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Kin17, a mouse nuclear zinc finger protein that binds preferentially to curved DNA

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ABSTRACT

Kin17 is a 45 kDa protein encoded by the KIN17 gene located on mouse chromosome 2, band A. The kin17 amino acid sequence predicts two domains, which were shown to be functional: (i) a bipartite nuclear localization signal (NLS) that can drive the protein to the cell nucleus, (ii) a *bona fide* zinc finger of the C_2H_2 type. The zinc finger is involved in kin17 binding to double-stranded DNA since a mutant deleted of the zinc finger, kin17Δ1, showed reduced binding. Single-stranded DNA was bound poorly by kin17. Interestingly, we found that kin17 protein showed preferential binding to curved DNA from either pBR322 or synthetic oligonucleotides. Binding of kin17 to a non-curved DNA segment increased after we had inserted into it a short curved synthetic oligonucleotide. Kin17Δ2, a mutant deleted of 110 amino acids at the C-terminal end, still exhibited preferential binding to curved DNA and so did kin17Δ1, suggesting that a domain recognizing curved DNA is located in the protein core.

INTRODUCTION

A 45 kDa mouse protein is expressed by KIN17, a gene located on mouse chromosome 2, band A, near the vimentin gene (1). The *KIN17* cDNA sequence (EMBL accession number: X58472) codes for a 391 amino acid protein corresponding to a 44 643 Da molecular weight (1). The kin17 amino acid sequence displays two putative regions of interest: (i) a bipartite nuclear localization signal, and (ii) a zinc finger motif. The two motifs are similar to those observed in some other nuclear proteins (2), one of which is poly(ADP-ribose) polymerase (PARP), an enzyme involved in the mammalian cell response to DNA damage (3).

It was of interest to investigate whether the two *in scripto* motifs were functional. Indeed, we have shown here that the bipartite nuclear localization signal can drive the protein to the cell nucleus. The zinc finger is also functional and is required for kin17 binding to double-stranded DNA.

Since we observed that the mouse protein kin17 was likely located in the cell nucleus, we sought to determine the binding of kin17 protein to various DNA substrates. After having shown that kin17 binds to single-stranded poorly but to double-stranded DNA efficiently, we asked whether the binding occurred at random or was targeted to specific segments.

Interestingly, we found that kin17 binds preferentially to curved DNA, its binding efficiency being correlated with the magnitude of DNA curvature.

The next question was: is the zinc finger or another domain involved in preferential binding to DNA? In order to answer this question, we constructed two plasmids that were able to produce two altered proteins: (1) kin17Δ1, a mutant deleted of the zinc finger located at the N-terminus, and (2) kin17Δ2, another mutant deleted of 110 amino acids at the C-terminus.

As kin17, the two proteins kin17Δ1 and kin17Δ2 bind preferentially to curved DNA, suggesting that: (i) the zinc finger is dispensable for preferential binding to curved DNA and (ii) another domain recognizing curved DNA is involved. This domain is located outside of the two deletions in the core of the protein.

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli K12 strains used in this work are described in Table 1. GY7648, a Δ*recA* derivative of BL21 (4), was constructed by A.Bailone. This strain carries the T7 RNA polymerase gene under the control of the IPTG-inducible *lacpUV5* promoter. Human HeLa S3 cells were from the de Murcia's laboratory stock (5).

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Plasmid pKGC102 carries *KIN17* cDNA, which can be expressed under T7 promoter control (1). From pKGC102, we derived two other plasmids, pKGC103 and pKGC104.

Plasmid pKGC103 encodes kin17Δ1, which is deleted of amino acids 6–70. Plasmid pKGC102 was digested with *Nco*I and *Eco*RI restriction endonucleases, the 211 bp long *Nco*I–*Eco*RI fragment encompassing the 5'-region of *KIN17* cDNA was removed and replaced with a synthetic oligonucleotide; the first strand was 5'-CATGGGCAAGTCGGATG-3' and the second 3'-CCGTTCAGCCTACTTAA-5' (*Nco*I and *Eco*RI sticky ends are underlined). KIN17Δ1 open reading frame (ORF) codes for a protein of 326 amino acids with a calculated molecular weight of 36 938 Da. Electrophoresis of kin17Δ1 gives a 37 kDa value in agreement with the theoretical value.

