The BRCT domain from the large subunit of human Replication Factor C

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Chapter 2

Characterization of the DNA Binding and Structural Properties of the BRCT region of the p140 subunit of human Replication Factor C

Abstract

BRCT domains, present in a large number of proteins that are involved in cell-cycle regulation and/or DNA replication or repair, are primarily thought to be involved in protein-protein interactions. The large (p140) subunit of Replication Factor C contains roughly ~100 amino acids in the N-terminal half, which has been shown to bind DNA. This domain also contains sequences distantly related to known BRCT domains. Here we demonstrate that a region of p140 that encompasses the BRCT domain contains two distinct DNA binding activities. Residues 375 to 480, which include 28 amino acids N-terminal to the conserved BRCT domain, contain a binding activity specific for 5'-phosphorylated dsDNA while a non-sequence or structure specific dsDNA binding activity requires an additional 65 amino acids C-terminal to the BRCT domain (residues 480-545). The N-terminal extension provides an extra α-helix to an otherwise conserved BRCT domain. However, the extended domain is flexible in the absence of a DNA ligand. Although the 65 amino acid region C-terminal to the BRCT domain is unstructured when expressed by itself, a larger polypeptide incorporating these sequences (375-545) is well ordered even in the absence of the DNA ligand. This suggests that the complete structural unit in the intact protein includes amino acids 375-545.

Introduction

The <u>BR</u>CA1 <u>C-</u>terminal homology (BRCT) domain is an abundant structural unit found in more than 50 nonorthologous proteins (1;2). Nearly all of these proteins are involved in the cell cycle checkpoint response to damaged DNA and/or more directly in DNA replication or repair. The BRCT superfamily has been further classified into three subsets. The first consists of a core of highly conserved domains found in proteins such as BRCA1 itself, the *Saccharomyces cerevisiae* Rad9 protein and the p53 binding protein 53BP1. A second distinct and more distantly related set can be found in DNA binding enzymes such as the bacterial NAD-dependent ligases and poly (ADP-ribose) polymerase (1). Finally, the retinoblastoma tumor suppressor and related proteins may contain a very distant member of the BRCT family(1).

BRCT domains are responsible for a number of important homo- and heterotypic protein-protein interactions. For instance, XRCC1, a protein involved in repair of single-stranded DNA breaks, binds DNA ligase III *via* its C-terminal BRCT domain (3) while its N-terminal BRCT domain specifically binds poly-ADP Ribose polymerase (4). Other BRCT domains, such as those in the *S. cerevisiae* protein Rad9, function in homotypic (self) protein binding (5). Recently, it has been shown that many BRCT domains posses a phosphoserine specific, protein binding function (6-8). The serine phosphorylations that have been investigated to date are performed by either ATM or ATR, two protein kinases involved in the early steps of signal transduction generated by damaged DNA (6). Although there are reports that some BRCT domains can bind DNA, the binding requirements have not been defined and it therefore remains unclear whether binding is a property of the BRCT domain itself in these cases (20).

BRCT domains consist of approximately 95 amino acid residues with a strictly conserved fold. The first 3D structure elucidation was that of the C-terminal BRCT domain of XRCC1 (9). The structure consists of a central 4-stranded parallel β-sheet surrounded by three α-helices. Recently the structures of the BRCT domain from DNA ligase III (10), and the tandem BRCT domains from BRCA1 (11) and from the p53 binding protein p53BP1 (12) have also been solved. In addition, a low-resolution structure of the BRCT domain from the DNA-ligase of *Thermus filiformis* (13), a member of the second class of BRCT domains has been solved. The structural conservation from bacteria to humans indicates that this is likely an ancient fold.

Replication Factor C (RFC) is a five protein complex involved in both the replication and repair of chromosomal DNA (14-16). The primary function of RFC appears to be to open the "sliding clamp" protein PCNA and "load" it onto DNA where it serves as a binding platform for a multitude of enzymes and regulatory proteins involved in the replication and repair of DNA. RFC consists of four subunits of between 35 and 40 kDa that share homology with a central region of the fifth subunit, which has a molecular mass of 140 kDa in mammals (referred to as p140). The N-terminal half of the p140 subunit contains sequences unique to RFC, including a region with DNA binding activity (17-21) that is not required for clamp loading (22;23) mediated by the DNA binding specific to the 3' end of the primer/template DNA. This region contains amino acid sequences which form part of the second distinct class of BRCT domains (1;2). Here we demonstrate that this region of p140 that includes the BRCT homologous sequences binds double-stranded DNA in both a 5' phosphate specific and a non-specific manner.

Materials and Methods

Gene Cloning

The plasmid containing a cDNA clone coding for residues 369 - 480 RFC p140 was a kind gift of Prof. Ullrich Hübscher (Universität Zürich, Switzerland). The three different constructs were generated using standard PCR based methods and were cloned into pET20b (Novagen) with a C-terminal 6-His tag (Fig 1A). The plasmid containing a cDNA clone encoding full length RFC p140 was a kind gift of Prof. Bruce Stillman (Cold Spring Harbor Laboratory). The two constructs encoding for residues 374-545 and 480-545 of RFC p140 were generated using PCR based methods and were cloned into pET20b(Novagen) with a C-terminal 6-His tag. A plasmid containing a cDNA clone of full length human C/EBPα was used to generate a DNA construct coding for residues 281 - 358 of C/EBPα in pET20b (Novagen) with a C-terminal 6-His tag. All PCR generated clones were sequenced bidirectionally.

Protein expression and purification

Proteins were expressed for 3 hours at 37 oC in BL21/DE3/pLysS cells (Novagen). Lysed cells were centrifuged at 20,000 x g for 30' at 4 oC and the supernatant applied to a 6 ml metal-chelate column (Novagen) charged with Ni2+. The column was developed as per the manufacturers recommendations. Fractions containing the His- tagged

protein, as judged by SDS-PAGE electrophoresis, were pooled and EDTA was added to the protein solution to a final concentration of 5 mM to chelate Ni2+ leached from the column. The protein solution was concentrated through an Amicon ultrafiltration device (YM10 membrane) to 5 ml volume. The protein was further purified to apparent homogeneity using a 140 ml (1.6 x 75 cm) column of Superose 12 (Amersham Pharmacia) equilibrated with 20 mM Tris pH7.5, 50 mM NaCl and 1 mM DTT. All proteins were stably stored at 4 °C. All three purified RFC p140 proteins (Figure 2.1A) were exchanged into the PBS buffer. Subsequently the secondary structure contents were evaluated by circular dichrosim spectra of 10 μM of each proteins in PBS solution by using Jobin Yvon CD6 instrument at 1 nm intervals over 190-240 nm wavelength at 21 °C in a 0.5 mm path-length cell. All three RFCp140 proteins were judged to be folded based on the circular dichroism spectra.

Stoichiometric titration

10 μM of the oligo 1 (in 25mM Tris-HCl pH 7.5, 50mM NaCl and 1mM DTT) was mixed with increasing amount of RFC p140 (375-480) (in the identical buffer condition) in the final volume of 250 μl. The DNA-protein complex was separated from the free DNA on the Superdex™ 10/300 GL Columns (Tricorn) which had been pre-equilibrated with 25mM Tris-HCl pH 7.5, 50mM NaCl and 1mM DTT. The volume of the absorbance peaks corresponding to the complex and free DNA were integrated and the fraction of the complex was calculated by dividing the volume of the complex peak by the sum of the volumes of the complex and the free DNA peaks.

