataxia-telangiectasia (ATM) and Rad3-related protein (ATR) kinases belong in this first group. The second group consists of transducers that convey the DNA damage signals received from the sensor group. This group includes mediator of DNA damage checkpoint protein 1 (MDC1), the product of breast cancer susceptibility gene 1 (BRCA1), receptor associated protein 80 (RAP80), p53-binding protein 1 (53BP1) and DNA topoisomerase II binding protein 1 (TopBP1). The checkpoint kinases 1 and 2 (Chk1 and Chk2) are members of the third group, which is comprised of effecter molecules (3).

Receptor associated protein 80 (RAP80) is a transducer of the DNA damage-induced checkpoint response, and regulates DNA damage signaling mediated by BRCA1. RAP80 recruits BRCA1 to DNA damage sites via a coiled-coil domain containing 98 (CCDC98) in a linkage-specific, polyubiquitin-dependent manner, and thus participates in G2/M checkpoint regulation (4-10). The linkage-specific polyubiquitin binding activity of RAP80 is mediated by two N-terminal tandem ubiquitin-interacting motifs (UIMs) that are considered crucial to the localization of BRCA1 to DNA damage sites. The RAP80 UIMs bind to lysine 63-linked and lysine 6-linked polyubiquitin chains *in vivo* and *in vitro*, but not to lysine 48-linked polyubiquitin chains (7, 8).

The UIM is a short helical motif that binds mono- and polyubiquitin, and is one of the best described ubiquitin-binding domains (UBDs). The first UIMs characterized were found in proteasomal subunit S5a (11). Tandem UIMs are mostly found in proteins associated with the intracellular trafficking and degradation of membrane proteins and the ubiquitin-proteasomal degradation of cytosolic proteins: Vacuolar protein sorting-associated protein 27 (Vps27p) (12), a neuronal heat shock protein (HSJ1A) (13), 26S proteosome subunit S3a (14), hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) (15), signal transducing adaptor molecule (STAM) (16), Epsin3 (17), and epidermal growth factor receptor pathway substrate 15 (Eps15) are examples (18). The UIM1-linker-UIM2 (RAP80 ULU) region of RAP80 is unique in that it is required for IR-induced foci formation, whereas the HJS1A UIM1-linker-UIM2 (HSJ1A ULU) region is not (7). This is due to point mutations in RAP80 UIM1 and 2 that prevent translocation to the DNA damage sites following IR treatment (7).

This RAP80 ULU region plays an important role in targeting

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the RAP80-CCDC98/Abraxas-BRCA1 complex to sites of DNA damage (7). Although convincing evidence has established that RAP80 is recruited to DNA breaks by binding to polyubiquitin chains through its UIMs, the regulation of this event in the cell remains obscure. Here, we demonstrate that the length of linker region connecting the tandem ubiquitin binding domains of RAP80 is a major regulator of specific binding with Lys63-linked polyubiquitin chains, and therefore regulates the translocation of RAP80 to sites of DNA damage.

RESULTS

RAP80 is known to translocate to sites of DNA damage following IR treatment, in contrast to the neuronal heat shock protein HSJ1A; The RAP80 ULU region could form DNA foci while the HSJ1A ULU region could not (7). Point mutations in RAP80 UIM1 or 2 prevented translocation to sites of DNA damage following IR treatment (7). These results generated the hypothesis that the RAP80 ULU region is critical factor for localization to sites of DNA damage. Therefore, RAP80 foci formation was checked using chimeric plamids in which either RAP80 UIM1 or 2 is exchanged with HSJ1A UIM1 or 2, respectively (RAP80-HSJ and HSJ-RAP80). The plasmid in which RAP80 UIM1 and 2 are removed (RAP80D1) was used as a negative control while a plasmid encoding wild-type RAP80 was used as a positive control (RAP80WT) (Fig. 1A). These plasmids were individually transfected into 293T cells, which 48 hours later were treated with 10 Gy of IR followed by immunofluorescence staining using anti-Flag and anti-γ-H2AX antibodies. Immunofluorescence imaging revealed that while RAP80WT and both chimeras (RAP80-HSJ and HSJ-RAP80) localized to nuclear foci following DNA damage, RAP80D1 failed to do so (Fig. 1B). These data therefore indicate that the presence of any tandem UIMs may be sufficient for the translocation of RAP80 to sites of DNA damage, but that that factors other tandem UIMs may contribute.

