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Structural Basis for the Interaction of Mutasome Assembly Factor REV1 with Ubiquitin

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Abstract

REV1 is an evolutionarily conserved translesion synthesis (TLS) DNA polymerase and an assembly factor key for the recruitment of other TLS polymerases to DNA damage sites. REV1-mediated recognition of ubiquitin in the proliferative cell nuclear antigen is thought to be the trigger for TLS activation. Here we report the solution NMR structure of a 108-residue fragment of human REV1 encompassing the two putative ubiquitin-binding motifs UBM1 and UBM2 in complex with ubiquitin. While in mammals UBM1 and UBM2 are both required for optimal association of REV1 with replication factories after DNA damage, we show that only REV1 UBM2 binds ubiquitin. Structure-guided mutagenesis in *Saccharomyces cerevisiae* further highlights the importance of UBM2 for REV1-mediated mutagenesis and DNA damage tolerance.

Keywords

translesion synthesis; human REV1; ubiquitin; NMR spectroscopy

Introduction

Cells have evolved a DNA damage response that includes DNA damage tolerance mechanisms contributing to cell survival and to mutagenesis [1-3]. Translesion synthesis (TLS) is an evolutionarily conserved DNA damage tolerance pathway operating with specialized DNA polymerases that bypass bulky adducts or abasic sites in damaged DNA [2,4-6]. Unlike high-fidelity polymerases that stall at DNA lesions, TLS polymerases can add nucleotides to replicating strands opposite lesions, albeit with low fidelity, which leads to mutagenesis. How DNA replication switches to TLS at damaged sites is poorly understood. In eukaryotes, mono-ubiquitylation of the replication clamp proliferating cell nuclear antigen (PCNA) on Lys164 by the DNA damage-triggered RAD18-RAD6 ubiquitin ligases controls the TLS switch[7-9]. Mono-ubiquitylation of PCNA is thought to activate REV1, a scaffold factor critical for the recruitment and assembly of other TLS polymerases

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Accession Numbers

Coordinates have been deposited in the Protein Data Bank with accession number 5VZM. NMR chemical shift assignments have been deposited in the Biological Magnetic Resonance Data Bank with accession number 30300.

Conflict of Interest

The authors declare no conflict of interest.

at DNA damage sites, which thereby permits bypass of a variety of lesions [10-25] and of replication disturbances in the absence of DNA damage [26]. Because of its function in TLS and associated mutagenesis, REV1 has been referred to as a mutasome assembly factor [27]. In addition to being a general activator of TLS, REV1 is a TLS polymerase that incorporates dCMP opposite dGMP or apurinic/apyrimidinic sites in DNA [28,29]. The enzymology of yeast and human REV1 is well understood from kinetic characterization and from the 3D structures of the catalytic domain of REV1 bound to lesion-bearing DNA [30-35]. REV1 also likely plays a role in post-replication DNA repair [36].

Activation of REV1 and TLS by ubiquitylation of PCNA likely involves direct recognition of ubiquitin in the context of ubiquitylated PCNA (PCNA^{ub}) [13, 37-39]. Indeed, REV1 harbors two putative C-terminal ubiquitin-binding motifs (UBMs) [37,40]. Similar UBMs were also identified in TLS polymerase ι (Pol ι) [39,41,42]. To better understand the mechanism of ubiquitin recognition by REV1 and of TLS activation, we used NMR spectroscopy to probe the interaction of ubiquitin with a 108-residue segment of human REV1 (residues 932–1039) encompassing the predicted UBM1 and UBM2 domains.

Results and Discussion

The 108-residue apo REV1 fragment (hereafter called REV1) was mostly disordered based on NMR chemical shift values [43], with the exception of predicted helical conformations that matched the UBM1 and UBM2 regions (Fig. 1a). Marked changes in the ^1H – ^{15}N heteronuclear single quantum coherence (HSQC) spectrum of REV1 after titration with ubiquitin were only detected for the UBM2 region indicating that UBM2, but not UBM1, contacted ubiquitin (Fig. 1b, c). Similarly, in the case of *Saccharomyces cerevisiae* REV1, only UBM2 bound ubiquitin [39]. The exchange between free and ubiquitin-bound human REV1 was fast to intermediate on the timescale of NMR chemical shifts allowing the dissociation constant (K_d) to be estimated. The NMR spectroscopy-derived K_d is $\sim 27\ \mu\text{M}$ (Fig. 2a), which is of the same order of magnitude as the isothermal titration calorimetry (ITC)-derived K_d of $\sim 49\ \mu\text{M}$ (Fig. 2b). The ITC stoichiometry number n is close to unity ($n = \sim 0.9$), consistent with only one ubiquitin molecule interacting with REV1 (Fig. 2b).