Preparation of phosphorylated oligonucleotides

The sequences of all oligonucleotides used in this study are presented in Figure 2.1. The oligonucleotides were synthesized using standard solid-state methods and contained a 5' PO4 (with the exception noted in Figure 2.1). Two pmol of 5' phosphorylated oligonucleotide were used in an exchange reaction employing the phage T4 polynucleotide kinase and 20 pmol of [γ-32P] ATP as substrate. In order to ensure a very high percentage of double stranded DNA, the hairpin oligonucleotides were subsequently denatured at 100 oC for 10 minutes and then slowly cooled to room temperature. All non-phosphorylated oligonucleotides (including the CREB oligonucleotide) were phosphorylated using a standard protocol for the forward reaction and the same amount of substrate and enzyme as described above.

Detection of protein-DNA complexes

DNA binding was detected using a gel retardation assay. The indicated amount of the various RFC p140 proteins were diluted in a buffer of 10 mM HEPES pH 7.8, 2 mM $MgCl_2$, 0.1 mM EDTA, 100 µg/ml BSA, 15 % glycerol, 0.8 µg/ml poly (dI-dC) and 2 mM DTT. Indicated amount and appropriate volume if 5'[32P] oligonucleotide was added to the mixture to the final volume of 12 µl, which was subsequently incubated on ice for 15 minutes, then applied to a non-denaturing 8% Tris-Glycine, acrylamide gel and electrophoresed at 150 V for 30 minutes at 4 °C in 25 mM Tris-HCl pH 8.5, 200 mM glycine, 1mM EDTA (TGE) buffer. Radioactivity was detected by exposing to a film (X-OMAT, Kodak) or to a phosphor-imager (BIORAD) after the gels was dried. Gel retardation was also used to determine the ligand (DNA) requirements for binding but in the form of a competition assay. In these experiments both labeled and unlabeled DNA were premixed and then added to the protein solution. The amounts of the competing oligonucleotides used in excess over the labeled oligonucleotide in the assay are indicated in the Figures.

Southern blotting and ECL-detection of protein-DNA complexes

The 5' biotinylated CREB oligonucleotide (CRE binding consensus sequence) was purchased from Microsynth GmbH, Switzerland. The CREB oligonucleotide was dissolved in the same buffer described above, heated to 100 oC and annealed by slow cooling. DNA binding of the RFC p140 proteins and/or C/EBPα DBD (DNA binding domain) was detected using 100 fmol of the CREB oligonucleotide in a gel retardation assay using the same buffer conditions described earlier (25). The biotinylated CREB oligonucleotide was then transferred to Hybond-N+ nylon membranes (Amersham Bioscience) at 4 °C at 200mV for 1 hour in 90 mM Tris - 90 mM borate - 2 mM EDTA (TBE) buffer. The wet membrane was exposed to UV (254 nm) for 3 minutes in order to cross-link DNA to the membrane. The membrane was incubated in 25 ml of TBE (with 5 % dry milk) for 1 hour and subsequently 15 µg of Streptavidin-HRP (horse radish peroxidase) conjugate (Zymed laboratories inc.) was added. CREB oligonucleotides were subsequently detected using the ECL reagent as described by the manufacturer (Amersham Bioscience).

NMR Measurements

All three proteins were concentrated to between 0.5 and 1 mM in 25 mM Tris-HCl, 100 mM NaCl, 1 mM DTT and NaN3 0.01 % (w/v) in 5% D2O where the final pH was adjusted to 7.5 (direct meter reading). The sample of 1:1 DNA-RFC p140(375-480) complex had a final concentration of approximately 0.5 mM in 25 mM Tris-HCl, 100 mM NaCl, 1 mM DTT and NaN3 0.01 % (w/v) in 5% D2O with the final pH adjusted to 7.5. All NMR spectra were recorded at 298 K on a 600 MHz Bruker DMX spectrometer. A standard [15N,1H] HSQC pulse sequence employing the WATERGATE solvent suppression method (24) was used. Spectra typically consisted of 128 increments of 64 transients each and were processed using TOPSPIN software. The sequential assignment was accomplished using 13C, 15N labeled 1:1 complex of RFC p140(375-480) and oligo 1 using standard through bond, triple resonance NMR experiments. Details will be published elsewhere. The consensus CSI score was generated with the program CSI version 1.0 (25) from $^{13}C\alpha$, $^{13}C\beta$ and $^{1}H\alpha$ chemical shifts values as inputs.

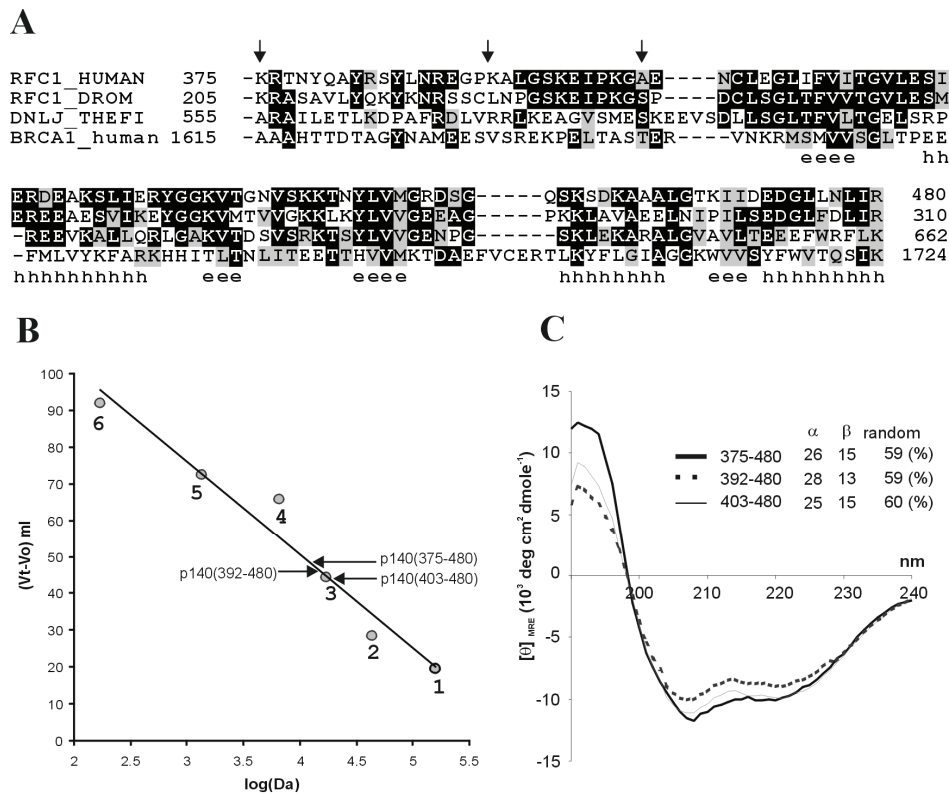
Results

Identification of protein domains required for DNA binding activity

The DNA binding domain of human RFC p140 was initially defined as consisting of residues 369 – 480 (17). Since this region includes sequences outside the conserved BRCT domain we were interested to find out whether just the BRCT domain (403-480) itself was sufficient for DNA binding. Based on the published sequence alignments (1;2) we made three N-terminal deletion constructs whose C-termini all coincide with the expected C-terminus of the BRCT domain (see arrows in Figure 2.1A). All three-deletion constructs were C-terminally tagged with six histidines, expressed in *E. coli* and subsequently purified using metal chelate and gel-filtration chromatography. A Superose 12 gel filtration column was calibrated with the proteins with known molecular weight. The resulting calibration curve was used to estimate the molecular weight of the purified p140 constructs (Figure 2.1 B). The estimated molecular mass of the p140(375-480) derived from the gel-filtration experiment, 14 kDa, was very close to the expected value of a monomer (12 kDa). p140 (403-480) eluted as a very broad peak and the estimated molecular weight (18 kDa) was twice the expected value. It is known that many isolated BRCT domains tend to form homodimers or multimers. These observations imply that this

construct is a mix of monomer and dimer or has an elongated shape. The elution of p140 (392-480) correlates with a molecular weight of (15 kDa) which is again slightly larger than the expected value (10 kDa). The secondary structure of the three constructs was analyzed using circular dichroism (CD) spectroscopy. Prediction of secondary structures from the CD spectra using the K2d program (26) shows that all of the peptide constructs contained a mixture of α and β - structures (Figure 2.1C).

