Chapter IV

Implications of BRCT Mediated DNA Binding:
Cooperative Binding of Two Primer Termini
Abstract.

Rev1 is a eukaryotic DNA polymerase of the Y family involved in translesion synthesis (TLS), a major damage tolerance pathway that allows DNA replication on damaged templates. Uniquely among the Y family polymerases, the N-terminal part of Rev1, dubbed the BRCA1 C-terminal homology (BRCT) region, includes a BRCT domain. While most BRCT domains mediate protein-protein interactions, Rev1 contains a predicted $\alpha$-helix N-terminal to the BRCT domain, which, was previously shown to endow the BRCT region of human Replication Factor C (RFC) with DNA binding capability [Kobayashi et al. 2006]. In the previous chapters it was shown that the BRCT region of Rev1 specifically binds to a 5’ phosphorylated, recessed, primer-template junction (5’ primer terminus). This DNA binding strongly depended on the extra $\alpha$-helix, N-terminal to the BRCT domain. Surprisingly, a stretch of 20 amino acids N-terminal to the predicted $\alpha$-helix confer specificity for the 5’ phosphate (Chapter II-III). This chapter shows that, in addition to the BRCT region, C-terminal regions of Rev1 efficiently and independently mediate binding to a recessed 3’ primer-template junction (3’ primer terminus). These DNA binding characteristics are discussed in view of the proposed recruitment of Rev1 by 5’ primer termini downstream of stalled replication forks.
Introduction.

Rev1 is a member of the Y-Family of polymerases, which function mainly in Translesion Synthesis (TLS) [Lawrence, 2004]. In the Y-family of polymerases, Rev1 is unique since the catalytic activity is limited to dCMP insertion [Nelson et al. 1996]. Despite its ability to bypass different DNA lesions [Waters et al. 2009; Otsuka et al. 2004; Lin et al. 2006], the polymerase activity is not essential for most TLS mediated by Rev1 [Otsuka et al. 2004; Ross et al. 2005; Nelson et al. 2000]. In addition to the deoxycytidyl transferase domain, mammalian Rev1 includes three other functional domains: an N-terminal BRCA1 C-terminal homology (BRCT) domain, two ubiquitin binding motifs (UBM) and a C-terminal region that binds to other Y family polymerases and Polζ [Waters et al. 2009; Guo et al. 2003]. Both the BRCT domain and the C-terminal region are essential for a proposed regulatory role of Rev1 in TLS, involving selection and stimulation of other TLS polymerases at template lesions during TLS. [Otsuka et al. 2005; Wood et al. 2007; Guo et al. 2006]. Although the BRCT domain is not essential for bypass of lesions by Rev1 in TLS, the absence of a functional BRCT domain strongly reduces the capability of Rev1 to stimulate the bypass of lesions [Otsuka et al. 2005; Jansen et al. 2005].

BRCT domains form a wide superfamily and are mainly found in proteins involved in DNA repair or cell cycle checkpoints [Callebaut and Mornon, 1997; Bork et al. 1997]. Although the majority of BRCT domains mediates protein-protein interactions via BRCT-BRCT, BRCT-non BRCT and BRCT-phosphopeptide interactions [Glover et al. 2004], previous work showed that the Rev1 BRCT region, similar to RFC, is specifically capable of binding to a synthetic 5’ phosphorylated primer terminus. (Chapter II-III.)

Recently, a role for mouse Rev1 was suggested in filling of post-replicative gaps that oppose damaged nucleotides [Jansen et al. 2007] in addition to direct lesion bypass. Post-replicative gap filling has been proposed as a mechanism that converts
relatively small DNA strands into DNA of high molecular weight in replicating bacteria and eukaryotic cells exposed to ultraviolet (UV) light [Lopes et al. 2006; Lehmann and Fuchs, 2006]. A model in which the 5’ primer terminus at a post-replicative gap acts as a docking site for Rev1 has been proposed [Jansen et al. 2007]. In this model it is thought that Rev1 subsequently migrates to the 3’ end of the stalled replication fork to mediate gap filling TLS [Masuda and Kamiya, 2006; Jansen et al. 2007].

This chapter demonstrates that Rev1 displays binding of both the 3’ and the 5’ primer terminus. While binding of the phosphorylated 5’ primer terminus solely depends on the BRCT region, binding of the 3’ primer terminus occurs independent of the BRCT region. These studies support a role of the Rev1 BRCT region in recruitment of Rev1 to 5’ primer termini at post-replicative gaps.
Methods.

