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The C-terminal domain of human Rev1 contains independent binding sites for DNA polymerase η and Rev7 subunit of polymerase ζ

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ABSTRACT

Human Rev1 is a translesion synthesis (TLS) DNA polymerase involved in bypass replication across sites of DNA damage and postreplicational gap-filling. Rev1 plays an essential structural role in TLS by providing a binding platform for other TLS polymerases that insert nucleotides across DNA lesions (polη, polι, polκ) and extend the distorted primer-terminus (polζ). We use NMR spectroscopy to demonstrate that the Rev1 C-terminal domain utilizes independent interaction interfaces to simultaneously bind a fragment of the 'inserter' polη and Rev7 subunit of the 'extender' polζ, thereby serving as a cassette that may accommodate several polymerases making them instantaneously available for TLS.

Structured summary of protein interactions:
REV1, **REV3** and **REV7** physically interact by nuclear magnetic resonance (View interaction), molecular sieving (View interaction) and isothermal titration calorimetry (View interaction).
REV3 and **REV7** bind by molecular sieving (View interaction)
REV1 and **Polη-RIR peptide** bind by nuclear magnetic resonance (View interaction)
REV1, **REV3**, **REV7** and **Polη-RIR peptide** physically interact by nuclear magnetic resonance (View interaction).

1. Introduction

Sites of DNA damage (lesions) encountered by replication machinery in the process of cell division stall replication fork progression, threatening the survival of dividing cells. DNA damage tolerance pathways provide the means to bypass DNA lesions while temporarily leaving them unrepaired, thus rescuing replication from coming to a halt [1,2]. Bypass DNA replication in human cells is carried out by low-fidelity translesion synthesis (TLS) polymerases, including Rev1, polη, polι, polκ (Y-type) and polζ (B-type), which constitute a mutagenic branch of DNA damage tolerance [1,2]. Recent reports suggest that TLS enzymes also play a role in postreplicational gap filling [3]. TLS polymerases represent the primary source of mutations introduced into the genome, with Rev1 and polζ responsible for most damage-induced and spontaneous mutagenesis [4].

Rev1/polζ-dependent TLS allows bypass replication across bulky DNA lesions such as N^2-benzo[*a*]pyrene-dG and cisplatin adducts, or [4–6] photoproduct, involving the concerted action of several TLS polymerases [5–8]. In this process the 'inserter' TLS enzyme replaces the replicative DNA polymerase (polδ, polε) at stalled replication fork in response to Rad6/Rad18-dependent mono-ubiquitination of PCNA [9] and inserts a nucleotide across from the lesion. Subsequently, the 'inserter' enzyme is replaced by 'extender' TLS polymerase (most frequently polζ) capable of extending the distorted DNA primer terminus [5,6]. The recruitment of TLS polymerases to sites of DNA damage and polymerase switching upon DNA lesion bypass are mediated by a variety of protein–protein interactions [2]. In spite of their major role in TLS, the key interactions of TLS polymerases that govern the selection of lesion-specific enzymes, and determine the order of polymerase switching events are not yet structurally characterized.

Besides exhibiting limited catalytic activity, Rev1 serves as an interaction platform for other major TLS polymerases, thereby playing a key role in coordinating the TLS pathway [2]. Unlike other Y-type polymerases, Rev1 possesses two unique interaction domains: an N-terminal BRCT domain that binds PCNA and a C-terminal domain (Rev1-CT) responsible for Rev1 interaction with major TLS polymerases [2]. Specifically, in vertebrates the Rev1-CT

Abbreviations: TLS, translesion synthesis; Rev1-CT, C-terminal domain of Rev1; RIR, Rev1 interacting region; NMR, nuclear magnetic resonance; TROSY, transverse relaxation optimized spectroscopy; ITC, isothermal titration calorimetry; aa, amino acid

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domain, encompassing the C-terminal ~100aa of Rev1, is required for binding polη, polι, polκ and Rev7 subunit of polζ [2,10]. Considerable evidence suggests that Rev1 and polζ (a complex of accessory Rev7 and catalytic Rev3 subunits) act together in mutagenic DNA lesion bypass [4,8]. Thus, cells deficient in Rev1, Rev3 or Rev7 proteins exhibit increased sensitivity to DNA damage and substantially reduced rate of mutations [4]. It is notable that Rev1 mutants lacking the C-terminal domain fail to complement Rev1 deficiency [8], suggesting that protein–protein interactions mediated by Rev1-CT are critical for Rev1/polζ-dependent TLS.