Next, we explored how the linker region connecting the tandem ubiquitin binding domains of RAP80 contributes to the translocation of RAP80 to double stranded DNA breaks. We constructed a series of plasmids in which the linker between the tandem ubiquitin binding domains of RAP80 are progressively deleted starting from the N-terminus (Fig. 2A). We tested the abilities of these mutant proteins to translocate to double-stranded DNA breaks by transfecting 293T cells with plasmids encoding HA-Flag-tagged RAP80 proteins with linkers of different lengths. Forty-eight hours after transfection, cells were treated with 10 Gy of IR followed by immunofluoresence staining using anti-Flag and anti-γ-H2AX antibodies. The results showed that only wild-type (RAP80WT) and mutants with more than two residues truncated from the

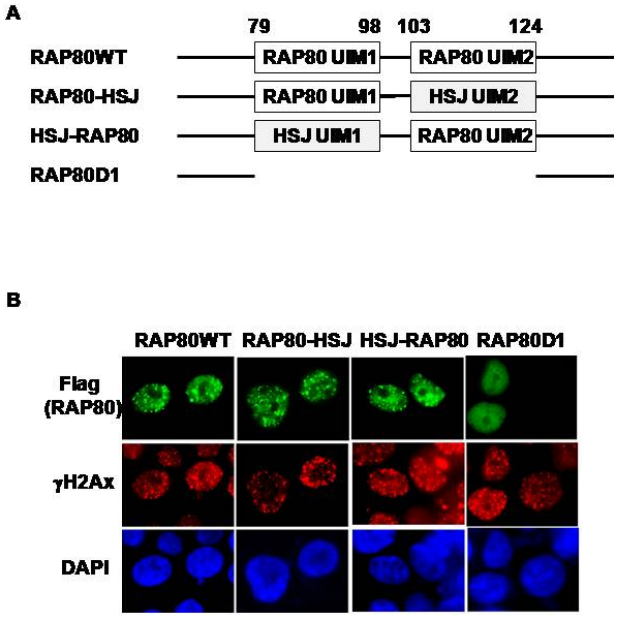

Fig. 1. Translocation of wild-type and RAP80-HSJ1A chimera mutants to the DNA damage sites. (A) Diagrams of the SFB-tagged wild type RAP80 and RAP80-HSJ1A chimera mutants. (B) DNA foci formation of the SFB-tagged wild type RAP80 (RAP80 WT), RAP80-HSJ and HSJ-RAP80 chimeras, as well as the UIM1 and 2 deleteion RAP80 mutants (RAP80 D1). Each expression plasmid was transfected into 293T cells. Forty-eight hours after transfection, cells were exposed to 10 Gy of IR. Cells were fixed 8 hours later and stained with Flag-specific monoclonal antibody and γ-H2AX-specific polyclonal antibody. DAPI staining indicates the nuclear region.

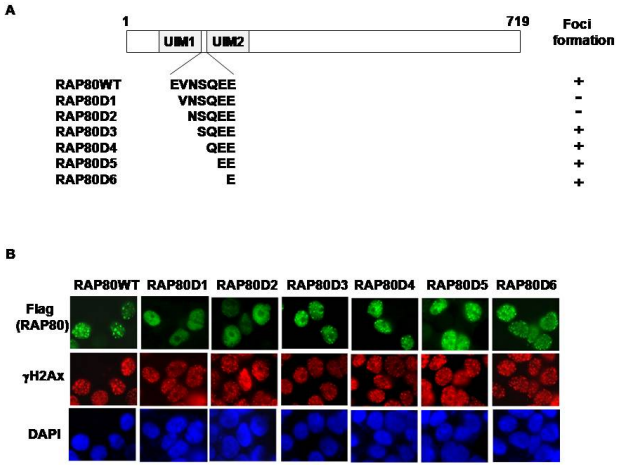

Fig. 2. The linker connecting the tandem ubiquitin binding domains of RAP80 is also important for localization to sites of DNA damage. (A) Diagrams of the HA-Flag-tagged wild type RAP80 and RAP80 UIM linker region deletion mutants. (B) DNA foci formation of the HA-Flag-tagged wild type RAP80 (RAP80 WT) and RAP80 UIM linker region deletion mutants (RAP80D1, 2, 3, 4, 5, or 6). Detection of DNA foci formation was performed the same as in Fig. 1.