Expression and purification of Rev1 Proteins

Full length Rev1 (mrev1(1-1249) and N-terminal truncations of full length mouse mRev1 (21-1249) and mRev1(41-1249) were kindly provided by Y. Masuda. All proteins were expressed and purified according to previous publication [Masuda and Kamiya, 2002]. mRev1(1-131) was purified and expressed as described previously (Chapter II).

5’ Radiolabeling.

2 pmol of the selected DNA substrate was treated with T4 polynucleotide kinase (New England Biolabs) at 37 °C in the presence of 10 µCi (6000 Ci/mmol) γ³²P-ATP. To ensure complete phosphorylation of the DNA substrate, unlabeled ATP was added to a final concentration of 50 µM after 90 minutes of incubation and the reaction was continued for 30 minutes. Non reacted radioactive nucleotides were removed using Sephadex G25 spin columns (GE Healthcare) following the standard protocol.

3’ Radiolabeling.

2 pmol of the selected DNA oligonucleotide was treated with Klenow fragment (New England Biolabs) at 37 °C with 10 µCi (6,000 Ci/mmol) α³²P-dCTP as a substrate. To ensure a high percentage of base pairing, the hairpin oligonucleotides, were denatured at 100 °C, and slowly cooled to room temperature. Non reacted radioactive nucleotides were removed using Sephadex G25 spin columns (GE Healthcare) following the standard protocol.
DNA binding experiments.

DNA binding was detected using a gel retardation assay or Electrophoretic Mobility Shift Assay (EMSA). The indicated amount of protein was diluted in a buffer consisting of 10 mM HEPES, pH 7.8, 2 mM MgCl₂, 0.1 mM EDTA, 100 µg/ml bovine serum albumin, 15% glycerol, (0-1.6) µg/ml poly(dI-dC) (Boehringer Mannheim) and 2 mM dithiothreitol in a total volume of 15 µl. 20 fmol of the ³²P-labelled GAP3 oligonucleotide (see Table 1) in 5 µl was added, incubated on ice for 30 minutes, and applied to a non-denaturing 8% or 4% Tris-glycine acrylamide gel containing 2% glycerol. Electrophoresis was performed at 80 V for 20 minutes and subsequently 120 V for 40 minutes at 4 °C in 25 mM Tris-HCl pH 8.5, 200 mM glycine. The gel was dried and radioactivity was detected using X-Ray film (BioMax, Kodak).

Quantification of DNA binding

Radioactivity was detected using a phosphorimager (Biorad). For each reaction, the amount of protein-DNA complex was calculated as a percentage: percent bound = ([counts in shifted DNA]/[total counts per lane])*100.

Dephosphorylation assay

The indicated amount of protein was incubated on ice with 20 fmol of ³²P GAP3 under the same conditions as the EMSA experiments in the absence of poly(dI-dC). After incubation, 3.33 units of Calf Intestinal Phosphatase (CIP, New England Biolabs) were added to the reaction mixture and applied on EMSA after incubation on ice for the indicated amount of time. Gel was dried, and radioactivity was detected using X-Ray film (BioMax XAR, Kodak).
Results

To investigate whether the DNA binding properties of the isolated Rev1 BRCT region are indicative of those of maintained in the full length Rev1 protein, full length mRev1, (mRev1(1-1249)) and two mutant mRev1 proteins that contain N-terminal deletions similar to those introduced in the isolated BRCT region, respectively mRev1(21-1249) and mRev1(41-1249), were obtained via a collaboration with Masuda and co-workers (Figure 4.1A). To discriminate between binding at the 5’ or 3’ terminus, the three DNA oligonucleotides described in Figure 4.1B were designed and synthesized commercially.

(Figure 4.1) Schematic overview of the Proteins and substrates used in this study. (A) Different versions of the Rev1 proteins, respectively full length Rev1 (mRev1(1-1249)), truncation of the 20NT (mRev1(21-1249)) and truncation of both the 20NT and α1’ helix. (B) Secondary structure (right: sequence; left: schematic) of the DNA substrates used in this study. Respectively a single hairpin DNA containing a 3’ primer terminus (3’SHP), a single hairpin DNA containing a 5’ primer terminus (5’SHP), and a double hairpin DNA containing both a 5’ primer terminus as a 3’ primer terminus, and 25 nucleotides of ssDNA flanked by 10 base pairs of dsDNA (GAP25).
Full-length Rev1 binds to both 5’phosphorylated and 3’OH primer termini.