Plasmid pKGC104 encodes kin17Δ2, a mutant of kin17 protein whose C-terminal part was truncated as follows. We removed from the KIN17 sequence an *Acy*I–*Sma*I restriction fragment from nucleotides 854 to 1034. Such a deletion led to a frameshift. Thus, the DNA sequence downstream the deleted region codes for five altered heterologous amino acids and a TAG stop codon. KIN17Δ2 ORF codes for a protein of 281 amino acids with a calculated molecular weight of 32 407 Da. In agreement with the theoretical value, electrophoresis of kin17Δ2 gives a 33 kDa molecular weight.

Plasmid pCHK, a eukaryotic translation vector, was constructed by inserting a *Kpn*I site into pCH110 (Pharmacia) just after the initiation codon of the *lacZ* gene. The kin17 putative nuclear localization signal (NLS) from nucleotides 727 to 818 was amplified by polymerase chain reaction (PCR) using two oligonucleotides harboring a *Kpn*I site at their ends. The forward primer was: 5'-TACGGTACCAAGCGCAGCATCCGGGAAACGC-3' and the reverse primer was 5'-TGAGGTACCGGCTCCATGATCTCATC-3' (the *Kpn*I sites are underlined). The resulting PCR product was cloned into the *Kpn*I site of plasmid pCHK in frame with the *lacZ* gene to produce plasmid pCHK-NLS_{kin17}-*lacZ*. Plasmids pCHK and pCHK-NLS_{kin17}-*lacZ* were used to transfect human HeLa S3 cells (5).

The single-stranded oligonucleotides C42: 5'-AAAAATCTCTAAAAAATCTCTAAAAAATCTCTAAAAAATCTCT-3' and NC42: 5'-TCTAATCTCTCTCTAATCTCTTCTAATCTCTCTCTAATCTCT-3' and their complementary strands were provided by Bonnefoy and Rouvière-Yaniv (6). Double-stranded oligonucleotides were made by heating the respective complementary strands at 65°C for 10 min in 0.1 kinase buffer (New England Biolabs) and cooling down to room temperature. Double-stranded C42 has a retarded migration on polyacrylamide gel as compared with NC42, displaying the very properties of curved DNA (7). Unless otherwise stated C42 and NC42 will designate the double-stranded form of the two oligonucleotides.

Insertion of curved C42 and non-curved NC42 into the pK42 polylinker *Sma*I gave rise to plasmids pK42-C42 and pK42-NC42, respectively. Plasmid pK42 is a derivative of pBR322 into which a 51 bp polylinker from pUC18 (New England Biolabs) was introduced into the *tet* gene (8).

Expression and purification of kin17, kin17Δ1 and kin17Δ2 proteins

GY7648 bacteria transformed with plasmids pKGC102, pKGC103 or pKGC104 were grown in LB medium plus 100 μg/ml ampicillin up to OD_{600} = 0.6. Then, IPTG was added (1 mM final concentration) to the cultures incubated for a further 3 h. Cell lysis and the first protein purification step on a heparin ultragel A4R column (IBF) were done as in Angulo *et al.* (1). The second purification step was done with an FPLC MonoS HR 5/5 column (Pharmacia) as follows. The kin17 heparin ultragel fraction was dialyzed overnight against 0.15 M NaCl, 20 mM Hepes, pH 7.8, 5 mM β-mercaptoethanol, 5% glycerol and loaded on a MonoS column equilibrated with the same buffer. Kin17 protein was eluted from the column with a 0.15–1.0 M NaCl gradient. Protein concentrations were measured as in Bradford (9). Low range (Bio-Rad) sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) standards were used as molecular weight markers. Protein preparations run on SDS–PAGE and stained with Coomassie blue displayed a major band corresponding to kin17 or its mutants and a few tiny contaminating bands (data not shown).

Kin17 blotting and probing for its specific properties

SDS–PAGE (10) was performed in the Mighty Small apparatus (Hoefer). Proteins were transferred on to a nitrocellulose membrane (S&S BA83, 0.2 μm) in a buffer containing 25 mM Tris–HCl pH 8.3, 192 mM glycine, 0.1% SDS and 20% ethanol at 200 mA for 1 h at 4°C utilizing a semi-dry electrotransfer cell (Touzart & Matignon, AE-6675) (11). Membranes with kin17 or kin17Δ1 or kin17Δ2 proteins were assayed as follows.