N-terminus of the linker (RAP80D3 through RAP80D6) localized to nuclear foci following DNA damage, whereas other mutants (RAP80D1 and RAP80D2) failed to do so (Fig. 2B). These data confirm that the length and/or the sequence of the linker region are pivotal in the Lys63-linked polyubiquitin- dependent localization of RAP80 to DNA damage.

The polyubiquitin binding activity of RAP80 is a prerequisite for its localization to DNA damage. Therefore, we investigated by GST pull-down assay whether tandem UIMs of RAP80 with altered linkers bind to Lys63-linked polyubiquitin chains (Fig. 3). We prepared GST fusion constructs encoding RAP80 UIMs with progressively deleted linker regions (GST-UIMD1 through D6; Fig. 3A) as well as a plasmid encoding a linear Ub_4, which is thought to mimic the topology of Lys63-linked polyubiquitin (19). Bacterially expressed and purified Ub_4 were recognized by commercial anti-ubiquitin antibodies in a dose-dependent manner (Fig. 3B). We observed that Ub_4 specifically interacts with GST-UIMWT, -UIMD5 and -UIMD6, but not with GST-UIMD1 and -UIMD2, correlating with its ability to localize to damage-induced foci *in vivo*. This again suggests that the length and/or sequence of the linker connecting the two UIMs of RAP80 are critical for the linkage-specific polyubiquitin binding activity of RAP80.

To further study the functional role of the linker connecting the tandem ubiquitin binding domains of RAP80, we prepared GST fusion constructs encoding RAP80 UIMs with amino acids of the linker region progressively added (GST-UIMA1 through A9; Fig. 4A). The polyubiquitin binding activities of these additional linker mutants to linear Ub_4 were then monitored (Fig. 4A). Fig. 4B shows that linear Ub_4 interacts specifically with GST-UIMWT, -UIMA1, -UIMA2, -UIMA3, -UIMA4, -UIMA5, -UIMA7 and -UIMA8, but not with GST-UIMA6 and -UIMA9, suggesting that the additional amino acids contribute to the polyubiquitin binding activity of RAP80.

DISCUSSION

It is well known that the tandem repeat UIM region of RAP80 plays a key role in DNA foci formation following IR treatment by binding with K63 and K6-linked polyubiquitin chains (7, 8). In addition, the data showed that this process is compromised by the deletion and addition of amino acids to the linker connecting the tandem ubiquitin binding domains of RAP80. These results suggest that the linker region may also be critical for the translocation of RAP80 to sites of DNA damage and for binding to Lys63-linked polyubiquitin chains.

Recent research by Sims and Cohen has found that the deletion of RAP80 UIM1 or 2 reduces its affinity for Lys63-linked polyubiquitin chains (20). This complements our previous data showing that point mutations in RAP80 UIM1 or 2 prevent translocation to sites of DNA damage following IR treatment (7). Together, these two studies emphasize the importance of