Recently, we have determined the solution NMR structures of human Rev1-CT domain (residues 1157–1251) alone and in complex with one of the two Rev1-interacting regions (RIRs) from polη (residues 524–539) (PDB: 2LSY,2LSK) [11]. The domain adopts a four-helix bundle fold, with the binding site for polη-RIR formed by the N-terminal β-hairpin and the following two α-helices. Human Y-type polymerases polη, polι, polκ contain RIR-motifs including two consecutive Phe residues that fit into a hydrophobic pocket in the N-terminal part of Rev1-CT [11,12], thus utilizing a similar mode for Rev1-CT binding. Moreover, recently reported spatial structure of mouse Rev1-CT in complex with polκ-RIR [13] revealed that polη and polκ utilize identical modes of Rev1-CT binding, suggesting that RIR-motifs of Y-type polymerases are likely to compete for the same binding site on the Rev1-CT domain.

In contrast, no RIR-motifs have been identified in Rev7 subunit of polζ, indicating that its mode of Rev1-CT interaction is different from that of polη, polι and polκ. The crystal structure of human Rev7 (R124A; PDB: 3ABD,3ABE) has been determined in complex with a peptide from the catalytic subunit of polζ, Rev3 (residues 1847–1898), and several residues of Rev7 involved in Rev1-CT interaction have been identified by mutational analysis [14]. This analysis revealed that Rev7 interacts with Rev1-CT and Rev3 through different interfaces and, therefore, can bind Rev1 and Rev3 simultaneously. Here we use NMR spectroscopy (i) to map previously undefined Rev1-CT binding site for Rev7 subunit of polζ, (ii) to show that this site is distinct from Rev1-CT binding interface for RIR-motifs of polη, ι, κ, and (iii) to demonstrate the possibility of assembling quadruple Rev1-CT/(Rev7/Rev3)/polη-RIR complex where Rev1-CT is bound to the fragments of polη and polζ. These results infer a possibility that Rev1-CT may serve as a scaffold that simultaneously binds one of the 'inserter' Y-type polymerases, polη, ι or κ, and the 'extender' polζ, thus facilitating the assembly of multi-polymerase complexes carrying out TLS.

2. Materials and methods

2.1. Protein expression and purification

$^{15}N/^{13}C/^{2}H$-labeled human Rev1-CT (11 kDa) was produced as described elsewhere [11]. In brief, the protein was over-expressed in *E. coli* BL21(DE3) cells transformed with pET-28b(+) vector harboring the Rev1-CT gene (residues 1158–1251 following cleavage site for TEV protease). Cells were grown in 100% $^{2}H_2O$ M9 medium using $^{15}NH_4Cl$ and U-$^{13}C/^{2}H$-glucose as the sole nitrogen and carbon sources, respectively, followed by protein purification by Ni^{2+}-affinity column, His-tag cleavage, and purification on a HiLoad Superdex-75 column.

The triple complex of $^{15}N/^{13}C/^{2}H$ Rev1-CT(1158–1251) and unlabeled Rev7(R124A)/Rev3(1847–1898) (below referred to as Rev7/Rev3) was obtained by HiLoad Superdex-200 size-exclusion purification of a mixture of the Rev1-CT (11 kDa) and an excess of Rev7/Rev3 (32 kDa), with subsequent collection of a peak corresponding to Rev1-CT/Rev7/Rev3 assembly (43 kDa). His-tagged Rev7(R124A) was co-expressed with Rev3(1847–1898) in *E. coli* BL21(DE3) cells transformed with pETDuet-1 vector harboring genes encoding both proteins [14]. Cells were grown on LB medium, followed by protein purification using Ni^{2+}-affinity and HiLoad Superdex-200 columns.