Using NMR spectroscopy-derived interatomic distances and dihedral angles, we determined the 3D structure of REV1 bound to ubiquitin (Figs. 2c and 3a). The structure calculation statistics are presented in Table 1. In the structure, the most ordered region of REV1 spans residues Ile1006 to Arg1038, corresponding to the UBM2 domain (Fig. 3a). On the basis of TALOS+ angle predictions [43], in the calculated structure, there are also short helical motifs in the region corresponding to REV1 UBM1 (data not shown). REV1 UBM2 is composed of an N-terminal loop region (Ile1007–Val1015) followed by two α -helices separated by a leucine and *trans*-proline residues (Leu1023–Pro1024) (Fig. 3b). The *trans* conformation is supported by predictions done on the basis of backbone chemical shift values and amino acid sequence using the program Promega [44]. The first α -helix ($\alpha 1$, Asp1016–Ala1022) consists of hydrophobic residues with the exception of Asp1016 and Glu1018. The helix is stabilized by a canonical N-cap motif (Asp1016–Pro1017) [45] with the carboxyl group of Asp1016 forming hydrogen bonds with the amide groups of Glu1018 and Val1019. The second α -helix ($\alpha 2$, Ala1025–Arg1038) is amphipathic. The two α -

helices are at an angle of $43^\circ \pm 4^\circ$ relative to one another. The overall UBM2 fold resembles a triangle with one site formed by the long-range interaction of Leu1009 with Ala1034. UBM2 contacts the surface of ubiquitin through hydrophobic residues from the N-terminal loop, the two α -helices, and the Leu1023–Pro1024 intervening motif (Fig. 3c). The UBM2 triangle is centered on ubiquitin Leu8, which has intermolecular contacts with Val1015, Val1019, Phe1020, Leu1023, and Leu1031. Other interfacial residues are ubiquitin Ile44, which contacts Pro1024 and Leu1027; ubiquitin His68, which contacts Val1019 and Leu1023; ubiquitin Val70, which contacts Leu1027; and ubiquitin Leu73, which contacts Leu1031, Glu1030, and Leu1009. In addition, ubiquitin Arg42 is favorably positioned to form a salt bridge with Glu1030.

There is no interaction between REV1 UBM1 and ubiquitin (Fig. 1c), which can be tentatively explained by a number of structural differences between UBM1 and UBM2 in human and *S. cerevisiae* REV1 (Fig. 4a). The putative helix α 1 may be less stable in REV1 UBM1 than in UBM2 as it lacks the N-terminal Asp-Pro capping motif. Yeast UBM2 also lacks this motif (Fig. 4a), but the negatively charged Glu-Glu sequence replacing Asp-Pro is expected to stabilize the helix dipole. In human UBM1, a proline residue follows the inter-helical Leu-Pro linker motif and may change the orientation of helix α 2 in comparison to UBM2. Human UBM1 also lacks the glutamate residue (Glu1030 in UBM2) involved in a salt bridge with ubiquitin Arg42 (Fig. 4a).

The key interacting residues in the structure of REV1 UBM2 bound to ubiquitin are conserved in the Pol η UBM2–ubiquitin structure [39,41,42] and are involved in the same intermolecular contacts in the two complexes. Conserved residues are as follows for REV1/Pol η : Val1019/Val687, Phe1020/Phe688, Leu1023/Leu691, Pro1024/Pro692, Leu1027/Val695, and Glu1030/Glu698. The helix-stabilizing N-cap motif is also conserved in Pol η (Asp1016–Pro1017/Asp684–Pro685). As expected, REV1 UBM2 and Pol η UBM2 have similar ubiquitin binding modes where ubiquitin Leu8 is at the center of the binding interface. The angle between the two UBM2 α -helices is a little larger in Pol η ($52^\circ \pm 2^\circ$) and the domain is slightly shifted relative to ubiquitin compared to REV1 (Fig. 3d). The difference between the two complexes is most pronounced toward the C-terminal end of the second α -helix of UBM2 including residues Leu699 and Trp703 in Pol η , corresponding to Leu1031 and Tyr1035 in REV1, and contact residue Leu73 at the C-terminus of ubiquitin (Fig. 3d). It is, however, not possible to conclude that these differences are significant owing to the experimental uncertainties inherent to NMR protein structure determination.