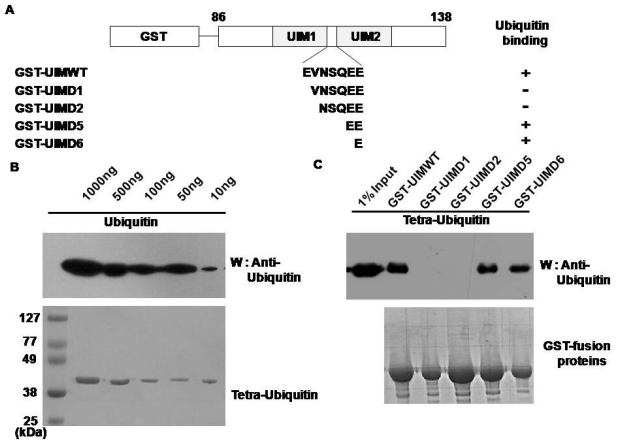

Fig. 3. Deletion of the linker connecting the tandem ubiquitin binding domains of RAP80 affects its binding to Ub_4. (A) Diagrams of GST-fused RAP80 wild type and the RAP80linker region deletion mutants. (B) Purification of Ub_4. The lower panel shows the coommassie staining and the upper panel shows the Western blotting analysis of purified Ub_4 using anti-ubiquitin antibody. (C) Binding of GST-RAP80 UIM or RAP80 UIM linker region deletion mutant fusion proteins (GST-UIMD1, 2, 5, and 6) to purified ubiquitin *in vitro*. Two μg of GST or GST-fusion proteins were incubated with 2 μg of purified ubiquitin for 2 hours 4°C. After extensive washing, bound ubiquitins were analyzed by immunoblotting with anti-ubiquitin antibody (upper panel). The amounts of GST fusion proteins are shown in the lower panel.

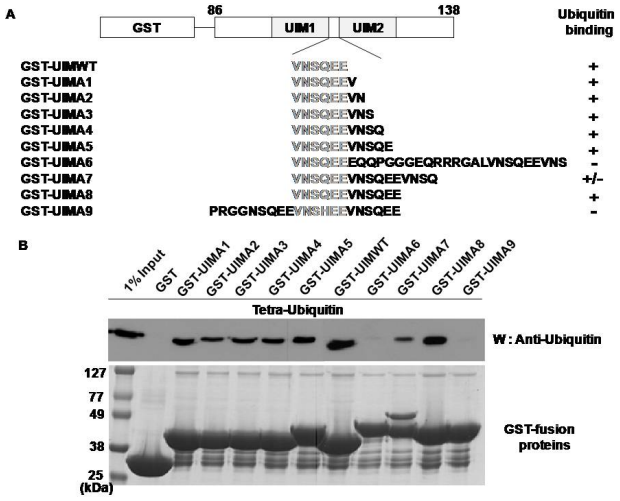

Fig. 4. Mutation of the linker region between the tandem ubiquitin binding domains of RAP80 affects its binding to Ub_4. (A) Diagrams of GST-fused RAP80 wild-type and additional RAP80 linker region mutants of. (B) Binding of GST-RAP80 UIM or RAP80 UIM linker region addition mutant fusion proteins (GST-UIMWT, -UIMA1-9) to purified Ub_4 *in vitro*. Two μg of GST or GST-fusion proteins were incubated with 2 μg of purified ubiquitin for 2 hours at 4°C. After extensive washing, bound Ub_4s were analyzed by immunoblotting with anti-ubiquitin antibody (upper panel). The amounts of GST fusion proteins are shown in the lower panel.

both UIM1 and UIM2 in the association of RAP80 with Lys63-linked polyubiquitin and in the translocation of RAP80 to DNA breaks following IR treatment. Interestingly, Fig. 1 in this study showed that wild-type RAP80, RAP80-HSJ1A and HSJ1A-RAP80 chimera mutants all localized to nuclear foci following DNA damage. Therefore, this data suggests that the presence of any tandem UIMs may be sufficient for RAP80 to translocate to sites of DNA damage, and that factors other than tandem UIMs may affect this process.

Our results in Figs. 3 and 4 are consistent with recent findings by Sims and Cohen (20) that found the linker connecting the tandem ubiquitin binding domains of RAP80, if modified, is altered in its binding to Lys63-linked polyubiquitin chains. These data suggest that the length of the linker region, including when altered by the deletion and addition of amino acids, may be critical for the association of RAP80 with Lys63-linked polyubiquitin chains, and that any modification may induce the linker to change structurally and therefore alter its affinity to Lys63-linked polyubiquitin chains. In addition, immunofluorescent assay showed that deletion of Glu or Glu-Val residues from the linker region is critical for translocation of RAP80 to DNA break sites (Fig. 2). It is also shown that the first and second amino acids of the linker are important for the association of RAP80 with Lys63-linked polyubiquitin chains as well as for translocation to sites of DNA damage. However, Sims and Cohen's data showed that replacing the linker region (REVNSQE) with 7 alanine residues does not affect binding affinity for Lys63-linked polyubiquitin chains (20), indicating that the length of the linker is more important than the amino acid composition.