Immuno-detection. Western blotting was performed with an anti-RecA antiserum (diluted 1:5000) (12). Note that originally kin17 was identified as a protein reacting strongly with purified anti-RecA antibodies (1,13). The kin17–anti-RecA complexes were visualized with alkaline phosphatase conjugate goat anti-rabbit

Table 1. Bacterial strains

Strain	Chromosomal markers used	Phage or Plasmid markers used	Origin, reference and alias
GY7648	Δ*recA306*	DE3: λ*int lacI lacpUV5 lacZ::*I_{T7}	(35)
GY8544	idem	KGC102	GY7648, this work
GY8559	idem	pKGC103	GY7648, this work
GY8811	idem	pKGC104	GY7648, this work
JC10287	Δ*recA304*		(36), GY8502
GY8841	idem	pK42	GY8502, (8)
GY8843	idem	pK42-NC42	GY8502, this work
GY8844	idem	pK42-C42	GY8502, this work
JM103	Δ(*lac-proAB*)	F'*lacI*^{q} *lacZ*Δ*M15*	(37)
IB103	idem	pCHK	JM103, (38)
IB104	idem	pCHK-NLS_{kin17}-*lacZ*	JM103, this work

IgG (Promega, S3731) in the presence of the color development substrates BCIP and NBT (Sigma).

Zinc binding. Before protein transfer on to a membrane, the gels were incubated 1 h at 37°C in a reduction buffer containing 25 mM Tris−HCl pH 8.3, 192 mM glycine, 0.1% SDS, 20% ethanol and 0.7 M β-mercaptoethanol (14). After transfer to the membrane, proteins were renatured for 1 h at 4°C in 50 mM Tris−HCl pH 7.5. Then, the membrane was washed for 15 min at 4°C in 5 ml of 50 mM Tris−HCl pH 7.0, 100 mM KCl and 1 μCi ^{65}Zn was added. The membrane was incubated for 15 min at 20°C (14) and washed three times for 10 min at 20°C in 50 mM Tris−HCl pH 7.0, 100 mM KCl and autoradiographed.

Binding to DNA. In order to eliminate the possible effects of a contaminating protein in our preparation, we took advantage of the resolving power of Southwestern blotting. Proteins after SDS−PAGE and transfer to a membrane were renatured for 30 min at 4°C in a binding buffer containing 50 mM Tris−HCl pH 8.0, 1 mM DTT, 0.3% Tween 20 and supplemented with an appropriate amount of NaCl. $ZnCl_2$ 20 μM or EDTA 5 mM was added if necessary (15). Then, ^{32}P labelled DNA (10^5-10^6 c.p.m./ml) was added and left for 1 h at 4°C. The membrane was washed in the binding buffer three times for 10 min and autoradiographed. When necessary the bound DNA was extracted from its complex with kin17 by incubating the membrane in 0.6 M NaCl, 0.2% SDS and 20 mM EDTA at 65°C for 10 min. DNA was deproteinized by phenol/chloroform (1:1) extraction and run on a polyacrylamide gel.

Analysis of kin17−DNA complexes by EMSA/Western blotting

We used the technique described by Khoury Christianson and Kafatos (16).

Binding mixtures of 10 μl containing kin17 (195 ng), end-labelled DNA (1.5 ng) and unlabelled DNA in increasing amounts (65−520 ng) in 45 mM Tris−HCl pH 8.0, 150 mM NaCl, 1 mM DTT were incubated for 15 min at 0°C. The mixtures were subjected to electrophoresis on a 7% polyacrylamide gel in 0.25×TBE (22.5 mM Tris, 22.5 mM borate, 0.5 mM EDTA, pH 8.3) at 4°C. Then, the entire gel contents was transferred on to two membranes: the first membrane below the gel was nitrocellulose (S&S BA83), the second was nylon (Hybond-N+, Amersham). Electroblotting was performed in 12.5 mM Tris−HCl pH 8.5, 95 mM glycine, 20% methanol for 1 h at 4°C in a semi-dry cell at 200 mA initial current and 100 V. Kin17 protein was revealed on the nitrocellulose membrane with anti-RecA antibodies, the labelled DNA bands on the nylon membrane were autoradiographed and quantified with a Bio-Rad GS-250 Molecular Imager.