Our *in vitro* studies identifying UBM2, and not UBM1, as a ubiquitin binding domain in human REV1 parallels previous work with *S. cerevisiae* where only REV1 UBM2 was found to bind ubiquitin and to be necessary for REV1-mediated mutagenesis [18,38–40]. In mammals, however, UBM1 and UBM2 are both required for optimal association of REV1 with replication factories in UV-irradiated cells [40]. The function of UBM1 remains unknown. Because the amino acid sequences of REV1 UBM2 are well conserved in *S. cerevisiae* and human (Fig. 4a), guided by the structure of human REV1 UBM2, we introduced mutations in yeast REV1 at the UBM2–ubiquitin interface to further probe the importance of UBM2 ubiquitin-binding function on UV-induced mutagenesis. We mutated to alanine Phe817 (Val1019 in human), Leu821 (Leu1023 in human), Ile825 (Leu1027 in

human), and Val829 (Leu1031 in human). Using an established assay, we examined the frequency of UV-induced *CANI^S* to *canI^r* forward mutations in an *S. cerevisiae rev1* strain carrying the wild-type or mutated *REV1* gene in a plasmid [46]. The rate of UV-induced mutagenesis was markedly reduced for cells expressing any of the four REV1 mutants, the most defective being F817A and L821A (Fig. 4b). The defects were statistically significant for all mutants (with *P* values less than 0.05). The corresponding amino acids in human REV1 (Val1019 and Leu1023) are both in the vicinity of Ile8 and His68, two residues at the center of the binding interface in ubiquitin. As a control, we verified that the G193R mutation, which unfolds the BRCA1 C-terminus (BRCT) domain in *S. cerevisiae* REV1, dramatically reduced UV-induced mutagenesis [10,38,47]. In parallel to investigating the mutagenesis frequency, we analyzed the survival of UV-induced cells. In comparison to UV-irradiated yeast cells expressing wild-type REV1, cell survival was decreased when REV1 was mutated in the UBM2 domain, with the strongest effect observed for the F817A and L821A mutants (Fig. 4b). Decrease in cell survival was statistically significant for G193R, F817A, L821A, and F825A (*P* values less than 0.05), but not for the V829A mutant. A similar trend in cell survival was obtained after exposing yeast cells expressing wild-type or UBM2-mutated REV1 to DNA damaging agent methyl methanesulfonate (MMS) (Fig. 4c).

Taken together, our structural and functional studies further highlight the key role of REV1 UBM2 in TLS. Nonetheless, the mechanism by which REV1 activates TLS remains unclear. For example, REV1 is thought to constitutively associate with PCNA independently of PCNA ubiquitylation at Lys164, but what drives this interaction is unknown and what role PCNA ubiquitylation would play in an already pre-formed REV1-PCNA complex is puzzling. REV1 does not harbor any PCNA-interacting peptide box, a motif found in virtually all other PCNA-binding proteins. It was suggested that the N-terminal BRCT domain of REV1 could mediate the interaction with PCNA [48,49], while other studies hinted that the N-terminal region of REV1, which includes the BRCT domain, bound DNA and not PCNA [50]. It was also reported that PCNA interactions require the REV1 C-terminal region [51] or catalytic core [52]. Future structural studies in the context of full-length REV1 will be required to understand how PCNA ubiquitylation and UBM2-mediated recognition of PCNA^{ub} triggers the activation of REV1. Much more work remains to be done to understand the mode of action of this fascinating protein.

Materials and Methods

Protein preparation

The REV1 (932–1039) construct included an N-terminal His₆ tag cleavable by TEV protease. Two ubiquitin constructs were used, one without a tag and another with an N-terminal non-cleavable His₆ tag. The proteins were purified using previously reported protocols [41,53].

NMR spectroscopy and structure determination

The NMR spectra were recorded using a Bruker Avance III 700 MHz NMR spectrometer equipped with a cryoprobe. The proteins were in 20 mM sodium phosphate buffer (pH 7.0), 30 mM NaCl, and 90% H₂O/10% D₂O. The NMR measurements were done at 30 °C using