Mutations in cell cycle checkpoint proteins often increase genomic instability and lead to tumor development. Since RAP80 is involved in controlling DNA damage-induced cell cycle checkpoints, it may be deregulated or mutated in human tumor tissue samples. Mutational studies focusing on the linker region connecting the RAP80 UIMs may provide additional tools to test this in the future.

MATERIALS AND METHODS

Plasmids

SFB (Streptavidin Binding Peptide)-tagged RAP80 WT and D1 expression plasmids were constructed as previously described (4, 7). RAP80-HSJ and HSJ-RAP80 chimera mutants were generated by PCR and subcloned into a modified pIRES-EGFP mammalian expression vector in order to create a S-Flag SBP-tagged expression plasmid. HA-Flag-tagged full-length RAP80 along with the UIM linker deletion and addition mutants were generated by PCR and subcloned into a HA-Flag tagged mammalian expression vector. The GST-fusion construct of RAP80-UIM was generated by PCR and subcloned into pGEX-4T-1 (Pharmacia), whereas various deletion and addition mutants of the RAP80 UIM linker region were subcloned into pGEX-4T-1 using a Quickchange site-directed mutagenesis kit (Stratagene).

Purification of the Ub_4

The gene encoding human Ub_4 was synthesized by a PCR-based method and was optimized for bacterial expression by DNAWorks (21). The synthesized Ub_4 gene contained BamHI and EcoRI restriction enzyme sites for later introduction into pGST-Parallel2 vector (22), resulting in the plasmid pGST-Ub_4. The GST-Ub_4 fusion protein was expressed in E. coli strain BL21 (DE3). Cells were grown at 37°C until reaching OD600∼0.5, induced by 1 mM IPTG and further grown at 20°C overnight. The cells were harvested and subsequently lysed by sonication. Cell lysates were centrifuged after which the supernatants were applied to glutathione-Sepharose resin (GE Healthcare) in buffer A (50 mM TrisHCl pH 7.5, 150 mM NaCl). After washing in buffer A, the Ub_4 was released from the resin by cleaving the fusion protein with tobacco etch virus protease. Released Ub_4 was further purified by size exclusion chromatography on a Sephadex-200 column (GE Healthcare) equilibrated with buffer A on an AKTA Prime-Plus system (GE Healthcare). Fractions containing Ub_4 were pooled and concentrated. Protein concentration was determined by measuring absorbance at 280 nm.

Cell culture and treatment with ionizing radiation

293T cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 (v/v). Cells were irradiated using JL Spepherd ^{137}Cs radiation at the indicated doses.

Antibodies and transfection

γ-H2AX antibodies were synthesized as previously described (7). Anti-Flag and anti-ubiquitin antibodies were obtained from Sigma Inc. A transient transfection was performed using Fugene 6 reagent (Roche. Inc) according to manufacturer's instructions.

GST pull-down assay

Two μg of GST-fusion protein were immobilized on glutathione-Sepharose 4B beads and incubated with 2 μg of ubiquitin in NETN buffer (0.5% Nonidet P-40, 20 mM Tris [pH 8.0], 50 mM NaCl, 50 mM NaF, 100 μM Na3VO4, 1 mM DTT and 50 μg/ml PMSF) for 2 hours at 4°C. After washing in NETN buffer, the samples were analyzed by Western blotting analysis.

Immunofluorescence staining

Cells grown on coverslips were fixed with 3% paraformaldehyde at room temperature for 15 min. Cells were then permeabilized with PBS containing 0.5% Triton X-100 at room temperature for 5 min and coverslips were blocked with PBS containing 5% goat serum at room temperature for 30 min. Coverslips were tnh incubated with primary antibodies at room temperature for 20 min. After washing with PBS, cells

were incubated with secondary antibodies, fluorescein isothio-cyanate-conjugated goat anti-mouse IgG, rhodamine- conjugated goat anti-rabbit IgG, or rhodamine-conjugated goat anti-mouse IgG (Jackson Immuno-Reserach Laboratories, Inc.) at room temperature for 20 min. 4, 6-diamidino-2-phenylindole (DAPI) was used to counterstain nuclei. Coverslips were washed a final time in PBS and then mounted with glycerin containing p-phenylenediamine. All images were obtained with a Nikon ECLIPSE E800 fluorescence microscope.

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