A. Sequences of NLS

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              240                           270
kin17   KLLGSAASGKRKESSQSSAQPAKKKKSALDEIMELEEEKKRTARTD-AW
          : :  . ::::     . .  ::::  .   .   ::   :  :  :
PARP    QLPGIKNEGKRKGDEVDGTDEVAKKKSRKETDKYSKLEKALKAQNELIW
                 210                           240
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B. Sequences of zinc fingers

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                                 20                          60
kin17      NH2-MGKSDF-LSPKAIANRIKSKGLQNVRWYCQMCQKQCRDENGFKCHCMSESHQRQL-LLASENPQQ
              . :..  :: : .      :. . :::    :    ::: ::.    : :     ::xx:  .
PARP    CKGCLEKIEKGQMRLSKKMVDPEKPQLGMID-RWYHPTCFVKKRDELGFRPEY-SASQLKGFSLLSAEDKEA
                   140                                 180
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Figure 1. Kin17 and PARP homologous sequences. (**A**) NLS sequences of kin17 and mouse poly(ADP-ribose) polymerase (PARP). The bipartite NLS consensus is underlined. (**B**) Zinc finger sequences of kin17 and PARP. The cysteine and histidine residues potentially ligating zinc ions are underlined. All peptides were cross-aligned using the Kanehisa program.

RESULTS

Kin17 nuclear localization signal can drive the protein to the nucleus

The kin17 amino acid sequence displays a putative (NLS) between residue 240 and 256 that looks similar to the bipartite class of NLS identified in some nuclear proteins (2), one of which is poly(ADP-ribose) polymerase (PARP), an enzyme involved in the mammalian cell response to DNA damage (3) (Fig. 1A).

We checked whether this nuclear localization signal was functional. For this purpose, we constructed plasmid pCHK-NLS_{kin17}-*lacZ* in which the NLS_{kin17} motif (235-SAASGKRK-ESSQSSAQPAKKKKSALDEIME-264) was fused to *lacZ* expressing β-galactosidase. When HeLa S3 cells were transfected with the pCHK-NLS_{kin17}-*lacZ* plasmid, the cell nuclei were stained blue with X-gal, indicating that the NLS_{kin17}-β-galactosidase fusion protein accumulated only in the cell nucleus (Fig. 2A). In contrast, when HeLa S3 cells were transfected with the pCHK vector β-galactosidase was expressed in the cytoplasm so that whole cells were stained blue (Fig. 2B).

This experiment demonstrates that the bipartite nuclear localization signal is functional and can be involved in driving kin17 protein to the cell nucleus.

The zinc finger is involved in kin17 binding to DNA

The kin17 amino acid sequence shows a single $CX_2CX_{12}HX_5H$ zinc finger motif (17) between residue 28 and 50 (Fig. 1B).

To assess that this motif is functional, we tested whether kin17 protein would bind (i) to zinc ions, and (ii) to DNA in a zinc-dependent manner. We constructed kin17Δ1, a mutant of kin17 deleted of amino acids 6−70 and thus devoid of its zinc finger (Fig. 1B). (i) We showed with a zinc blotting assay (14) that

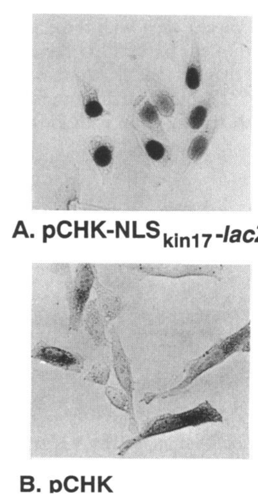

Figure 2. Kin17 protein nuclear localization signal is functional. HeLa S3 cells were transformed with pCHK-NLS_{kin17}-*lacZ* (**A**) or with pCHK (**B**) using the calcium phosphate method. The expression of β-galactosidase activity was detected by staining with X-gal (34).

kin17 binds ^{65}Zn whereas kin17Δ1 does not (Fig. 3). Note that albumin (66 kDa) and carbonic anhydrase (31 kDa) two molecular weight markers known to bind zinc ions gave also a positive signal. (ii) We observed that kin17 binding to double-stranded DNA was markedly decreased when EDTA was added (Fig. 4). This is likely due to the chelation of zinc ions and the corresponding inactivation of the zinc finger. Kin17 binding to DNA was restored by incubating the protein in a buffer containing zinc ions (Fig. 4). When kin17Δ1 was used instead of kin17 protein binding to DNA was severely reduced (Fig. 5).

In conclusion, kin17 binds efficiently to double-stranded DNA if its zinc finger is functional.

