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XRCC1 interaction with the REV1 C-terminal domain suggests a role in post replication repair

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Abstract

The function of X-ray cross complementing group 1 protein (XRCC1), a scaffold that binds to DNA repair enzymes involved in single-strand break and base excision repair, requires that it be recruited to sites of damaged DNA. However, structural insights into this recruitment are currently limited. Sequence analysis of the first unstructured linker domain of XRCC1 identifies a segment consistent with a possible REV1 interacting region (RIR) motif. The RIR motif is present in translesion polymerases that can be recruited to the pol ζ /REV1 DNA repair complex via a specific interaction with the REV1 C-terminal domain. NMR and fluorescence titration studies were performed on XRCC1-derived peptides containing this putative RIR motif in order to evaluate the binding affinity for the REV1 C-terminal domain. These studies demonstrate an interaction of the XRCC1-derived peptide with the human REV1 C-terminal domain characterized by dissociation constants in the low micromolar range. Ligand competition studies comparing the X1 RIR peptide with previously studied RIR peptides were found to be inconsistent with the NMR based K_d values. These discrepancies were resolved using a fluorescence assay for which the RIR – REV1 system is particularly well suited. The structure of a REV1-XRCC1 peptide complex was determined by using NOE restraints to dock the unlabeled XRCC1 peptide with a labeled REV1 C-terminal domain. The structure is generally homologous with previously determined complexes with the pol κ and pol η RIR peptides, although the helical segment in XRCC1 is shorter than was observed in these cases. These studies suggest the possible involvement of XRCC1 and its associated repair factors in post replication repair.

Keywords

XRCC1; REV1 C-terminal domain; post replication repair; REV1 interacting Region; NMR spectroscopy

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Introduction

DNA repair is generally a multistep process requiring the coordinated activities of multiple enzymes. This coordination is often achieved via the intermediacy of protein scaffolds that are characterized by both constitutive and conditional interactions with the individual repair enzymes. One such scaffold, X-ray cross complementing group 1 protein (XRCC1), consists of three globular domains: an N-terminal, pol β -binding domain and two BRCT domains, that are separated by two unstructured linker domains of ~ 140 residues each (Figure 1a). XRCC1 facilitates the coordination of the overlapping base excision repair (BER) and single strand break repair (SSBR) activities of the cell by binding to many of the enzymes involved in the repair process [1-4]. Recruitment of XRCC1 to sites of DNA damage appears to involve primarily the first linker and the first BRCT domain. These regions have been found to mediate the poly(ADP-ribose) (PAR)-modification-dependent recruitment to single strand breaks [5-7], the recruitment of XRCC1 by proliferating cell nuclear antigen (PCNA) to sites requiring processive repair [8], and the recruitment of the XRCC1 complex into the nucleus via a nuclear localization sequence [5, 9]. The interaction of XRCC1 with PCNA may form the basis for its reported involvement in the nucleotide excision repair pathway [10, 11].

The second XRCC1 linker, XL2, contains a segment that exhibits phosphorylation-dependent interactions with the FHA domains of several alternative binding partners, including polynucleotide kinase phosphatase (PNKP), aprataxin (APTX), and aprataxin and PNKP-like factor (APLF) [2, 12-14]. These interactions, along with constitutively bound LigaseIII α [15, 16], create a structurally heterogeneous module optimized for repair activities that require modification of the 3' and 5' termini of the break, so that polymerization and/or ligation can proceed.

The first linker domain of XRCC1 contains a pair of sequential phenylalanine residues that are typically present in the PCNA-binding PIP Box motif, while lacking the other residues typically involved in the interaction [8]. Recently, another similar motif, also characterized by consecutive phenylalanine residues, has been identified that mediates the recruitment of translesion polymerases to the C-terminal domain of REV1, an enzyme involved in translesion synthesis and post replication repair [17-19]. In the present study, we have evaluated affinity of an XRCC1-derived peptide containing the putative RIR motif for the REV1 C-terminal domain, and find it to be in the low micromolar range. NMR spectroscopy has been used to provide restraints for docking the XRCC1 RIR peptide (X1RIR) into the REV1 C-terminal domain. Furthermore, the putative X1RIR binding sequence includes one of three common XRCC1 polymorphisms, R194W [20, 21]. This polymorphism has been associated with an increased level of polycyclic aromatic hydrocarbon DNA adducts [22], and with various forms of cancer [21-24].