To probe the DNA substrate specificity of full length mRev1 [mRev1(1-1249)] and of truncated mRev1 proteins, binding to 5’SHP, containing only a 5’P primer terminus was assayed. The isolated BRCT region was shown to bind this substrate with good efficiency (Chapter II). While mRev1(1-131) and mRev1(1-1249), both of which contained the entire BRCT region, efficiently bound the 5’P SHP, substrate, neither

(Figure 4.2) DNA binding by the four different Rev1 proteins. (A) Respectively mRev1(1-131), Rev1(1-1249), Rev1(21-1249) and Rev1(41-1249) were incubated with 5’Phosphorylated (5’P) 5’SHP and run on an EMSA. Lane 1: no protein. Lane 2: mRev1(1-131). Lane 3: mRev1(1-1249). Lane 4: mRev1(21-1249). Lane 5: mRev1(41-1249). The experiment was repeated with respectively 3’SHP containing a 3’OH (B), GAP25 containing both a 3’OH and a 5’OH group (C) and GAP25 containing both a 3’OH and a 5’phosphate group (D).
of the N-terminally truncated proteins mRev1(21-1251) nor mRev1(41-1249) exhibited any DNA binding activity (Figure 4.2A). When binding to a recessed 3’OH terminus was assessed using the 3’SHP1 oligonucleotide (Figure 4.2B), only the mRev1(1-131) protein did not bind, suggesting that portions of the protein C-terminal to the BRCT region are required for binding to this substrate. These data indicate that the BRCT region is exclusively involved in binding a recessed, 5’ phosphorylated primer terminus whereas the remainder of mRev1 specifically binds to a recessed 3’ primer terminus (Figure 4.2A-B). Next the double hairpin substrates with a 25 nucleotide gap (GAP25) containing both 5’ and 3’ primer termini were assayed (Figure 4.2C). In agreement with the data in Figure 2.4A, mRev1(1-1249) as well as the N-terminally truncated mRev1 proteins bound GAP25 containing a non-phosphorylated 5’ terminus, while mRev1(1-131) did not bind. However, all four proteins bind a GAP25 substrate containing both a 3’OH and a 5’P terminus (5’P GAP25, Figure 4.2D).

To further characterize the difference in DNA binding by the various Rev1 proteins, increasing amounts of each protein were titrated into the indicated DNA substrates and DNA binding was quantified (Figure 4.3). In line with previous results, mRev1(1-131) binds 5’P SHP, and 5’P GAP25 (Figure 4.3A) with similar affinity (approximately 100 nM). mRev1(1-1249) binds the 3’SHP and 5’P SHP1 substrates with comparable affinities and displays a slightly higher affinity for unphosphorylated GAP25 (approximately 30nM). However, the DNA binding affinity of mRev1(1-1249) for 5’P GAP25 was highest (approximately 10 nM), suggesting that the full length protein binds to both 5’ phosphorylated and 3’ primer termini (Figure 4.3D). Both mRev1(21-1249) and mRev1(41-1249) also show a slightly higher binding affinity for unphosphorylated GAP25 in comparison to 3’SHP, but the increased binding affinity for a 5’P GAP25 is not observed (Figure 4.3C-D).
Quantification of three independent EMSAs in which respectively mRev1(1-131) (A), Rev1(1-1249) (B), mRev1(21-1249) (C), and mRev1(41-1249) (D) were titrated into a constant amount of the indicated substrate.

The significance of the 5′ phosphate in overall DNA binding by Rev1 is further demonstrated when the data in Figure 4.3 are presented in a different format. With respect to the 5′OH GAP25 substrate, DNA binding is identical for mRev1(1-1249), mRev1(21-1249) and mRev1(41-1249) (Figure 4.4A). However, for the 5′P GAP25 substrate the binding affinity is in the order mRev1(1-131) < mRev1(21-1249) = mRev1(41-1249) < mRev1(1-1249) (Figure 4.4B). Combined, these results suggest that, akin to the isolated Rev1 BRCT region, the BRCT region in full length mRev1 is essential for binding of mRev1 to the 5′ phosphorylated primer terminus and that binding of the two different primer termini appears to be cooperative.
Different representation of results from Figure 4.3 to visualize the differences between respectively full length mRev1, mRev1(1-1249), mRev1(21-1249) and mRev1(41-131) with respect to binding of either non-‘5 phosphorylated GAP25 (A) or binding of ‘5 phosphorylated GAP25 (B).