Low efficiency of kin17 binding to single-stranded DNA

We tested whether kin17 would show some binding to single-stranded DNA. For this purpose, we used the two complementary strands of the C42 synthetic oligonucleotide.

In a Southwestern assay kin17 was transferred on to a membrane that was probed with: (i) C42 light single-strand; (ii) C42 heavy single-strand; and (iii) double-stranded C42. Kin17 protein was barely able to bind to either piece of single-stranded DNA (Fig. 6). Kin17 binds more tightly to double-stranded than to single-stranded DNA.

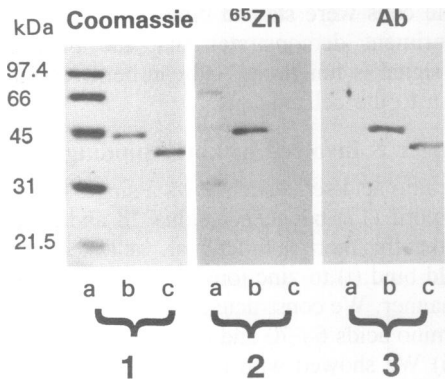

Figure 3. Kin17 protein binds zinc ions. Proteins were run on a 12% SDS–PAGE. The gel was cut into three pieces each with three lanes containing: molecular weight markers (lane a); kin17 (lane b); kin17Δ1 (lane c). The pieces were stained with Coomassie blue R-250 (panel 1), or electrotransferred on to a nitrocellulose membrane, incubated with ^{65}Zn and autoradiographed (panel 2) or probed with anti-RecA antibodies, which reacted only with kin17 and kin17Δ1 (panel 3).

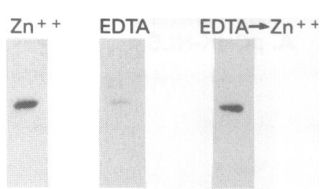

Figure 4. Kin17 binding to double-stranded DNA is zinc-dependent. Kin17 protein (3 mg per slot) was run on 12% SDS–PAGE and transferred on to a nitrocellulose membrane which was cut into three pieces. The first piece was incubated with 20 μM $ZnCl_2$ in a binding buffer supplemented with 150 mM NaCl and the two other pieces were incubated with 5 mM EDTA. Then, one of the last two pieces was washed thoroughly and incubated further with 20 μM $ZnCl_2$. Finally, all three pieces were probed with linear ^{32}P-end-labelled pBR322 double-stranded DNA (0.1 μg/ml).

Preferential binding of kin17 to pBR322 curved fragments

Does kin17 bind to DNA at random or at specific sites? To answer this question, we prepared three different sets of ^{32}P-end-labelled pBR322 restriction fragments (Fig. 7A) and with a Southwestern assay we tested to which fragments kin17 would bind. Then, we extracted DNA fragments from the complexes formed with kin17 which we then identified on a polyacrylamide gel. Note that the restriction fragments were produced in an equimolar mixture and were competing with one another in the protein binding assay.

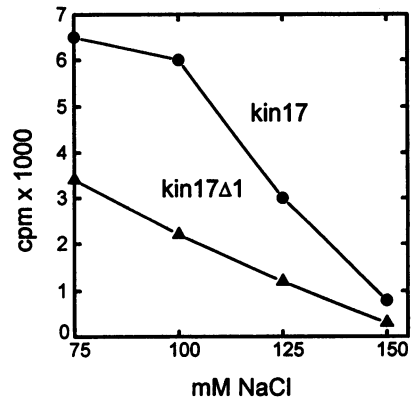

Figure 5. Absence of its zinc finger reduces kin17 binding to DNA. After SDS–PAGE of kin17 (circles) and kin17Δ1 (triangles) (5 μg per slot), the gel contents was electrotransferred on to a nitrocellulose membrane, which was cut into four pieces and placed in four tubes containing ^{32}P-end-labelled linear double-stranded pBR322 (0.1 μg/ml, 2×10^6 c.p.m./ml) in a binding buffer supplemented with various NaCl amounts which are plotted on the abscissa. After washing the membrane pieces, the radioactivity of the kin17–DNA complexes was counted and plotted on the ordinate.