A

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RFC1_HUMAN   375   -KRTNYQAYRSYLNREGPKALGSKEIPKGAE----NCLEGLIFVITGVLESI
RFC1_DROM    205   -KRASAVLYQKYKNRSSCLNPGGSKEIPKGSP----DCLSGLTFVVTGVLESM
DNLJ_THEFI   555   -ARAILETLKDPAFRDLVRRLKEAGVSMESKEEVSDLLSGLTFVLTGELSRP
BRCA1_human 1615   -AAAHTTDTAGYNAMEESVSREKPELTASTER----VNKRMSMVVSGLTPEE
                                                              eeee       hh

ERDEAKSLIERYGGKVTGNVSKKTNYLVMGRDSG-----QSKSDKAAALGTKIIDEDGLLNLIR   480
EREEAESVIKEYGGKVMTVVGKKLKYLVVGEEAG-----PKKLAVAEELNIPILSEDGLFDLIR   310
-REEVKALLQRLGAKVTDSVSRKTSYLVVGENPG-----SKLEKARALGVAVLTEEEFWRFLK   662
-FMLVYKFARKHHITLTNLITEETTHVVMKTDAEFVCERTLKYFLGIAGGKWVVSYFWVTQSIK  1724
hhhhhhhhhh    eee      eeee      hhhhhhhh    eee hhhhhhhhh
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B   **C**

(Figure 2.1) Characterization of the three purified constructs, p140(375-480), p140(392-480) and p140(403-480). **(A)** Alignment of the amino acid sequences of various BRCT domains including: the N-terminal BRCT domain of human BRCA1 (BRCA1_HUMAN1), the BRCT domain of NAD= dependent DNA ligase from *Thermus filiformis*, the BRCT domain of the p140 subunit of *Drosophila melanogaster* RFC (RFC1_DROM) and the BRCT domain of the p140 subunit of human RFC (RFC_HUMAN). The alignment is recreated according to the original alignment described by Bork *et al.*, 1997 (1) by using Clustal W. Identical residues are highlighted in black while similar residues are shown in grey. Gaps are shown as dashes. At the bottom of the alignment, the secondary structures found in the crystal structure of human BRCA1 BRCT1 are indicated in e (β-strand) and h (α-helix). The vertical arrows indicate the first amino acid of each of the three N-terminal deletion constructs. All three constructs included sequences up to residue 480. **(B)** The calibration curve for the Superose 12 column was generated with **1**. γ-globulin (670 kDa), **2**. ovalbumin (44 kDa), **3**. myoglobin (17 kDa), **4**. aprotinin (6.5 kDa), **5**. vitamin E (1.35 kDa) and **6**. tryptophan (0.17 kDa). The elution position of each protein is indicated with arrows. The estimated molecular mass of each is given in the text. **(C)** CD spectra were measured for the three constructs. The far UV-CD spectra of all three constructs have characteristic minima at 208 nm and 220 nm, reflecting α-helical and β-strand contents. The percentages of each secondary structure were predicted by the K2d program (26).

Protein-DNA complex formation was detected using a gel retardation assay in which purified proteins were titrated into a constant amount of a 5' [32P] labeled, 10 bp, hairpin oligodeoxynucleotide (Table I, Figure 2.2A). Only the protein containing 28 residues N-terminal to the predicted boundary of the BRCT domain (p140(375-480)) demonstrated DNA binding activity. The CD spectra of both RFC p140(403-480) and p140(392-480) were similar to that of the p140(375-480) protein indicating that the lack of DNA binding is likely not due to gross structural rearrangement or inability to fold (Figure 2.1C).
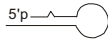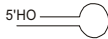
A

(403-480) (392-480) (375-480)

B

wt   R436G

10   5   10   20   100   200   (DNA:Protein)
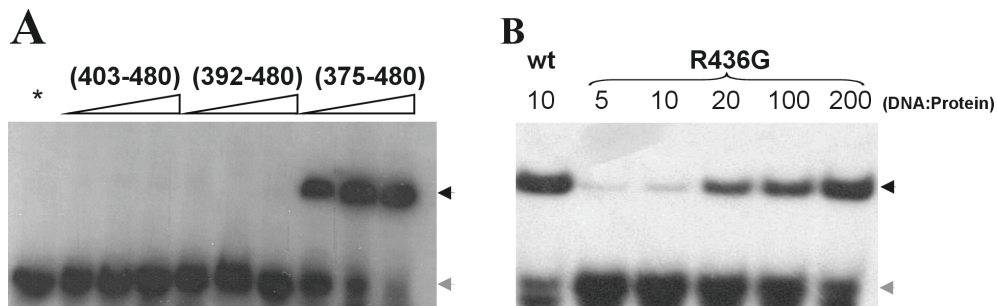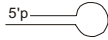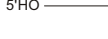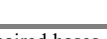
(Figure 2.2) Extent of the protein domain required for DNA binding.
(A) Gel retardation experiments were used to detect DNA binding to the 5' ^{32}P labeled oligodeoxynucleotide (oligo 1, Figure 2.1). Increasing amounts (40fmoles, 200fmoles, and 400fmoles) of the indicated constructs of hRFC p140 were titrated into 40fmoles of DNA. The dash indicates a no protein control. The black arrow indicates the DNA-protein complex and the gray arrow indicates the unbound DNA. **(B)** Amino acid substitution at the conserved glycine 435 to arginine (G435R) reduced the DNA binding activity of the p140(375-480). The numbers above the gel lanes indicate the protein to DNA ratio. The amount of DNA used was 40fmoles. To achieve a similar amount of DNA-protein complex observed for wt in G435R mutants, roughly 20 fold higher protein:DNA ratio was required (lane DNA:protein ratio of 200).

An amino acid substitution at the conserved G435 to R in p140(375-480) reduced DNA binding by almost 20 fold in comparison to the wildtype (wt) protein (Figure 2.2B). This reduced DNA binding due to the mutation suggests that dsDNA binding is contributed by the both BRCT domain and the N-terminal sequence outside of the domain. The mutated glycine occurs within the most conserved GG repeat found in the BRCT family and forms a tight turn between the α1 and β2 structures of the X-ray crystal structure of the BRCT domain from XRCC1 (27). The substitution of a bulky, charged residue like arginine could potentially result in destabilization of the fold of the protein, which may have been indicated by the precipitation behavior of the G435R mutant observed at room

temperature and the lower yield during purification[1] compared to the wild type (data not shown).

(Table 2.1) The sequence of oligonucleoides used in this study.

Name	Duplex size	DNA sequence	Structure
Oligo 1	10 bp	pCTCGAGGTCG**TCAT**CGACCTCGAGATCA	
	9 bp	pCTCGAGTCG**TCAT**CGACTCGAGATCA	
	8 bp	pCTCAGTCG**TCAT**CGACTCAGATCA	5'p⎯◯
	7 bp	pCTAGTCG**TCAT**CGACTAGATCA	
	6 bp	pCTGTCG**TCAT**CGACAGATCA	
Oligo 2	9 bp	pCTCGATGTCG**TCAT**CGACCTCGAGATCA	5'p⌢◯
5'OH	10 bp	CTCGAGGTCG**TCAT**CGACCTCGAGATCA	5'HO⎯◯
5'Pb	10 bp	pCTCGAGGTCG**TCAT**CGACCTCGAG	5'p⎯◯
5'B	20 bp	bTGCAGATTGCGCAATCTGCA	5'b ═══ 5'b
Ss	Single stranded	TGGGGTGGGGT	5'HO ⎯⎯
5'Ps	Single stranded	pTGGGGTGGGGT	5'p ⎯⎯

[1.] For hairpin oligonucleotides, the hairpin sequence is highlighted with bold letters. The unpaired bases of oligo 2 are indicated in italics. A "p" indicates 5' PO$_4$ while "b" indicates a 5' biotinylated. [2.] The consensus sequence for the C/EBP DBD (bZIP) binding site.