Next it was investigated whether the DNA binding kinetics observed in the isolated BRCT region were retained in the context of the full-length protein. Thus, mRev1(1-131), mRev1(1-1249) and mRev1(21-1249) were preincubated with $^{32}$P labeled 5’SHP and subsequently subjected to phosphatase treatment (Figure 4.5A), similar to the experiment depicted in Chapter II. When mRev1(21-1249) was preincubated with $^{32}$P labeled 5’SHP, the DNA was completely dephosphorylated within the time required for the DNA to enter the gel, whereas both mRev1(1-131) and mRev1(1-1249) displayed an identical protective effect against dephosphorylation of 5’SHP (Figure 4.5A). When the experiment was repeated with 5’P GAP25 as a substrate, mRev1(1-1249) initially showed a faster release from the 5’ phosphate than mRev1(1-131) but subsequently the release was similar (Figure 4.5B & C). This initial faster release is likely explained by binding of mRev1(1-1249) to either the 5’ or 3’ termini, leaving a greater portion of 5’ termini unprotected. To test whether dephosphorylation of 5’P GAP25 affects DNA binding of mRev1(1-131), mRev1(1-1249) and mRev1(21-1249), the experiment was repeated using a 5’P GAP25 substrate that was labeled by incorporation of an α-$^{32}$P nucleotide at the 3’ terminus using Klenow fragment (Figure 4.5D). This labeling is refractory to
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phosphatase treatment, allowing an analysis of protein-DNA binding, even after removal of the 5’P. The rate of loss of DNA binding by mRev1(1-131) is the same as the rate of loss of $^{32}$P label (Figure 4.5A), confirming that binding of this protein is entirely dependent on the 5’P at the primer terminus. In contrast, the mRev1(21-1249) protein binds a smaller fraction of the input DNA. Since this binding is entirely independent of the loss of 5’P, this result suggests that the mRev1(21-1249) protein stably associates with the 3’ OH terminus of the DNA. Full length mRev1(1-1249) however, exhibits a very different behavior. mRev1(1-1249) binds nearly all of the input DNA and undergoes an initial slow loss of DNA binding upon phosphatase treatment. After approximately 15 minutes of phosphatase treatment, DNA binding by mRev1(1-1249) reaches a plateau that precisely matches that of the mRev1(21-1249) protein. The additional binding, compared to mRev1(21-1249), and slow release upon phosphatase treatment suggests that initially either the 3’OH or the 5’P terminus is bound by mRev1(1-1249). Since a significant release of 5’P is observed (Figure 4.5B), the slow release from 3’ labeled GAP25 (with respect to mRev1(1-131)) indicates that Rev1, after release from the 5’P, transiently remains tethered to the single-stranded region before releasing the DNA. These results confirm that the BRCT region of full length Rev1 preferentially binds to phosphorylated 5’ termini of gapped substrates. Independent of the BRCT region, other portions of Rev1 are important for stable binding to 3’ termini.

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Protection of the 5’ phosphate is maintained in context with the complete Rev1. (A) mRev1(1-131), Rev1(1-1249) and mRev1(21-1249) were incubated with phosphorylated 5’SHP1 for 30 minutes. Subsequently, calf intestinal phosphatase was added for the indicated time before running the gel. (B) Similar experiment as in (A) with respectively mRev1(1-131), mRev1(1-1249) and mRev1(21-1249) incubated with 5’ labeled 5’ phosphorylated GAP25. (C) Data from figure B plotted on logarithmic scale, showing the rate of dephosphorylation. (D) Similar experiment as in (B) and (C) with 3’ labeled 5’ phosphorylated GAP25, visualizing the amount of DNA bound during dephosphorylation. The ratio of protein to DNA is the same for each of the three different protein constructs.
Discussion

It was found that, in addition to the N-terminal BRCT region that directs binding of mRev1 to a 5’ phosphorylated primer terminus in a gapped DNA substrate, other portions of the protein mediate mRev1-binding to the 3’ OH terminus of a gap. Interestingly, binding at the 3’ OH, but not at the 5’ phosphate, is dependent on the length of the ssDNA (compare binding of 3’SHP with binding of GAP25 in Figure 4.3). A possible explanation is the reported ssDNA binding property of the polymerase domain region [Masuda and Kamiya 2002], which may slightly increase the affinity for the gapped substrate in comparison to the single hairpin substrate. The affinity of mRev1(1-1249) for a 5’ phosphorylated gapped DNA is significantly greater than for a non-phosphorylated DNA, while the stoichiometry remains unchanged as judged by migration in the EMSA. This suggests that in the assay mRev1(1-1249) binds to either end of the DNA compared with mRev1(21-1249) that only binds at the recessed 3’ primer terminus (Figure 4.2C-D).