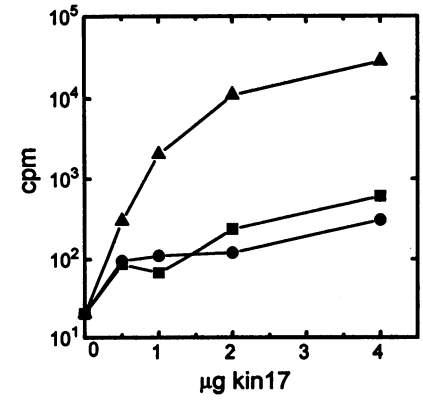

Figure 6. Kin17 binds poorly to single-stranded DNA. Kin17 in increasing amounts, plotted on the abscissa, was run on a 12% SDS–PAGE and electrotransferred on to nitrocellulose. Then, membranes were incubated with 5′-end-labelled single-stranded C42 (heavy strand, solid squares; light strand, solid circles), or double-stranded C42 (solid triangles). The reactions were carried out in 1.8 ml of binding buffer supplemented with 90 mM NaCl and 3 pmol (5′-ends) of oligonucleotides with a total radioactivity of 2.4×10^6 c.p.m. The membranes were washed, then the radioactivity of kin17–DNA complexes was counted and plotted on the ordinate.

When we used a mixture of *Hin*fI restriction fragments, we found that kin17 was tightly bound to a 1632 bp fragment (Fig. 7B, panel 1b) which carries C8, a DNA segment displaying the highest curvature (18) (Fig. 7A). Using various sets of restriction enzymes we narrowed down the region of kin17 preferential binding to a 777 bp fragment spanning C8 (Fig. 7B, panels 2 and 3). We also detected in slightly less stringent conditions kin17 binding to two other *Hin*fI restriction fragments, 517 and 506 bp long, spanning the C7 and C4 curved segments respectively (Fig. 7B, panel 1b').

In short, kin17 binds preferentially to pBR322 fragments carrying curved segments with an efficiency that seems correlated with the magnitude of DNA curvature.

Preferential binding of kin17 and of its mutants to a reconstructed curved DNA segment

We tested whether kin17 binding to non-curved DNA would be enhanced if we made it curved by inserting into it a short curved oligonucleotide.

To do this, we took the non-curved pK42 *tet* gene into which we inserted the C42 curved oligonucleotide or the non-curved NC42, which was used as a control sequence (Fig. 8A). Two *Nhe*I–*Nru*I restriction fragments, 876 bp long, spanning the inserted oligonucleotides migrated differently on a 5% polyacrylamide gel (Fig. 8B, lane a). The curved and non-curved *Nhe*I–*Nru*I restriction fragments were clearly distinguishable and easily separated.

When kin17 binding was assayed by Southwestern blotting with an equimolar mixture of the curved and non-curved 876 bp fragments, we observed that kin17 did bind more tightly to curved than to non-curved DNA (Fig. 8B).

Kin17Δ2 which migrates faster on a gel was tightly bound to the 876 curved fragment as was kin17 (Fig. 8B). This result eliminates the possibility that a contaminating protein of identical electrophoretic mobility as kin17 would bind curved DNA. The absence of the zinc finger in kin17Δ1 decreased its binding to DNA but it did not eliminate it (Fig. 5). We checked whether kin17Δ1 would still distinguish between curved and non-curved DNA. Indeed, it does but less efficiently than kin17 and kin17Δ2 proteins (Fig. 8B).

In sum, kin17 and its mutants recognize a short synthetic curved DNA fragment inserted into a non-curved region.