Specificity of DNA binding

To determine the stoichiometry of the DNA-protein complex, a titration experiment was performed by adding an increasing amount of p140(375-480) to a constant amount of oligo 1 (Figure 2.1). The protein-DNA complex was separated from unbound DNA through an analytical scale gel filtration column and the absorbance peaks corresponding to the free DNA and DNA-protein complex were integrated and the fraction of the DNA–protein complex was calculated. The fraction of DNA bound to protein

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[1] The quantity of the mutant G435R was carefully measured by using both the UV absorbance and the Bradford protein assay prior to use. Due to the poor stability, the mutant could not be reached to the suitable concentration for CD measurement.

increased almost linearly until a DNA-protein ratio of 1.25:1 and starts to saturate after a ratio of 1:1.2 (Figure 2.3A). The experimental titration curve closely resembles an ideal titration curve of a DNA-protein complex ratio of 1:1. We also analyzed the 5' PO4 specific DNA binding activity using the gel shift assay (Figure 2.3B). A unique DNA-protein complex was formed between p140(375-480) and oligo 1 under all titration conditions tested. Relative amounts of free and bound DNA were quantified using a phosphor-imager and the data was analyzed to yield a KD ~ 10 nM. The fit of the binding isotherm was performed by assuming that one p140(375-480) binds to a single DNA binding site (See Methods). Using a protein concentration close to KD,, competition binding between two different oligonucleotide ligands was performed by mixing the 5'[32P] labeled oligo 1 (10 base pairs) with an excess of various oligonucleotides (Figure 2.1) prior to the addition of p140(375-480). Competitive binding to the protein is detected as a reduction in the amount of protein-DNA complex observed in the gel retardation assay (Figure 2.3C).   As expected from previous studies (17;18), binding to the 5' phosphorylated hairpin oligonucleotide could not be competed with either a 5' OH hairpin oligonucleotide, a ds 5' biotinylated, or 5' OH single stranded oligodeoxynucleotide (Figure 2.3C). Competition was slightly more effective with dsDNA with a recessed 5'-phosphate end than with blunt ended DNA (Figure 2.3C) correlating with the earlier report that the *Drosophila* RFC p140 (18) binds 5' end recessed dsDNA better than blunt ended DNA. DNA binding was also insensitive to salt up to 500 mM NaCl and not dependent on Mg2+ (data not shown).

In order to precisely determine the binding determinants of the DNA, we synthesized a series of 5' phosphorylated oligonucleotides in which the ds region was successively shortened by one base pair. As seen in Figure 2.3D, 8-10 base pairs compete effectively to the protein binding to the oligo 1 indicating that 8-9 base pairs are required for optimal binding of RFC p140 (375-480). Binding to oligonucleotides with duplex regions longer than 10 base pairs was not more efficient than binding to oligo 1(data not shown). In addition, fully base-paired DNA forms the best ligand since a mismatched-Watson-Crick base pair 6 nucleotides downstream from the 5' phosphate (oligo 2) reduced the affinity of the protein-DNA interaction (Figure 2.3D).

A

B

Ratio RFC p140(375-480) to DNA

C

D
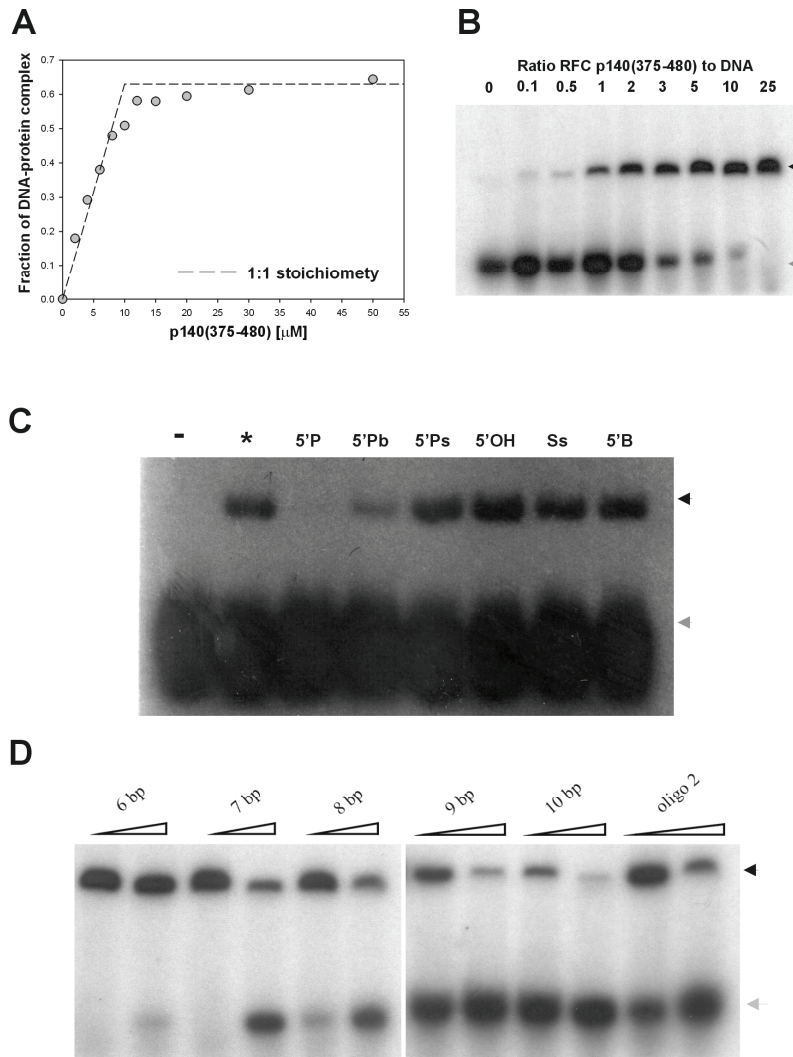
(Figure 2.3) Characterization of the DNA binding requirements of RFC p140(375-480). **(A)** Stoichiometric titration of protein-DNA complex. A constant amount of oligo 1 (10 μM) was titrated with increasing amount of RFC p140(375-480) and the resulting DNA-protein complex was separated from the unbound DNA through an analytical gel filtration column. **(B)** Titration of protein-DNA complex. A constant amount 40fmoles of 5' ^{32}P labeled oligo 1 was titrated with increasing amount of RFC p140(375-480) in a gel retardation assay. The protein:DNA ratio is indicated at the top of each lane. Only one DNA-protein complex (black arrow) was observed over the titration range. **(C)** A competition gel retardation assay was used to delineate the chemical features of DNA required for binding by RFC p140(375-480). 40fmoles of 5' ^{32}P labeled oligo 1 was mixed with a 50 -fold excess of the competing DNA and subsequently 200fmoles of protein was added. The competing DNA was a unlabeled oligo 1 (5'P), 5' PO_4 blunt end (5'Pb) hairpin-oligonucleotide, 5' PO_4 single stranded (5'Ps) oligonucleotide, 5'OH version of oligo 1 (5'OH),), a single stranded (Ss) and the 5' biotinylated C/EBP oligonucleotide (5'B) oligonucleotide (see details on the Table I). **(D)**. Competition gel retardation assay to characterize the extent of dsDNA required for binding. A constant amount 40fmoles of ^{32}P labeled oligo 1 was mixed with a 10 or 60 fold excess of the indicated unlabeled DNA (see Figure 2.1) and allowed to bind to 200fmoles of protein. The black arrow indicates DNA-protein complexes and the grey arrow indicates unbound DNAs.