Materials and Methods

Cloning, Overexpression and Purification

The C-terminal domain of human Rev1 (R1CTD, residues 1158-1251) preceded by a tev cleavage site (ENLYFQG) was ordered from GenScript (Piscataway, NJ). The construct was

optimized for *E. coli* expression, and cloned into a pUC 18 vector. PCR primers designed to incorporate an N-terminal hexa-histadine tag along with restriction sites for sub-cloning into a pET21a plasmid, were designed using Geneious software (vendor), then synthesized by IDTI (Coralville, IA).

The first linker region from mouse XRCC1 (XL1, residues 166-310) was cloned from a full length mXRCC1 gene obtained from GenScript. Primers designed with an N-terminal hexa-histadine tag and restriction sites, were then used to sub-clone the construct into a pCOLADuet-1 vector. The residues present in the putative X1RIR sequence, x-x-x-F-F-y-y-y-y, where y is restricted to residues consistent with a helical geometry, appear to be reasonably well conserved, although there is some variation in the identity of the y-residues that succeed the two phenylalanines. The corresponding peptide sequence of the mouse XRCC1 diverges from the sequence of the human protein near the ends of the RIR sequence used in the present study. R186 is replaced by K and the terminal P²⁰⁰VT sequence is replaced by SAS. Neither of these differences involves residues found to interact with the R1CTD.

Plasmids were transformed into BL21-DE3-RIL cells. Cells were grown in 2XYT media to an OD₆₀₀ ~0.7, induced with 1mM IPTG, and protein was expressed for ~16 h at 20 °C. Uniformly-¹⁵N-labelled R1CTD was grown in M9 minimal media supplemented with ¹⁵NH₄Cl and 2.5ml/L ¹⁵N-Bioexpress (Cambridge Isotopes, Andover, MA). Doubly labeled U-[¹³C, ¹⁵N]R1CTD was produced by growth in M9 minimal media supplemented with U-¹³C-glucose, ¹⁵NHCl₄, and 2.5 ml/L ¹³C-¹⁵N-Bioexpress.

Cells were pelleted (5,000 g/20min at 4°C), re-suspended in a buffer containing 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.6), lysed by sonication, then centrifuged at 30,000 g for 20 min at 4°C to produce a clear lysate. The His-tagged proteins were purified using immobilized metal affinity chromatography. Lysate was loaded onto a 3ml bed of Ni⁺² charged NTA-resin (Amersham/GE Healthcare). Protein was eluted with a stepped gradient of 5, 20, 45, 400 mM imidazole buffer containing 500 mM NaCl, 20mM Tris (pH 7.6). Proteins eluted with the 400mM imidazole. R1CTD was dialyzed into buffer: 50 mM NaCl, 50 mM Tris (pH 7.5), 1 mM DTT, then digested with TEV protease for ~48 hr at room temp to remove the His tag. The Rev1 was then re-run over an IMAC column to separate un-tagged protein from any remaining tagged protein. Cleavage at the TEV site resulted in an untagged R1CTD(1158-1251) with an additional glycine residue on the N-terminus. Yield of unlabeled and labeled Rev protein was 16-22 mg/L. Yield of XL1 linker was at least 8 mg/L.

Peptides

The X1RIR peptide: NSLRPGALFFSRINKSPVT, and its fluorinated analog, F-X1RIR: NSLRPGALFXSRINKSPVT, where X = 4-fluorophenylalanine, were obtained from Pi Proteomics (Huntsville, AL). Other peptides listed in Table 1 were obtained from Pi Proteomics (Huntsville, AL), Atlantic Peptides, or Genscript, and used without additional purification.

Fluorescence Assays

Fluorescence assays of RIR peptide binding to R1CTD were run on an Amino-Bowman AB2 Fluorometric spectrometer with excitation set to 295 nm and emission recorded at 350 nm, in order to bias the observation toward the intrinsic tryptophan fluorescence [25, 26]. Buffer consisted of 150 mM NaCl, 20 mM Tris-HCl (pH 7.2), 0.1 mM EDTA. R1CTD concentration was typically 5 μ M, but for the tighter binding pol κ peptide, we used 2 μ M. Data was collected at an ambient temperature of 26°C. 2.0 mM peptide stock solutions were prepared in DMSO. We also determined that the maximum DMSO concentrations present in the sample did not appear to significantly affect the fluorescence signal.

The K_d value for the tryptophan-containing peptide corresponding to one of the common XRCC1 polymorphisms, X1RIR(R194W), was estimated by analysis of the fluorescence quenching of the R1CTD signal by the pol κ RIR in the presence of varying concentrations of the R194W peptide, and determining the fraction of R1CTD occupied by the R194W peptide based on the reduced quenching effect of the pol κ peptide.