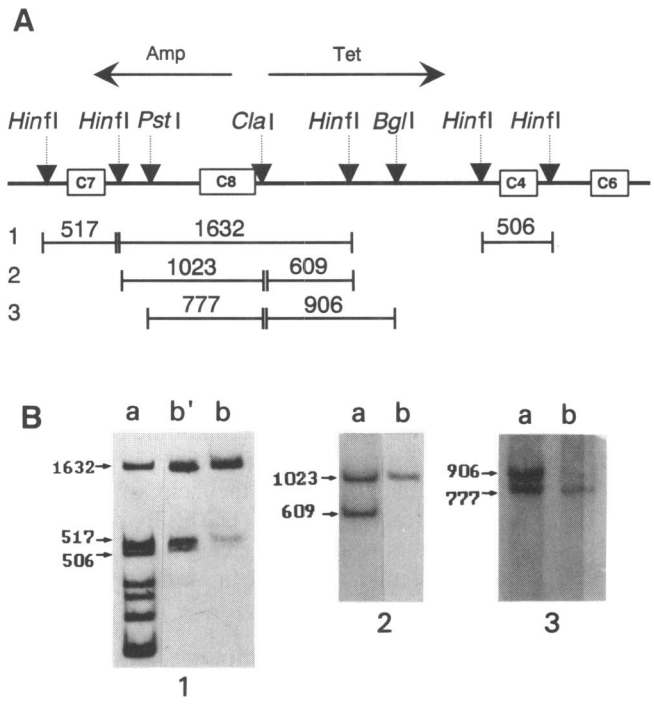

Figure 7. Kin17 binds preferentially to curved pBR322 fragments. (**A**) Size and mapping of three sets of pBR322 restriction fragments. We used three sets of pBR322 fragments resulting from the digestion with: (1) *Hin*fI that were end-labelled using Klenow fragment and [α-^{32}P]dATP (only some of *Hin*fI sites are shown and only the three largest fragments are indicated); (2) *Cla*I that were labelled as above with [α-^{32}P]dCTP and redigested with *Hin*fI; (3) *Cla*I that were end-labelled and redigested with *Pst*I and *Bgl*I. The restriction fragment lengths are in base pairs. C4, C6, C7 and C8 designate the curved DNA segments (18). Arrows indicate the position and orientation of Amp^R and Tet^R genes. (**B**) Kin17 trapping of curved pBR322 fragments. The kin17 protein preparation (3 μg per slot) was run on a 11% SDS–PAGE and transferred on to a membrane. The membranes with kin17 were incubated with ^{32}P-end-labelled DNA restriction fragments (0.1 μg/ml) (the three sets of fragments mentioned in (A) are present in sub-panels 1, 2, and 3, lines a). Then, DNA was extracted from the kin17–DNA complexes and run on a polyacrylamide gel (lanes b and b'). The reactions were done in a binding buffer supplemented with NaCl 150 mM (lanes b) or 140 mM (lane b').

Identification of complexes formed between kin17 and curved or non-curved oligonucleotides

Kin17 preferential binding to curved DNA was confirmed with a test, other than Southwestern blotting, called EMSA/Western. A constant amount of kin17 was incubated with a constant amount of radiolabelled curved C42 or non-curved NC42 to which

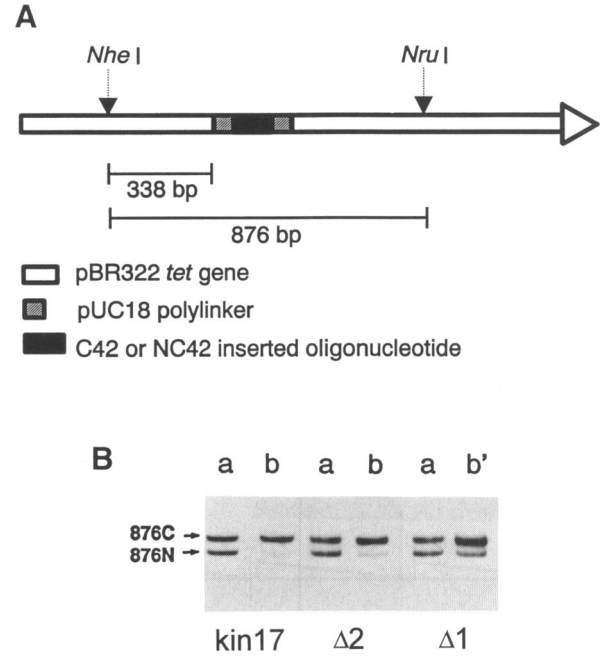

Figure 8. Insertion of curved oligonucleotide C42 into a non-curved fragment enhances kin17, kin17Δ1, and kin17Δ2 binding. (**A**) Location of C42 and NC42 inserted into the pK42 *tet* gene. (**B**) Release of curved and non-curved fragments bound to kin17 or to its mutants. The ^{32}P-end-labelled *Nhe*I–*Nru*I fragments from pK42-C42 and pK42-NC42 were mixed in an equimolar concentration (0.4 μg/ml) (lanes a) in a binding buffer supplemented with NaCl 150 mM (lanes b) or 130 mM (lane b'). Kin17, kin17Δ2, kin17Δ1 protein preparations (10 μg per slot) were run on a 12% SDS–PAGE and transferred on to a membrane and incubated with the *Nhe*I–*Nru*I fragments, which were then extracted from the protein–DNA complexes and run on a polyacrylamide gel (lanes b and b').

increasing amounts of the corresponding cold oligonucleotides were added to facilitate immunodetection. The mixtures were subjected to gel electrophoresis followed by autoradiography of the oligonucleotides or by immunodetection of kin17. We were thus able to identify complexes between kin17 protein and C42 or NC42 oligonucleotides.