Altered DNA binding activity in p140(375-545)

Since sequences N-terminal to the conserved BRCT domain were required for DNA binding, it was logical to determine the effect of sequences C-terminal as well. Accordingly a fragment of RFC p140 including amino acids 375 to 545 was cloned. The p140(375-545) contained a c-terminal six histidine tag and was purified to apparent homogeneity in the same manner as the wildetype protein. Using the gel shift assay, we titrated increasing amounts of the protein into the same 5' phosphorylated, hairpin oligonucleotide. As shown in Figure 2.4A, the C-terminally extended RFC p140(375-545) behaves differently than the shorter RFC p140(375-480). First, the affinity for DNA binding as a whole is reduced. Second, a single complex is never observed, rather two complexes with slightly different mobility (Figure 2.4, arrows), yet with similar apparent DNA binding affinities can be observed. The sum amount of the two complexes reached a maximum ~25% of the total labeled oligo1 when the p140(375-545) concentration was 150nM (Figure 2.4A, lane marked 20). As the ratio of protein to DNA is further increased, the amount of these two complexes decreases and broad bands migrating significantly slower are observed (see Figure 2.4A lane marked 200). In order to better understand the basis of these differences we determined which characteristics of the DNA ligand were necessary for binding using the competition gel shift assay (Figure 2.4B).
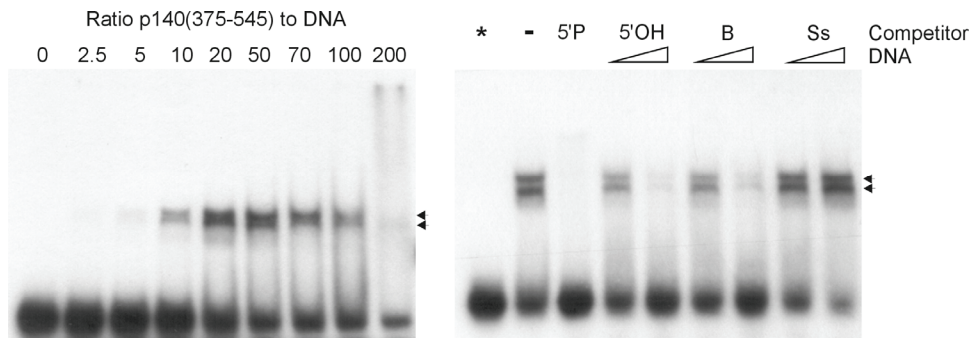
(Figure 2.4) Characterization of DNA binding requirements of RFCp140 (375-545). **(A)** Titration of DNA-protein complex. 90fmoles (7.5 nM) of 5' ^{32}P labeled oligo 1 was titrated with increasing amount of p140 (375-545). The numbers o the lanes indicate the ratio of protein :DNA. **(B)** A competition gel retardation assay was used to delineate the chemical features of DNA required for binding by RFC p140(375-545). 90fmoles of 5' ^{32}P labeled oligo 1 was mixed with a 10 or 20 fold excess (only 10 fold for oligo 1) of the competing DNA and subsequently 1.8pmoles of protein was added.

In contrast to the RFC p140(375-480) fragment, in the absence of poly(dI-dC)[2] in the reactions, a 5' OH hairpin oligonucleotide competed with the 5' phosphorylated hairpin oligonucleotide for protein binding in both complexes. Similarly, a double stranded 5' biotinylated oligonucleotide competed as efficiently as the 5' OH hairpin oligonucleotide. However competition by these alternative ligands was not as effective as that by the 5' phosphorylated hairpin, where only a 10 fold excess was necessary to completely block binding to the labeled oligonucleotide (Figure 2.4B).

In order to assess whether the non-specific dsDNA binding by p140(375-545) is due to an independent binding activity of the C-terminal aa's 480-545 alone or requires the BRCT domain, two further proteins consisting of RFC p140 residues 480-545 and 403-545 were expressed and purified. These two soluble proteins, p140(403-545) and p140(480-545) were purified but did not bind oligo1 in the gel shift assay (Figure 2.5). This observation suggests that p140(480-545) does not bind DNA independently but that an alternative DNA binding site maybe created by a combination of the p140(375-480) and the aa's 480-545.
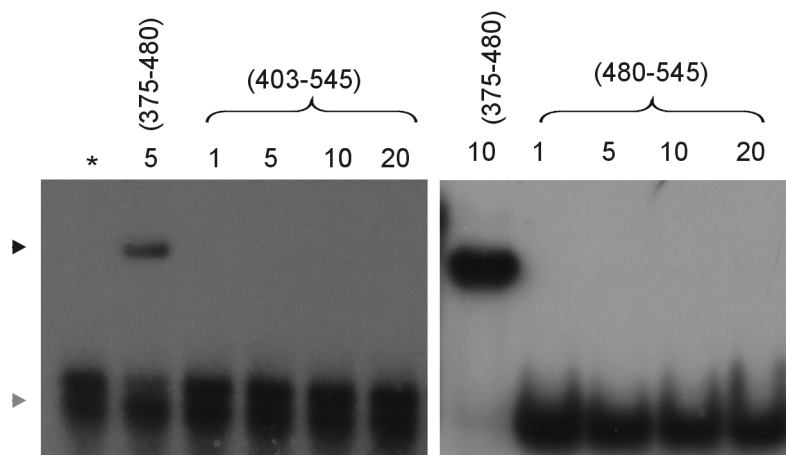
(Figure 2.5) Analysis on DNA binding activity of p140(403-545) and p140(480-545). Constant amount, 40 fmoles of 5' [^{32}P] oligo 1 was titrated with various peptide constructs of p140 (the residue numbers are described on the lanes). The number on each lane indicates the ratio of protein : DNA concentrations in each lane. Asterisk indicates lane with only oligo 1. DNA-protein complex formed by p140(375-480) and unbound oligo1 are indicated by black and grey arrows respectively. No apparent formation of DNA-protein complexes were observed for p140(480-545) and (403-545).

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[2] An excess amount of Poly(dI-dC) was used to reduce non-specific protein-DNA interaction and maintain a constant ratio of protein to DNA (see Materials and Methods).

As expected, p140(375-545) was also capable of binding to the 5' biotinylated ds oligonucleotide (5'B in Figure 2.1) when the DNA-protein complex was detected with streptavidin-HRP (Supplementary materials Figure S1). This biotinylated oligonucleotide contained a consensus binding sequence for the bZIP domain of C/EBP-α, which is also known to interact with the p140(375-480) while binding to its cognate DNA to form a ternary complex (28). However no such complex were observed in the presence of bZIP and p140(375-545), and instead we observed two separate DNA-protein complexes that appear to be the result of independent DNA binding by bZIP or p140(375-545) (Supplementary materials Figure S1A). The ternary complex was only observed when the 5'-biotin was replaced by a phosphate. The simplest explanation for the ternary complex is that bZIP binds its cognate sequence simultaneously to p140(375-545) binding to the 5' phosphorylated terminus (Figure S1B).

Structural Characterization of the RFC p140 BRCT Region

We used NMR to characterize the folded state of the three different fragments of RFC p140. The [^{15}N,^{1}H] HSQC experiment, which detects the 1 bond correlation within an amide moiety, is particularly useful for this purpose. Since 1 peak should be observed for every non-prolyl residue, the folded state of a protein can rapidly be determined by analyzing the dispersion and linewidth of the peaks in the spectrum (Figure 2.6). The spectrum in Figure 2.5 implies that large portions of the p140(375-480) protein are conformationally flexible in the absence of DNA as indicated by the poor dispersion of resonances (see the overlapped peaks in the region from 7.8 to 8.6 ppm) and by the presence of very broad peaks indicative of exchange that is intermediate on the NMR time scale (e.g. kex ~ Δδ (Hz)[3], indicated by arrows in Figure 2.6).