NMR studies

Fluorine-19 NMR studies were performed on a Varian INVOA 600 NMR spectrometer operating at a frequency of 564.279 MHz. Measurements were performed at 25 °C using a triple resonance ^1H - ^{19}F { ^1H - ^{19}F , ^{13}C } 5 mm PFG variable temperature probe. The NMR buffer was 90/10% $\text{H}_2\text{O}/\text{D}_2\text{O}$, 100 mM NaCl, 50 mM NaHPO_4 (pH 7.1), 5 mM DTT, 0.25 mM EDTA, 0.25 mM sodium azide, and a protease inhibitor cocktail (Ameresco), as well as 0.2 mM 4-fluorobenzamide (Sigma-Aldrich) as a chemical shift and intensity standard.

For the solution characterization of the X1RIR-R1CTD complex, NMR experiments were performed at 25°C using either a Varian 800 MHz INOVA spectrometer equipped with a 5 mm Varian ^1H { ^{13}C , ^{15}N } triple resonance probe or a Varian 600 MHz INOVA spectrometer equipped with a 5 mm Varian ^1H { ^{13}C , ^{15}N } triple resonance Cold Probe. The 2D and 3D NMR data were processed with NMRPipe [27], and the spectra were assigned with NMRView [28]. 2D $^1\text{H}/^{15}\text{N}$ HSQC spectra were acquired using the WATERGATE 3919 pulse sequence [29] for water suppression, with ^1H and ^{15}N sweep widths of 14.0 ppm and 29.6 ppm, respectively. The 2D matrices contained 1024 complex points in the ^1H dimension and 128 complex points in ^{15}N dimensions. The spectra were acquired with a 1 second delay between scans.

Intermolecular NOE distance restraints were measured from a 3D $^{15}\text{N}/^{13}\text{C}$ F1-filtered, F3-edited NOESY-HSQC spectrum [30] obtained at 800 MHz with a mixing time of 250 ms, using a sample containing 0.7 mM U- ^{13}C , ^{15}N R1CTD and 0.7 mM unlabeled XRCC1 peptide. The NMR samples used for structural analysis were dissolved in 50 mM sodium phosphate (pH 7.1), 100 mM NaCl, 5 mM DTT, 0.25 mM EDTA, 0.25 mM sodium azide, in 90%/10% $\text{H}_2\text{O}/\text{D}_2\text{O}$. Nineteen intermolecular NOEs were assigned from the 3D NOESY spectrum. The assignment of the intermolecular NOEs was facilitated using the R1CTD chemical shift assignments of Pozhidaeva et al. [31] and Wojtaszek et al. [32] and an initial model of the complex based on the structure of Rev1-CTD-Pol κ RIR complex described in the Wojtaszek study [32]. The NOE assignments were confirmed by comparing a

3D $^{15}\text{N}/^{13}\text{C}$ F3-edited NOESY HSQC spectrum [33] acquired with a mixing time of 150 ms at 600 MHz of the complex with a second NOESY spectrum obtained without ^{13}C -refocusing and ^{15}N -decoupling during the t_1 proton evolution period. The intermolecular NOE cross-peaks appear as singlets in the F_1 dimension of the decoupled spectrum and doublets in the $^{15}\text{N}/^{13}\text{C}$ -coupled spectrum.

Data Analysis

K_d values were obtained from NMR titration studies that utilized a fluorinated peptide analog and report directly on the fraction of peptide involved in the R1CTD complex. The fraction of bound peptide is given by the relation:

$$p_B = \frac{([R1CTD]K_d + [Pep]) + \sqrt{([R1CTD]K_d + [Pep])^2 + 4[Pep][R1CTD]}}{2[Pep]} \quad (1)$$

Ligand competition studies were fit using the relation that assumes full occupancy of the receptor by the two ligands present in the study [34]:

$$p_B = \frac{([R1CTD](1/R)S_0 + I_0) + \sqrt{(S_0I_0([R1CTD](1/R))^2 + 4R(1/R)I_0[R1CTD])}}{2I_0(1/R)} \quad (2)$$

where $[R1CTD]$ is the concentration of the REV1 C-terminal domain (R1CTD), I_0 is the probe ligand concentration (in our study, the F-X1RIR peptide, that is present at a fixed concentration), S_0 is the second ligand titrated into the sample, p_B is the fraction of probe ligand in the bound state, and $R = K_d(S_0)/K_d(I_0)$ is the ratio determined from the titration study.

Analysis of the fluorescence quenching data for the R1CTD followed a similar approach. However in this case, the fraction of R1CTD occupied by the RIR peptide is required, rather than the fraction of peptide that is bound. Thus, the peptide concentration in the denominator, $[pep]$ is replaced by the protein concentration, $[R1CTD]$.