The quadruple complex of $^{15}N/^{13}C/^{2}H$ Rev1-CT(1158–1251), unlabeled Rev7(R124A)/Rev3(1847–1898), and unlabeled polη-RIR peptide (residues 524–539) was obtained by gradually titrating a concentrated 25 mM solution of custom synthesized (GenScript) peptide into Rev1-CT/Rev7/Rev3 sample. The final samples of triple Rev1-CT/Rev7/Rev3 (43 kDa) and quadruple Rev1-CT/Rev7/Rev3/polη-RIR (44 kDa) complexes contained 0.4 mM protein, 50 mM NaH_2PO_4, 100 mM NaCl, 1 mM EDTA, 5 mM DTT, 0.05% NaN_3, 10% D_2O, pH = 7.0.

2.2. NMR spectroscopy

Nearly complete $^{1}H/^{15}N/^{13}C$ NMR resonance assignments for the free Rev1-CT and Rev1-CT/polη-RIR complex (15 °C) were reported elsewhere (BMRB: 18455,18434) [11]. The backbone $^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$ and $^{13}C^{O}$ assignments (35 °C) for $^{15}N/^{13}C/^{2}H$ Rev1-CT in complex with unlabeled (i) Rev7/Rev3 and (ii) Rev7/Rev3/polη-RIR were obtained in this work from a series of 2D $^{1}H–^{15}N$ HSQC, $^{1}H–^{15}N$ TROSY, and 3D TROSY-HNCO, TROSY-HNCA and ^{15}N-edited NOESY-HSQC spectra [15,16] recorded on 18.8T Agilent VNMRS spectrometer equipped with cold-probe.

The residues on Rev1-CT interaction interfaces with Rev7/Rev3 and polη-RIR were identified based on chemical shift changes for the backbone nuclei upon the formation of the complexes. Specifically, normalized chemical shift differences were calculated for each residue [17]:

$$\Delta\varpi' = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(\frac{\Delta\varpi_i}{\varpi_{std,i}}\right)^2} \quad (1)$$

where index i runs over $N = 4$ nuclei used for the comparison: $^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$ and $^{13}C^{O}$, $\Delta\varpi_i$ is chemical shift change for a nucleus i, $\varpi_{std,i}$ is nucleus/residue specific normalization value equal to 1 standard deviation of chemical shift distribution in BMRB database (www.bmrb.wisc.edu). The chemical shift differences, $\Delta\varpi'$, between (i) Rev1-CT/polη-RIR complex and the free Rev1-CT (15 °C), and between (ii) Rev1-CT/Rev7/Rev3/polη-RIR and Rev1-CT/Rev7/Rev3 complexes (35 °C) were used to assess Rev1-CT – polη-RIR binding, while $\Delta\varpi'$ between (iii) Rev1-CT/Rev7/Rev3 complex and an isolated Rev1-CT were used to map the Rev1-CT – Rev7 interaction interface. For the later comparison we used $^{1}H^{N}$ and ^{15}N chemical shifts obtained at 20 °C, assigned from a temperature series of $^{1}H–^{15}N$ HSQC spectra recorded for the free domain and the complex, and $^{13}C^{\alpha}$ and $^{13}C^{O}$ shifts obtained at 15 °C (free Rev1-CT) and 35 °C (Rev1-CT/Rev7/Rev3). Note that at temperatures above 20–25 °C spectra of the free Rev1-CT deteriorate due to μs-ms exchange line-broadening [11].

^{15}N R_1 and R_2 relaxation measurements for the $^{15}N/^{13}C/^{2}H$ Rev1-CT in complex with Rev7/Rev3 and Rev7/Rev3/polη-RIR (35 °C, 18.8T) were performed using the pulse-sequences of Farrow et al. [18]. Overall rotation correlation times, τ_R, for the complexes were calculated from ^{15}N R_2/R_1 ratios for residues from regular secondary structure elements of Rev1-CT (α-helices H1-H4), and were used to monitor the change in molecular size upon the formation of the complexes. ^{15}N relaxation measurements (15 °C, 11.7T) and the analysis of molecular overall rotation for the free Rev1-CT and Rev1-CT/polη-RIR complex were reported previously [11].

2.3. Isothermal titration calorimetry

Rev1-CT - Rev7/Rev3 binding was additionally assessed by ITC performed at 30 °C using nano-ITC low-volume unit (TA Instru-