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[3] When a nucleus experiences two or more states with different chemical properties, it is in exchange. For example a nucleus experiencing two different environments due to conformational exchange between A and B states would have two different chemical shifts (δ_A and δ_B). Then the NMR signal(s) of the nucleus depend on the exchange rate (k), relative to the difference in the chemical shifts of the two states ($\Delta\delta = \delta_A - \delta_B$ in frequency unit s^{-1}). The exchange rate can be used to describe three extreme regimes; fast exchange (k << Δδ), intermediate (k ~ Δδ), and slow (k >> Δδ). In the slow exchange regime, two distinctive resonances representing separate conformational states can be observed. In the fast exchange regime, a single chemical shift, representing the stoichiometrically averaged resonance, is observed. In the intermediate state, the two resonances coalesce resulting in a single broad, often undetectable, resonance.
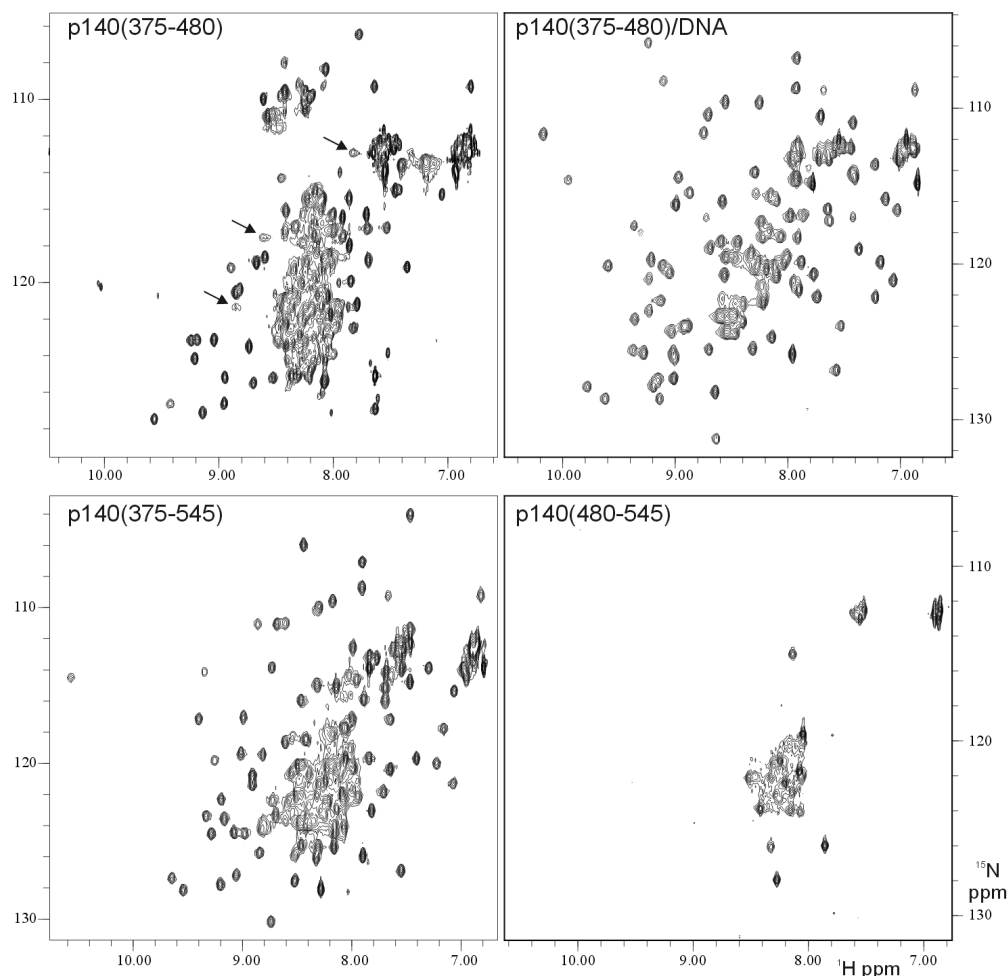
(Figure 2.6) [^{15}N,^{1}H] HSQC NMR spectra of the various constructs of RFC p140. Across the top the spectra of the free protein and the complex formed between p140(375-480) and oligo 1 are shown. The HSQC spectra of RFC p140 (375-545) and p140(480-545) are presented across the bottom. NMR spectra were measured at 298 K at 600 MHz. Examples of some of the resonances with unusually broad lines indicating intermediate exchange behavior are shown with arrows. The spectra of p140(480-545) is clearly indicative of an unstructured protein.

Sharp peaks deriving from the backbone amide moiety of the unstructured 6 His tag can also be seen (^{1}H 7.4 –7.7 ppm, ^{15}N 124-128 ppm). In contrast, the spectrum of the DNA bound protein in Figure 2.6 shows good peak dispersion and more uniform linewidth. The [^{15}N,^{1}H] HSQC spectrum of RFC p140(375-545), with its good peak dispersion and uniform linewidth, indicates a well ordered protein, even in the absence of DNA (Figure 2.6). However, the [^{15}N,^{1}H] HSQC spectrum of RFC p140(480-545), characterized by very poor peak dispersion and narrow linewidths, suggests that this protein fragment is completely unstructured in solution (Figure 2.6).

53

Structure of DNA bound p140(375-480)

Since the minimal DNA binding domain includes the N-terminal sequences outside of the conserved BRCT domain, we wished to ascertain whether or not it contained elements of regular secondary structure. As a first approach we used the PSIPRED secondary structure prediction service (29). The N-terminal sequences of the BRCT region were predicted to form an α-helix with a high degree of confidence (Figure 2.7). As a control we performed similar analysis on other BRCT domain sequences with known structures and obtained a close correlation between the predicted and experimentally determined elements of secondary structure (Figure 2.7). As an example we show the alignment of the N-terminal BRCT domain of XRCC1, which forms specific, heterologous protein-protein complexes. The PSIPRED (v. 2.4) predicted secondary structure of the XRCC1 BRCT domain closely matches the secondary structure elements defined by the crystal structure (not shown). However, the region N-terminal to the BRCT domain is predicted to be in a random coil conformation.

Once the sequential chemical shift assignment of a protein is known, the chemical shift index (CSI) is a useful means of correlating the so-called secondary chemical shift (the difference between the observed chemical shift and that expected for the same residue in random coil) with the secondary structure of a protein (30). We have used 13C,15N labeled protein in conjunction with standard triple resonance NMR experiments, to determine the essentially complete sequential assignment of the p140(375-480)-DNA complex (Chapter 4 supplementary materials Table 1S) (31) and have obtained a preliminary backbone assignment of the free protein. CSI analysis of the NMR data (Chapter 4 supplementary materials Figure 1S) was used to determine the secondary structure of the DNA bound RFC p140(375-480) (Figure 2.7). The secondary structure of residues 410 to 480 is consistent with that of other BRCT domains whose 3D structure has been determined by X-ray crystallography and closely matches that predicted by PSIPRED. Importantly, the α-helix between residues 379 and 386 that is predicted by PSIPRED is experimentally confirmed by the CSI analysis of the backbone chemical shifts.