Structure Calculations

The structures of the Rev1-CTD-XRCC1 linker peptide complex were generated following the rapid docking method described by Clore [35] and Wang et al. [36], utilizing the intermolecular NOE distance restraints and rigid body minimization. The calculation was carried out using the prot-prot/sa_cross_tor.inp XPLOR script provided with the XPLOR-NIH 2.33 distribution [37]. In the present calculation, the rigid residues of the Rev1-CTD were held fixed in Cartesian space, but the rigid residues of the XRCC1 linker peptide were only grouped, so that they were free to move with respect to the Rev1-CTD domain during the structure refinement. Residues Ala1160, Gly1161, Glu1174, Trp1175, Ile1179, Met1183, Glu1185, Asp1186, and Val1190 of the Rev1-CTD that exhibited intermolecular NOEs with

the XRCC1 peptide were allowed to be completely flexible. Only residues Leu190-Asn196 of the XRCC1 linker that showed intermolecular NOEs with the Rev1-CTD were grouped; all other XRCC1 peptide linker were allowed to be completely flexible. All side-chain atoms of the Rev1-CTD and the XRCC1 linker peptide, excluding C β , including the fixed and grouped residues were allowed to move freely during the structure refinement.

Results

A 20 residue peptide corresponding to residues 183-202 of XRCC1, containing a putative Rev1 interacting region motif (X1RIR), was used for the initial evaluation. Addition of 0.7 mM X1RIR to a sample containing 0.7 mM U-[¹⁵N]R1CTD produced shift perturbations of the ¹H-¹⁵N HSQC spectra qualitatively similar to those reported previously for the R1CTD in the presence of the pol η RIR peptide [31]. In order to determine whether the binding interaction could be significant, we initially utilized an F-19 labeling strategy as described below.

Fluorine-19 NMR binding studies

In order to have a convenient NMR probe for evaluation of the binding interaction between the postulated XRCC1 REV-interacting region (X1RIR) and the REV1 C-terminal domain (R1CTD), we obtained an analog of the XRCC1(183-202) peptide in which Phe192 was replaced with a p-fluorophenylalanine residue (F-X1RIR). This analog was selected based on structural data for the complexes of R1CTD with the pol ϵ and pol η RIR peptides (pdb codes: 2LSJ,2LSK,4FJO, [31, 32, 38], indicating that the corresponding phenylalanine extends into the protein making contact with Trp1175 and other residues. It was thus anticipated that the ¹⁹F shift would be sensitive to complex formation. A series of F-19 spectra obtained at [R1CTD] = 0.3 mM as a function of the F-X1RIR concentration is shown in Figure 1b. It is immediately apparent from this study that the interaction of F-X1RIR with R1CTD must correspond to a dissociation constant well below 0.3 mM. Thus, the exchange rate between the free and bound peptide is slow on the chemical shift time scale, and at equimolar concentrations, the resonance arising from uncomplexed F-X1RIR is barely visible. The chemical exchange behavior is typical of a reversibly complexed molecule, with the exchange contribution to the linewidth of the bound species independent of concentration and the exchange contribution of the free peptide proportional to the bound/free ratio:

$$\Delta\nu_B \propto \frac{1}{\Delta\nu_B} \Delta\nu_F \quad \Delta\nu_F \propto \frac{1}{\Delta\nu_F} \frac{1}{\Delta\nu_B} \frac{P_B}{P_F}$$

This measurement was repeated twice and fit to the relation described in Methods to yield K_d values of 4.6 and 3.9 μ M (Figure 2a). Since these K_d values are below the K_d values previously reported for the RIR peptides of the translesion polymerases [19], it was initially concluded that the X1RIR peptides appeared to be the preferred ligand of R1CTD.

In order to further characterize the affinity of R1CTD for the F-X1RIR peptide, we also performed a ligand competition study. The pol κ RIR peptide, corresponding to human pol ϵ

(560-575), was titrated into a sample initially containing 0.3 mM R1CTD, 0.4 mM F-X1RIR. In contrast with expectations based on the NMR-determined K_d value for F-X1RIR and the reported K_d value of 7.5 μ M for pol κ RIR, the ligand competition study indicated much greater affinity of R1CTD for the pol λ peptide than for F-X1RIR. The $K_d(\text{F-X1RIR})/K_d(\text{pol } \lambda \text{ RIR})$ ratio obtained using this approach was 15.2 (Figure 2c).

In order to determine whether more consistent results could be obtained using another RIR peptide, we performed a similar ligand competition study with F-X1RIR and the pol λ RIR peptide previously studied [19]. The NMR study yielded a K_d ratio, $K_d(\text{F-X1RIR})/K_d(\text{pol } \lambda \text{ RIR}) = 1.0$ (Figure 2d). Combining this result with the reported $K_d = 69 \mu\text{M}$ for the pol λ RIR again leads to a result that is inconsistent with the ^{19}F NMR-determined K_d of 4.5 μM .