It has also been shown that binding of both mRev1(1-131) and mRev1(1-1249) to a 5’ phosphorylated primer terminus protects it from dephosphorylation. Interestingly, following removal of the 5’ phosphate, mRev1(1-1249) did not immediately dissociate but remained transiently bound to the DNA (Figure 4.5D). In contrast mRev1(1-131) releases dephosphorylated DNA with the same kinetics as the dephosphorylation itself. This observation may reflect mRev1(1-1249) binding to, and possibly sliding along, ssDNA at the gapped molecule towards the 3’ OH terminus. This is in agreement with the observation that Rev1 is sequestered on long stretches of DNA [Masuda and Kamiya, 2006]. Also, the elucidated structure of the Rev1-DNA complex reveals a complete encirclement of the ssDNA by the PAD and N-digit of Rev1 [Swan et al. 2009]. The topological constraint of completely encircling the single stranded DNA is likely to significantly reduce dissociation from the DNA (Figure 4.6B). This tethering likely enables Rev1 to move from the 5’ primer terminus
Different modes of binding to a gapped DNA substrate by Rev1. (A) Rev1 is bound at the 5’ phosphorylated primer terminus. (B) In an intermediate state Rev1 is bound to ssDNA and able to slide to either primer end. The N-Digit, is located between the PAD and Palm domain, and completes the fully encircling of the ssDNA, which reduces dissociation from the DNA and enables Rev1 to move to the 3’ primer terminus (C). Given the high flexibility of ssDNA it is possible that both primer ends are bound (D).

(Figure 4.6A) to the 3’ primer terminus (Figure 4.6C). Formally, the possibility should be considered that Rev1 is able to bind both primer termini simultaneously (Figure 4.6D). However, no evidence for this was observed in the EMSA. In vivo, the binding of RPA to ssDNA should also be considered, which could interfere with simultaneous binding at both primer termini by Rev1.

Recent studies have suggested repriming of replication downstream of replication fork-blocking DNA lesions in eukaryotes [Lopes et al. 2006, Lehmann and Fuchs, 2006]. Such repriming would result in single-strand gaps between the site of reinitiation and the lesion. Alternatively, these gaps will also be generated upon firing of an (otherwise dormant) origin of replication downstream of the fork-blocking DNA lesions. At least some of these gaps seem to persist to the end of S
phase and into early G$_2$ [Lopes et al. 2006] where in yeast, surprisingly, expression of Rev1 is maximal [Waters and Walker, 2006]. The ssDNA gaps are indeed substrates for post-replicative TLS [Karras et al. 2010, Daigaku et al. 2010]. In mammalian cells, Rev1 deficiency leads to the accumulation of post-replicative gaps opposing photolesions, in agreement with an essential function of Rev1 in post-replicative TLS [Jansen et al. 2009]. The different binding modes of Rev1 are consistent with a recent model on Rev1-mediated DNA damage bypass, whereby, at sites of stalled replication, Rev1 is recruited to the 5’ primer terminus of a single stranded gap generated via either downstream repriming or alternative origin firing [Jansen et al. 2007]. The present study indicates that such recruitment may be mediated by the BRCT region by direct binding to the 5’ phosphorylated terminus of the downstream primer. Subsequently, Rev1 may translocate to the 3’ end of the gap [Jansen et al. 2007; Masuda and Kamiya, 2006]. However, this function of the Rev1 BRCT region may only be important for “early stage” lesion bypass [Jansen et al. 2009]. Rev1-mediated DNA damage bypass can also occur independently of the Rev1 BRCT region [Jansen et al. 2009], implicating other modes of recruitment of Rev1 to stalled replication forks. For example, the DNA embracing heterotrimeric clamp Rad9-Rad1-Hus1 (9-1-1), located preferentially at the 5’ primer terminus [Ellison and Stillman, 2003], may recruit Rev1 via an interaction with Rev7, a binding partner of Rev1 [D’Souza and Walker, 2006]. Interestingly, the 9-1-1 clamp, bound to the 5’ primer terminus, may activate the checkpoint kinase Mec1/Atr [Majka et al. 2006] that, in yeast, is required for phosphorylation of the BRCT region of Rev1, which is important for DNA damage-induced mutagenesis [Pages et al. 2009].
References