Our preliminary backbone resonance assignment of RFC p140(375-480) in the absence of DNA indicates that the secondary structural elements are retained but they are shorter, that is all 4 helices are present but each is reduced by one or two residues at either end as are the strands of the central β-sheet (data not shown). This analysis however is

complicated by the dynamic behavior of the unbound protein, a significant portion of which is either in intermediate conformational exchange on the NMR time scale or in chemical exchange with 1H's of water. As a result, there are gaps in the sequential assignment of the isolated p140 (375-480). The reduced secondary structure contents in the free protein suggested by NMR is also supported by the secondary structure contents derived from the CD of the free p140(375-480) (Figure 2.1C), which predicts 26% and 15% for α-helices and β-strands respectively in comparison to the CSI predicted values of 31 % and of 21% for α-helices and β-strands respectively in the DNA bound p140(375-480) (Figure 2.7).
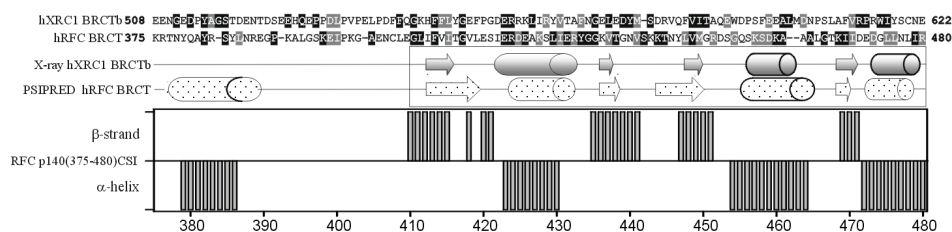
(Figure 2.7) Secondary structure analysis of RFC p140(375-480). The sequence alignment of human XRCC1 BRCTb and human RFC p140 BRCT region was performed as described in Figure 2.1A. The secondary structure elements, arrows for a β-strands and cylinders for α helices, are aligned with the corresponding amino acid sequences based on the X-ray crystal structure of XRCC1 (11) and PSIPRED (26) prediction of hRFCp140 (375-480). The consensus secondary structure predictions of hRFC p140 (375-480) by the chemical shift index method was generated from NMR Assignment data (31) using the Cα, Cβ, and Hα chemical shifts as input.

Discussion

Domain Boundaries required for DNA binding

The results presented here (summarized in Figure 2.8) confirm and extend the work of others (1;2;17;18) concerning this interesting region of the p140 subunit of RFC. A minimal DNA binding domain appears to comprise residues 375 to 480 of human RFC p140, a region that contains 28 more residues at the N-terminus than the conserved BRCT domain. The importance of the N-terminal extension was indicated by the complete absence of DNA binding of N-terminally truncated proteins (Figure 2.8). Furthermore a mutation at one of the highly conserved glycine repeats found in the BRCT domain resulted in a reduction in DNA binding activity of p140(375-480) indicating that the binding necessitates both the intact N-terminal sequence and the BRCT domain. In yeast,

an equivalent single amino acid substitution at the conserved G193 to R in the BRCT domain of Rev1 reduced the efficiency of the lesion T−T pyrimidine pyrimidinone adduct bypass *in vivo* (32). Rev1 is a deoxycytidyl transferase, which adds dCMP opposite an abasic (apyrimidinic/apurinic) site generated by glycosylase activity at sites of DNA damage to bypass the damaged site. In addition, participation of Rev1 in bypass of a T−T pyrimidine pyrimidinone adduct has been reported (32). While the dCMP transferase activity is not required for bypass of T-T lesions, the G193R mutation suggests that the BRCT domain is (32).

Fragment	DNA binding
375-545	5'PO$_4$/general dsDNA
375-480	5'PO$_4$ dsDNA
403-480	N/D
392-480	N/D
403-480	N/D
480-545	N/D
375-480	Reduced 5'PO$_4$ dsDNA

375   403   480   545
BRCT

G435R

(Figure 2.8) Summary of DNA binding study. N/D means "Not Detected". * indicates the amino acid substitution. The data of DNA binding by 403-545 and 480-545 are presented in the Appendix A1.

Specificities of DNA binding by p140(375-480)

Secondary structure based on NMR data indicates that the DNA bound p140(375-480) consists of an extra α-helix at the N-terminus followed by a loop that connects to the BRCT domain which consists of three α-helices and four β-strands. The p140(375-480) protein fragment has an absolute requirement for a free 5' phosphorylated terminus, and optimal binding requires 10 base pairs of fully duplex DNA. The binding stoichiometry of the DNA-protein complex is 1: 1, which reflects its specific binding to the single available 5'-phosphate on the hairpin oligonucleotide. However, there appears to be no restriction on the sequences that form the duplex region. Thus it is likely that the majority of protein contacts are made to the backbone and 5' phosphate of the DNA. Whether or not the BRCT domain actually contacts all ten base pairs of DNA awaits the elucidation of the structure of the complex. Alternatively, it is possible that stability of the duplex became limiting in our gel-shift experiments as the number of base pairs was reduced. This does not seem

likely however, since the entire assay was performed at 4 °C, a temperature at which even the short 6 bases duplex should be stable in the context of a hairpin oligonucleotide.

DNA binding model of p140(375-545)

Expression of a larger fragment of RFC p140 encompassing residues (375-545) resulted in a protein with different structural and DNA binding properties. RFC p140(375-545) forms two different complexes with oligo 1 that are resolvable in the gel retardation assay however with a much reduced DNA binding compared to that of the p140(375-480). The two complexes can be competed by DNA containing either a non-natural biotin or a hydroxyl group at the 5' position and therefore likely result from non-specific, dsDNA binding. This non-specific DNA binding of p140(375-545) is not due to the independent activity of the C-terminal aa's 480-545 because the isolated p140(480-545) is inactive in DNA binding. Instead the C-terminal aa's 480-545, may sterically hinder access to the putative 5' phosphate binding motif present in the residues 375-480 resulting in the reduced 5' phosphate-dependent DNA binding. Meanwhile the non-specific dsDNA binding is likely mediated by the aa's in the N-terminus 375-403 and the C-terminus 480-545, since the preferential non-specific dsDNA binding is present only in p140(375-545) and neither in p140(403-545) or p140(375-480) (Figure 2.8). However this model does not explain the observed two distinct DNA-p140(375-545) complexes. Further study is therefore necessary to understand in how the non-specific dsDNA binding and two distinct complexes are formed by p140(375-545).

While the p140(480-545) does not have a fold by itself, it is likely to take a part in the overall structure of p140(375-545), which is structurally better defined than p140(375-480), as judged by the good peak dispersion and relatively homogenous linewidth distribution exhibited in the [^{15}N,^{1}H] HSQC spectrum. Consequently the participation of aa's 408-545 in the structure of p140(375-545) may result in a preformed non-specific dsDNA binding site, which lacks in the p140(375-480). In contrast, a significant fraction of the shorter p140(375-480) peptide is likely in conformational exchange in the absence of its ligand but becomes more rigid upon binding DNA. Therefore it seems that the complete structural unit in the intact protein comprises residues 375-545.

The dual modes of DNA binding helps to explain observations that made by others working on RFC. The groups of Burgers and Hurwitz observed that 5 subunit complexes of both yeast (22) and human (23) RFC formed with an N-terminally truncated

p140 subunit (trRFC), in which the BRCT region was deleted, supported PCNA dependent DNA replication by polδ more efficiently than complexes with full-length p140. The difference in DNA synthesis activity between the native RFC complex and the trRFC was greater for the synthetic substrate poly dA-oligo dT than for singly primed M13 DNA. Since neither substrate provides a ds, 5' PO4 terminus it is likely that the non-specific dsDNA binding activity of the amino acid region 375-545 "trapped" RFC away from the 3' terminus where it is required for PCNA loading. This was further supported by the observation that trRFC exhibited a significantly lower non-specific dsDNA binding activity than the native RFC (33). Since poly dA-oligo dT has considerably more dsDNA than singly primed M13, non-productive binding would be expected to be greater on this substrate.

It was recently shown that hRFC p140 encompassing residues 361-545, which includes the sequences used in this study, can physically and functionally interact with the bZIP domain of transcription factors, C/EBPα, C/EBPβ, CREB, Jun and ATF2 (28). We have been unable to detect any evidence of direct protein-protein interaction between the BRCT region of the RFC p140 and the bZIP domain of C/EBPα using essentially the same proteins and solution conditions described in this work (28). We have however, demonstrated simultaneous binding of both proteins to short oligonucleotides bearing the C/EBPα recognition sequence and a 5' PO$_4$ (Supplementary materials S1) thereby providing an alternate explanation for the results described earlier (28).