Based on the above studies, we concluded that there are significant discrepancies between the K_d values we obtained using NMR spectroscopy and the previously reported values determined using surface plasmon resonance. Although the conditions of these studies were not identical, both were determined at 25 $^{\circ}\text{C}$ at similar ionic strength.

Fluorescence assay for peptide binding

In order to resolve the significant binding discrepancies noted above, we sought an alternative basis for evaluating the RIR-R1CTD K_d values. Based on the structural studies reported for the complexes of R1CTD with the pol κ and pol λ RIR peptides, the system seems to be very well suited for a fluorescence assay that utilizes the intrinsic fluorescence of the R1CTD. In particular: 1) Trp1175 of hR1CTD is located at the RIR binding site and interacts directly with the second phenylalanine residue of the RIR motif, so that there is a likely to be a binding-dependent effect on the fluorescence intensity, and 2) None of the peptides of interest contains a tryptophan residue or other significant fluorophore that would interfere with the measurement. For all of the RIR peptides studied, a significant quenching of the intrinsic fluorescence intensity measured at 350 nm was observed, and the concentration dependent data were adequately described by a simple binding equation that assumes a 1:1 binding stoichiometry. Each of the RIR peptides was found to quench the fluorescence emission signal measured at 350 nm by $\sim 40\%$ under the conditions studied, however a significantly greater quenching effect of $\sim 60\%$ was obtained for the fluorine-containing X1RIR analog. Fluorescence titration studies corresponding to the pol λ RIR, pol λ RIR, X1RIR, and F-X1RIR peptides are shown in Figure 3. Averaging three determinations for pol λ RIR:R1CTD binding gives $K_d = 0.28 \mu\text{M}$, about 27-fold stronger than the previously reported value of 7.6 μM . The K_d ratio relative the fluorinated F-X1RIR peptide, $K_d^{\text{F-X1RIR}}/K_d^{\text{pol } \kappa} = 16.1$, is in good agreement with the ligand competition study which gave a ratio of 15.2 (Figure 2c). A summary of the K_d values obtained in the present study is given in Table 1. Similarly, a comparison of the K_d values for F-X1RIR and pol λ RIR using the NMR or fluorescent assay results in Table 1 is in good agreement with the results of the ligand competition study described above. Thus, the use of a second binding assay method appears to resolve the apparent discrepancies among the different K_d values obtained. We note, however, that the binding trends are consistent with the earlier studies [17, 19]

Interaction of the REV1 C-terminal domain with XRCC1 Linker 1—In order to explore whether the interaction identified for the XRCC1-derived peptides is retained for the complete linker, we expressed the first linker from mouse XRCC1, residues 166-310 (XL1), uniformly labeled with ^{15}N . The limited spectral dispersion of the ^1H - ^{15}N HSQC spectrum (Figure 4) is consistent with expectations for an unstructured protein. Addition of an equimolar concentration of R1CTD resulted in selective disappearance of several resonances (highlighted in Figure 4 by arrows) and smaller shift perturbations of others. The perturbed resonances are expected to be shifted and broadened due to the larger size of the complex and/or chemical exchange contributions to the linewidth. This pattern is consistent with the involvement of a short peptide segment within the XRCC1 linker domain. Thus, the interaction with the R1CTD is not limited to a short peptide sequence, but is apparently maintained for the full linker.

Effect of R194W polymorphism: The putative RIR sequence in XRCC1 includes one of the three common XRCC1 polymorphisms, R194W [20]. In order to evaluate the effect of the R194W mutation on binding, we performed a ligand competition study using F-X1RIR, as described above. However, the poor solubility of the X1RIR(R194W) peptide and the high concentrations required for the NMR study produced inconsistent data. Alternatively, the presence of a Trp residue in the X1RIR(R194W) peptide precludes the use of the simple fluorescence assay used for the other peptides. An estimate of the K_d for the X1RIR(R194W) peptide was obtained by titrating solutions containing R1CTD and varying concentrations of X1RIR(R194W) with the pol κ RIR peptide. The presence of X1RIR(R194W) results in an increased initial fluorescence signal despite some (presumed) quenching of the protein fluorescence signal. The partial occupancy of the R1CTD binding site by X1RIR(R194W) reduces the fractional loss in fluorescence intensity that accompanies the subsequent pol κ RIR titration (Figure 5). The reduced magnitude of fluorescence quenching provides a value for the fraction of the R1CTD binding site initially occupied by the X1RIR(R194W) peptide. The fractional occupancy of the R1CTD was then used to fit a titration curve (Eq. 1 normalized by the [R1CTD] concentration rather than the peptide concentration), and provided an estimate of 32 μM for the K_d of the R194W analog. From these results, we estimate that the mutation reduces the binding affinity of X1RIR(R194W) by a factor of 4 relative to the wild type (wt) analog (Table 1).