samples of the REV1–ubiquitin complex composed of $^{15}\text{N}/^{13}\text{C}$ -labeled REV1 at 1 mM and unlabeled ubiquitin at 3 mM, and $^{15}\text{N}/^{13}\text{C}$ -labeled ubiquitin at 1 mM and unlabeled REV1 at 3 mM. A series of standard 2D and 3D spectra were recorded to generate the data required for structure determination [41,54,55]. For structure determination, nuclear Overhauser enhancement (NOE)-based restraints were categorized into distance ranges with upper limits of 2.8, 3.5, 4.0, 4.5, 5.0, and 5.5 Å. For very weak signals, an upper limit of 7.5 Å was used to take into account possible spin diffusion. Backbone hydrogen bond distance restraints were included in the calculations on the basis of amide $^1\text{H}/^2\text{H}$ exchange measurements. The upper limit was set to 3.2 Å for the distance between N and O, and 2.2 Å for the distance between HN and O. Dihedral angle restraints ϕ and ψ were used in the calculations with a 30° tolerance based on TALOS+ predictions [43]. The NMR spectra were processed using NMRPipe [56] and analyzed using NMRView [57]. The structures were calculated with CYANA 2.1 [58] and refined with AMBER 8 [59] using previously reported protocols [41,60].

ITC

The ITC measurements were done at 10 °C in 50 mM Tris–HCl (pH 7.0) and 30 mM NaCl using a VP-ITC instrument (Malvern MicroCal). Ubiquitin was in the injection syringe at a concentration of 1.5 mM. REV1 was in the calorimeter cell at an initial concentration of 100 μM .

UV irradiation-induced mutagenesis and cell survival in *S. cerevisiae*

To study the effect of single-point mutations in *S. cerevisiae* REV1 on cell survival and mutagenesis after UV irradiation, we used a *rev1*[−] strain derived from the EMY74.7 (*MATa* his3–1 leu2–3 leu2–112 ura3–52) to generate YREV1.14 in which deletion of the *REV1* gene is marked by the URA3+ gene [46]. Wild-type and mutant REV1 proteins were expressed from plasmid pVP21, which carries the *LEU2* gene [46]. Yeast cells were cultured as previously reported [46]. To probe cell viability following UV irradiation, serial dilutions of yeast cultures were spread on plates prepared with synthetic defined (SD) media lacking leucine (SD – leu) for plasmid selection. The frequencies of *CAN1*^S to *can1*^r mutations caused by UV exposure were determined by plating the cells on SD media lacking leucine and arginine, but containing canavanine (SD – leu – arg + can). The plates were UV-irradiated at 125, 150, 175, and 200 J/m², using a Stratalinker 2400 irradiator (Stratagene) operated in energy mode. Plates were incubated in the dark at 30 °C for 3 to 5 days. Three independent experiments were conducted with the assays done in triplicate. The cell survival and *CAN1*^S to *can1*^r mutation assays were done in parallel. The *P* values were calculated using the multiple-comparisons Dunnett's test implemented in GraphPad Prism 6.

To test the viability of yeast cells expressing wild-type or mutated REV1 in the presence of MMS, the cells were spotted onto SD – leu media prepared without and with MMS. Plates were incubated in the dark at 30 °C for 3 to 5 days.

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Abbreviations used:

BRCT	BRCA1 C-terminus
HSQC	heteronuclear single quantum coherence
ITC	isothermal titration calorimetry
PCNA	proliferating cell nuclear antigen
TLS	translesion synthesis
UBM	ubiquitin-binding motif

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Fig. 1. Interaction of human REV1 (932–1039) with ubiquitin. (a) Prediction of α -helical (red) and β -sheet (blue) regions in apo REV1 (top) and in REV1 titrated with threefold molar excess ubiquitin (bottom) based on TALOS+ analysis of $\text{C}\alpha$, $\text{C}\beta$, C' , HN and N NMR chemical shifts [43]. The UBM1 and UBM2 regions are shown in orange. (b) Chemical shift perturbations in the ^1H - ^{15}N HSQC spectrum of ^{15}N -labeled REV1 (1 mM concentration) caused by addition of threefold molar excess of unlabeled ubiquitin. The normalized chemical shift changes δ in parts per million (ppm) for the ^1H - ^{15}N correlation signals are defined as $\Delta\delta = \sqrt{(\Delta\delta_{\text{H}})^2 + (\Delta\delta_{\text{N}}/5)^2}$. (c) Overlay of the ^1H - ^{15}N HSQC spectra of ^{15}N -labeled REV1 at 0.5 mM concentration before and after addition of threefold molar excess of unlabeled ubiquitin. (d) Overlay of the ^1H - ^{15}N HSQC spectra of ^{15}N -labeled ubiquitin at 0.3 mM concentration before and after addition of 5-fold molar excess of unlabeled REV1.

Fig. 2.