The function of the entire N-terminal half of the p140 subunit of RFC, including the DNA binding domain discussed here, remains unclear. Deletion of this region in yeast did not result in an observable phenotype (22). *In vitro*, the DNA binding region of RFC p140 is not required for loading PCNA onto DNA nor for the elongation of primed DNA template (23;34). In light of recent observations indicating that many or possibly all, BRCT domains are phosphoserine binding modules, it is fair to ask the question as to whether the observed DNA binding activity is not an artifact of a binding site designed to interact with a phosphate group. While it is not yet possible to give a definitive answer to this question, the weight of evidence does not support it. DNA binding is tight (KD ~10 nM) and specific for 5' phosphorylated dsDNA rather than the single stranded DNA. Both 5' PO4 specific and non-specific, dsDNA binding modes are observed in the full-length protein suggesting that they are not an artifact of the truncated proteins used in this study. It is difficult to

rationalize the non-specific dsDNA binding as a byproduct of the phosphate specificity. Further, the 5' PO4 specific mode of binding could be biologically relevant since a substrate would be available on the lagging strand upon encountering a previously completed Okazaki fragment. Interestingly, recent data in prokaryotes suggests that stalled replication forks reinitiate daughter strand synthesis downstream of blockage sites thereby providing another potential source of 5' phosphorylated DNA termini (35;36). Of course, what the function of a 5' PO4 bound RFC complex would be remains pure conjecture and therefore the focus of ongoing studies.

Supplementary materials

S1.

Interaction with the DBD (DNA binding domain) of C/EBPα

It has been reported that RFC can modulate the activity of transcription factors of the C/EBP family (28). In this report, the authors suggest a direct protein-protein interaction between the BRCT region of RFC and the bZIP DNA binding domain of C/EBPα. In order to investigate this interaction further we cloned the bZIP domain of C/EBPα and expressed it in *E. coli* with a His6 tag. We used 5' biotinylated oligonucleotides containing the C/EBP consensus binding sequence in a gel shift assay to detect binding to the purified bZIP protein (See Methods). As expected, a unique bZIP-DNA complex was observed (Figure S1A lane 3). 5' biotinylation was used to prevent interaction with RFC p140 (375-480) *via* 5' phosphate specific binding. As expected from the data in Figure 2.4, RFC p140(375-545) is also capable of binding the 5' biotinylated oligonucleotide (Figure S1A, lane 1) while RFC p140(375-480) did not (data not shown). When both the bZIP DBD of C/EBPα and RFC p140(375-545) were added, no additional, slowly migrating band was observed. Instead two distinct bands migrating at the same rate

as when the two proteins were added individually (Figure S1A, lane 5) were observed, suggesting that no ternary complex is formed between DNA, bZIP DBD and p140(375-545).

However, when the 5' biotin was replaced with a 5' phosphate while keeping the C/EBPα recognition sequence, very different results were obtained (Figure S1B). In this case all three proteins bound the CREB oligonucleotide (RFC p140(375-480), p140(375-545) and C/EBPα DBD) and migrated at different rates (lane 2, 3 and 4 from the left). When the C/EBPα DBD is combined with either RFC p140(375-480) or RFC p140(375-545), multiple complexes were observed. First, a band corresponding to DNA-DBD and DNA-RFCp140 complexes can be discerned individually. Second, a more slowly migrating complex is observed. The amount of the slow migrating complex increases with the amount of RFC p140 added. This slowly migrating complex likely corresponds to one RFC p140 and one C/EBPα DBD bound to the same oligonucleotide. Taken together with the observation made in Figure S1A, the slowly migrating complex appears to be the result of two separate events: one, that the C/EBPα DBD binds its cognate DNA sequence while two, RFC p140(375-480) or p140(375-545) binds the 5' phosphorylated terminus. The data clearly indicate that the slowly migrating band is not the result of a physical interaction between the two different proteins. At the bottom of the gel, the free DNA migrates as two distinct complexes. These complexes correspond to a majority of intermolecular (upper band) and a minority of intramolecular (lower band) annealed strands deriving from the self-complementarity of the oligonucleotide.
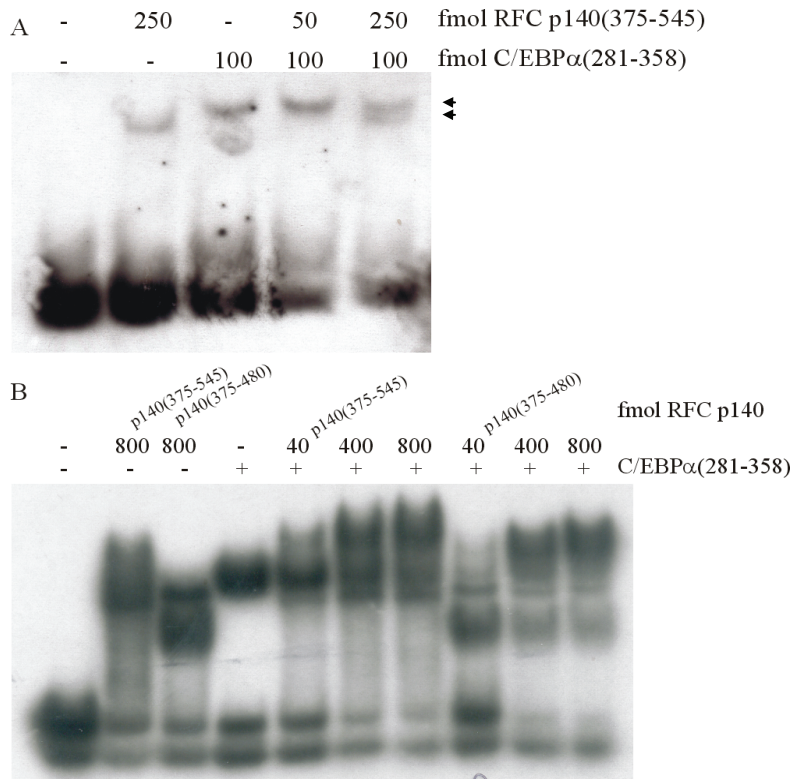
A   -   250   -   50   250   fmol RFC p140(375-545)

     -      -   100   100   100   fmol C/EBPα(281-358)

B   p140(375-545)   p140(375-480)   p140(375-545)   p140(375-480)   fmol RFC p140

    -     800   800   -   40   400   800   40   400   800

    -     -   -   +   +   +   +   +   +   +   C/EBPα(281-358)

Figure S1. RFCp140 (375-545) does not interact with bZIP domain of C/EBPα.
(A). A gel retardation assay was used to investigate the interaction between RFCp140 (375-545) and the bZIP domain (281-358) of c/EBPα. 100fmoles of a 5' biotinylated oligonucleotide (5'B in Table I) containing the C/EBP binding sequence was mixed with the indicated amounts of either RFCp140 (375-545), C/EBPα (281-358) or both proteins together (black arrows). A complex containing both RFC and C/EBPα is not observed. **(B)**. For comparison a similar experiment was performed using the same oligonucleotide as in A that was 5' labeled. The quantity of the C/EBPα bZIP domain (790 fmoles) and DNA (40 fmoles) was held constant, while the indicated amount of either of the two RFC p140 constructs was titrated in. The free DNA migrates as two bands indicating both inter- and intra-molecular hybridization of the self-complementary sequence. When a sufficient quantity of either of the two RFC p140 constructs is added, a band corresponding to just RFC bound DNA as well as a super-shifted band that appears to contain both RFC and C/EBPα can be observed.

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