Structural characterization of the hR1CTD•X1RIR complex—In order to determine the structure of the X1RIR•R1CTD complex, the unlabeled X1RIR peptide was docked into the U- ^{13}C , ^{15}N R1CTD based on a series of isotope edited/filtered NOE studies. The resulting structure shows clearly that the R1CTD -X1RIR complex is homologous with the recently determined complexes involving the pol κ and pol η Rev1 interacting regions [31, 32, 38]. However, we observed significantly fewer peptide-protein NOEs compared with the analogous study of pol η RIR. This resulted in a shorter, 6-residue helix, compared with the 9 residue helix formed by pol η [31]. This difference is not surprising, given that the Ser, Pro, and Thr residues at positions 199-201 of XRCC1 are known to disfavor helical structures [39, 40]. Alternatively, there were extensive NOEs connecting the residue preceding the two phenylalanines, Leu190, with Met1183, Glu1185, and Asp1186, suggesting that a hydrophobic residue preceding the two phenylalanine residue can partially

compensate for the reduced interactions of residues following the helix. The lowest energy docked NMR structure is shown in Figure 6, with R1CTD residues showing significant NOE interactions with the peptide in orange.

Conclusions

REV1 is a dCMP transferase that plays central roles in translesion synthesis during replication and post replication DNA repair [41-43]. It also has been identified as a component of the Fanconi anemia complex [43, 44]. The translesion synthesis functions of REV1 are critically dependent on a C-terminal domain that interacts with pol ζ . In higher eukaryotes, the REV1 C-terminal domain also possesses a second binding site characterized by lower specificity and micromolar affinity for the REV1 interacting regions (RIR) that have been identified in unstructured segments of other translesion polymerases: pol κ , pol η , and pol θ [18, 19]. These characteristics of limited binding affinity and specificity will result in a heterogeneous set of complexes in which the relative fraction of any specific complex will be dependent on the ratio of ligand concentration divided by its dissociation constant. In the case of the R1CTD complex, these fractions will thus be determined by: $[\text{pol } \kappa]/K_d(\text{pol } \kappa)$, $[\text{pol } \eta]/K_d(\text{pol } \eta)$, and $[\text{pol } \theta]/K_d(\text{pol } \theta)$, corresponding to the three translesion synthesis polymerases demonstrated to form physiologically important complexes. This strategy appears to favor heterogeneity of potential responses over a complex that is optimized for a specific type of DNA damage.

The presence of sequential phenylalanine residues in an unstructured peptide segment often corresponds to a PCNA-binding PIP Box motif [8, 45, 46]. However the XRCC1 linker 1 sequence studied here lacks the other residues that characterize this motif, and preliminary studies of PCNA binding showed the interaction to be very weak (unpublished results). This preliminary result is consistent with recent NMR studies indicating that slight deviations from the consensus PIP-Box sequence can dramatically reduce the strength of PCNA binding [47]. Since the same XRCC1 sequence meets the apparent requirements for a REV1-interacting region (RIR) [19], we evaluated this possibility and find the affinity to be higher. The data summarized in Table 1 indicate that, in the absence of significant concentration differences, pol θ represents the preferred REV1 C-terminal domain binding partner, while the binding affinity for an X1RIR RIR peptide is on the order of that characterizing the pol θ and pol θ /RIR interactions. Hence, in the absence of alternative binding partners it is likely that small amounts of each of these complexes will be present as well. As discussed above, these fractions will be altered by conditions that influence the levels of these proteins in the nucleus. For example, nuclear XRCC1 levels increase in response to hydrogen peroxide [48]. The X1RIR is also subject to post-translational modifications, particularly the high levels of phosphorylation characterizing the two XRCC1 linker domains [49-51]. Ser193 within the X1RIR motif appears not to be significantly phosphorylated, and in general, the other residues reported to be phosphorylated are probably too distant from the binding site to exert a significant perturbation, although this remains to be demonstrated.