Estimation of the binding affinity of human REV1 (932–1039) for ubiquitin. (a) NMR spectroscopy-based titration of ^{15}N -labeled REV1 with non-labeled ubiquitin. REV1 was at initial concentration of 0.5 mM. Multiple apparent K_d values were determined from the changes in chemical shifts for five REV1 residues by nonlinear least-squares analysis using

$$\text{equation: } \Delta\delta = \Delta\delta_{\max} \left(\frac{[L] + [P] + K_d - \left([L] + [P] + K_d \right)^2 - 4[P][L]}{2[P]} \right) \text{ where } [L] \text{ is the concentration}$$

of ubiquitin, $[P]$ is the concentration of REV1, δ is the observed normalized chemical shift change as defined in Fig. 1b, and $\delta_{\max}$ is the normalized chemical shift change at

saturation. (b) ITC of REV1 with ubiquitin. The integrated heat measurements from raw titration data and curve fitting using a one-site binding model are shown. The K_d and stoichiometry (n) values are indicated with the standard deviations determined by nonlinear least-squares fitting. (c) Representative intermolecular NOE signals identified in F1 ^{13}C -filtered, F2 ^{13}C -edited NOESY-HSQC spectra. The resonance labels are colored orange for REV1 and blue for ubiquitin.

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Fig. 3.

Three-dimensional structure of human REV1 (932–1039) in complex with ubiquitin. (a) Cartoon representation of the overlay of 20 REV1–ubiquitin complex structures representing the NMR ensemble (left) and a cartoon representation (right) of REV1 UBM2 in complex with ubiquitin. While structure calculations were done with REV1 (932–1039), the N-terminal disordered region is not shown. Only REV1 (1000–1039) is shown. (b) Cartoon and stick representation of the UBM2 structure highlighting negatively charged (blue), positively charged (red), neutral (orange), and hydrophobic (yellow) amino acid side chains. (c)

Cartoon and stick representation of the REV1 UBM2–ubiquitin complex. Selected amino acid side chains at the binding interface are shown and labeled. (d) Overlay of the ubiquitin structures in the REV1 UBM2–ubiquitin and Pol η UBM2–ubiquitin (PDB accession number 2KTF) complexes. Ubiquitin bound to REV1 is shown in blue, while ubiquitin bound to Pol η is shown in gray.

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Fig. 4.

Effects of single-point mutations in REV1 UBM2 using *S. cerevisiae* as a model system. (a) Amino acid sequences of human and *S. cerevisiae* REV1 UBM2 and UBM1 regions. Similar and identical residues are highlighted in blue and yellow, respectively. (b) Frequencies of mutagenesis (top) and cell viability (bottom) following UV irradiation of yeast cells expressing wild-type or mutated REV1. (c) Viability of yeast cells expressing wild-type or mutated REV1 in presence of different concentrations of MMS.

Table 1.

NMR and refinement statistics for human REV1 (932–1039)–ubiquitin

NMR distance and dihedral angle restraints ^a	
Distance restraints	
Total NOE	5903
Intra-residues	902
Inter-residues	
Sequential ($ i-j = 1$)	851
Medium ($ i-j < 5$)	606
Long-range ($ i-j > 4$)	995
Ambiguous	2379
Inter-molecular	170
Hydrogen bond restraints	57
Total dihedral angle restraints	183
φ	90
ψ	93
Structure statistics	
Violations (mean $\pm$ SD)	
Distance restraints (Å)	0.11 $\pm$ 0.03
Dihedral angle restraints (°)	2.51 $\pm$ 0.75
Max. dihedral angle violation (°)	3.40 $\pm$ 0.46
Max. distance violation (Å)	0.22 $\pm$ 0.01
Deviations from idealized geometry	
Bond lengths (Å)	0.0086 $\pm$ 0.0001
Bond angles (°)	2.09 $\pm$ 0.02
Impropers (°)	0.22 $\pm$ 0.01
Average pairwise r.m.s. deviation (Å) ^b	
Heavy	1.00 $\pm$ 0.11
Backbone	0.35 $\pm$ 0.08
Ramachandran plot (%) ^b	
Most favored	91.1
Additionally allowed	8.6
Generously allowed	0.3
Disallowed	0

^aThe number of NOEs corresponds to the non-redundant distance restraints generated from all NOESY spectra.

^bThe r.m.s. deviations and Ramachandran plot parameters were calculated for residues REV1 (1009–1039) and ubiquitin (1–73) using an ensemble of 20 structures.