As presented above, the results of ligand competition studies were found to be inconsistent with the previously reported K_d values, while the order of binding affinities determined here

is consistent with the previous study by Ohashi et al. [19]. The fluorescence assay introduced here appears to represent an extremely attractive approach for characterizing these R1CTD binding interactions, since the use of the intrinsic R1CTD fluorescence eliminates the need for further labeling. The lower value of 5.5 μM obtained for the pol δ RIR K_d value, compared with the previously reported value of 69 μM , is more consistent with a weak [17] but nevertheless significant physiological interaction. Interestingly, substitution of the second phenylalanine with a 4-fluorophenylalanine analog significantly enhanced the affinity of the peptide for the X1RIR as well as the fluorescence quenching effect of the bound peptide. This functionality may thus prove useful if analogs that more potently target the R1CTD are sought.

The K_d variations indicated in Table 1 are generally not easily understood in terms of the corresponding peptide-protein interactions. It is possible to rationalize some of these variations on the basis of effects that alter the solution conformation of the peptide and thus influence the entropic penalty associated with the transfer of the peptide from a dynamic ensemble of conformations in the uncomplexed state to the single preferred bound conformation. The helical segment of pol κ RIR contains residues that can form salt bridges consistent with the helical structure, while the other peptides do not [52]. Structural characterizations of the pol δ and pol δ complexes with R1CTD show that a salt bridge involving the highly conserved Lys residue at position 5 (where the first phenylalanine residue is assigned to position 1), helps to stabilize the complex. In addition to this contribution, this lysine appears to lie against the sidechain of Phe1, helping to stabilize the helical conformation of the peptide and presumably to pre-form this structure in the unbound state. The X1RIR sequence contains an Ile residue at the corresponding position that cannot form a salt bridge with the protein, but which can help to stabilize the helical conformation based on hydrophobic interactions with the Phe sidechains. The interaction of the R1CTD with X1RIR confirms the analysis of Ohashi et al. [19] that a lysine at this position, while beneficial for binding, is not strictly required. The effects of the R194W substitution on binding are difficult to predict, but one likely effect would be introduction of alternative hydrophobic interactions that could stabilize conformations other than the α -helix. Similarly, the three leucine residues in the pol δ RIR sequence may also introduce additional hydrophobic interactions that influence the conformation of the unbound peptide. Thus, effects of these residues on the unbound peptide conformation may be important determinants of their R1CTD binding affinities.

The structure of the R1CTD-RIR complex is analogous to the structures reported recently for the R1CTD complexes with the pol δ and pol δ RIR binding peptides. However, the XRCC1 helix is less extended, consistent with the presence of Thr198, Ser199, and Pro200 which will strongly disfavor helical structure beyond six residues after Phe192 [39]. Residues Ser (pol δ , pol δ), Pro (pol δ) or Leu (XRCC1) occupy the position preceding the first phenylalanine residue. Interestingly, extensive NOE interactions between the R1CTD residues and the Ser or Leu residue preceding the first phenylalanine are observed in the corresponding structures, while no NOEs were reported for the Pro residue at the analogous position of pol δ . These results suggest that the Leu residue in X1RIR may contribute significantly to the binding interaction.

Recruitment of XRCC1 to the REV1 complex extends the ability to deal not only with non-standard bases, but could introduce a capability to deal with problematic 3' and 5' termini. Furthermore, the occurrence of the common XRCC1(R194W) polymorphism in the identified X1RIR may provide a basis for understanding some of the reported disease correlations identified for this polymorphism [21-24, 53]. Interestingly, epidemiological studies of the R194W mutation report both positive and negative correlations with various cancers. The R194W mutation was associated with a marginal reduction in the risk of breast cancer [21, 53]. In view of the weaker binding of the X1RIR(R194W) with REV1, this correlation suggests that the XRCC1-REV1 interaction may be undesirable, introducing competition with physiologically more important ligands such as the translesion polymerases. Additional studies will be required to confirm whether the interaction between XRCC1 and the REV1 identified here is present under physiologically relevant conditions and leads to the functional consequences.

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List of Abbreviations

R1CTD	REV1 C-terminal domain
PCNA	proliferating cell nuclear antigen
XRCC1	X-ray cross complementing group 1 protein
RIR	REV interacting region
X1RIR	peptide corresponding to a putative REV1 interacting region in XRCC1, centered on Phe200-Phe201
F-X1RIR	X1RIR containing a 4-fluorophenylalanine substitution for the second phenylalanine residue in the RIR motif

Highlights

XRCC1 contains a REV1 interacting sequence with micromolar binding affinity for REV1

A fluorescence quench assay provides a direct, simple method for RIR binding affinity

The structure of REV1-bound XRCC1 peptide is characterized by a shorter α -helix

Figure 1.

XRCC1 schematic and F-19 NMR spectra of titration of R1CTD with F-X1RIR. A) Schematic illustration of the XRCC1 structure showing the N-terminal, pol β binding domain, the two BRCT domains, linker L1 locations of the putative RIR sequence and the nuclear localization sequence (NLS) and linker L2, showing the location of the phosphorylated FHA domain binding sequence (PFBS). B) F-19 NMR spectra showing the resonances of F-X1RIR (NSLRPGALFXSRINKTSPVT, X = 4-fluoro-L-phenylalanine) at the peptide concentrations indicated. Sample contained 0.3 mM R1CTD in D₂O, 100 mM NaCl, 50 mM NaHPO₄ pH 7.1 (uncorrected for isotope effect), 5 mM DTT, 0.25 mM EDTA, 0.25 mM sodium azide, and a protease inhibitor cocktail (Amersco), as well as 0.2 mM 4-fluorobenzamide as a chemical shift and intensity standard. Shifts are referenced to external trifluoroacetate and internal 4-fluorobenzamide at -32.3 ppm. NMR parameters were 30° pulse-width, 1.1s recycle time, 42K sweep width, 45min acquisition time, T = 25 °C.

Figure 2.

Analysis of titration and ligand competition studies. a) Fraction of bound F-X1RIR peptide as a function of peptide concentration in a sample containing 0.3 mM R1CTD. b)

Calculations illustrating the dependence of f_B on the dissociation constant, with the R1CTD concentration set at 0.3 mM. c) Data derived from ^{19}F NMR ligand competition study with initial concentrations: [R1CTD] = 0.3 mM; [F-X1RIR] = 0.4 mM, and the competing pol ι RIR concentrations indicated. d) Data from a similar study in which pol κ RIR was the competing ligand. In all studies, buffer and temperature conditions were as indicated in Figure 1.

Figure 3.

Fluorescence assays of RIR peptide-R1CTD binding. Peptides titrated were: a) pol κ /RIR; b) F-X1RIR; c) pol ι /RIR; d) X1RIR; The K_d values corresponding to the curves shown are: 0.28 μ M, 3.3 μ M, 6.0 μ M, and 8.7 μ M, respectively. Average K_d values for multiple determinations are given in Table 1. The pol ι and pol κ /RIR peptides were those previously studied [19]. Emission intensities were measured at 350 nm, with excitation at 295 nm. The pol κ titration (panel a) used 5 μ M R1CTD, while the studies corresponding to panels b, c, and d used 5 μ M R1CTD. Buffer: 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.1 mM EDTA. $T = 26^\circ\text{C}$. Peptide stock solutions were made up in DMSO, but the final DMSO concentration in the sample was less than 1%, and this DMSO concentration was found not to interfere with the fluorescence measurement.

Figure 4.

Effect of R1CTD on the HSQC spectrum of XRCC1 L1. Overlay of ^1H - ^{15}N HSQC spectrum of 100 μM U- ^{15}N mXRCC1 Linker 1 (XL1) in the absence (black) or presence (red) of excess of R1CTD. The limited dispersion is consistent with the random coil structure for this section of the protein, and the highly selective resonance perturbation is consistent with the specific interaction between the R1CTD and a very short RIR region of the XL1. Buffer as in Figure 1, except that the buffer was made up in 90% H_2O /10% D_2O .

Figure 5.

Estimation of the X1RIR(R194W) K_d value. a) Fluorescence signals of R1CTD as a function of X1RIR(R194W) in the absence (circles) or presence (squares) of saturating pol I RIR. b) Binding curve for X1RIR(R194W) determined using the fractions of R1CTD bound to X1RIR(R194W) determined from the data in panel a. The samples contained 5 μM R1CTD in the fluorescence buffer (Figure 3).

Figure 6.

Structure of the R1CTD•X1RIR complex. A ribbon diagram for the solution structure of the R1CTD(green)•X1RIR(cyan) complex determined on the basis of isotope-edited:isotope filtered NOE restraints, is illustrated. R1CTD residues for which NOE interactions were observed are indicated and annotated in orange. NOE restraints were observed for five X1RIR residues: Leu190, Phe191, Phe192, Ile195, and Asn196.

Table 1
K_d values for the interaction of RIR peptides with R1CTD

Peptide	Sequence	K _d μM	No. expts	Method
F-X1RIR	NSLRPGALFXSRINKTSPVT	4.3	2	NMR
		4.6	4	Fluorescence
X1RIR	NSLRPGALFFSRINKTSPVT	7.9	3	Fluorescence
X1RIR(R194W)	NSLRPGALFFSWINKTSPVT	32	1	Fluorescence/competition
pol κ RIR	EMSHKKSFFDKKRSE	0.28	3	Fluorescence
Pol λ RIR	ASRGVLSFFSKKQMQD	5.5	3	Fluorescence
pol η RIR	QSTGTEPFFKQKSLLL	4.4	3	Fluorescence