Bands F1 and F2 (Fig. 9A) correspond to free oligonucleotides which are properly or improperly annealed (see lanes 1 and 7 devoid of kin17). The original single-stranded C42 and NC42 oligonucleotides contain four repeated sequences. Upon annealing the single strands can be mispaired and this sliding will generate a DNA structure made of partly double-stranded DNA with protruding single-stranded ends.

Bands denoted Cx1, Cx2 and Cx3 (Fig. 9A) correspond to various protein–oligonucleotide complexes. Cx1 was likely formed with a contaminating protein since it did not react with anti-RecA antibodies (Fig. 9B). The Cx2 and Cx3 complexes were formed with kin17 as shown by immunoblotting (Fig. 9B). The Cx2 and Cx3 complexes were produced optimally at an oligonucleotide concentration of 260 ng. At a higher oligonucleotide concentration formation of the Cx2 and Cx3 complexes decreased. This possibly resulted from the formation of multi-complexes that could not enter the gel.

The Cx2 and Cx3 kin17–oligonucleotide complexes were formed two to four times more efficiently with curved C42 than with non-curved NC42 (data deduced from scanning bands with a Molecular Imager).

Clearly, kin17 protein can recognize a curved from a non-curved DNA structure.

DISCUSSION

Kin17 NLS and zinc finger are functional

The NLS located between residue 240 and 256 has two basic elements, 240-KRK-242 and 253-KKKK-256, similar to the bipartite NLS described by Robbins *et al.* (2). As was done before for the PARP bipartite NLS (5), we engineered a protein fusing a peptide made of the putative kin17 NLS residues with a reporter enzyme β-galactosidase. Since the fused protein accumulated in the nucleus of HeLa cells, it means that the kin17 NLS has the ability to drive kin17 to the cell nucleus as does the PARP bipartite NLS (5).

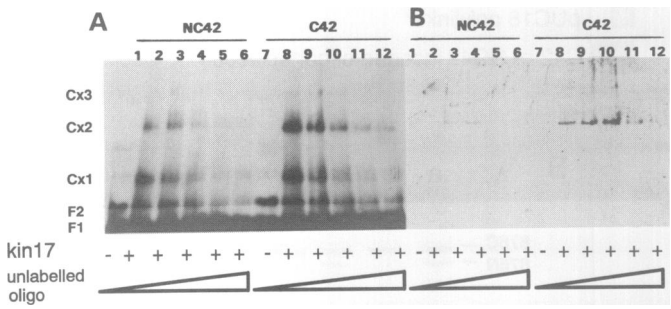

Figure 9. Kin17 binds more tightly to curved C42 than to non-curved NC42. Kin17 (195 ng) was incubated in 10 μl 45 mM Tris–HCl, pH 8.0, 150 mM NaCl and 1 mM DTT with 5′-end ^{32}P-labelled double-stranded C42 or NC42 (1.5 ng) and with the same cold oligonucleotide at increasing concentration in ng: 65 (lanes 2 and 8), 130 (lane 3 and 9), 260 (lane 4 and 10), 390 (lane 5 and 11) and 520 (lanes 6 and 12). Lanes 1 and 7 were without kin17. The reaction mixtures were run on a 7% polyacrylamide gel and the whole gel contents was electrotransferred on to itrocellulose and nylon membranes simultaneously. (**A**) Autoradiography of the nylon membrane containing radiolabelled oligonucleotides. Bands Cx1, Cx2 and Cx3 correspond to three protein–oligonucleotide complexes. F1 and F2 bands are made of free DNA since they are seen in lanes 1 and 7 devoid of kin17. (**B**) Western blotting of the proteins on the nitrocellulose membrane. Kin17 was detected with anti-RecA antibodies in the Cx2 and Cx3 complexes.

Kin17 protein also displays a zinc finger motif, a major determinant of DNA-binding proteins. The kin17 zinc finger motif $CX_2CX_{12}HX_5H$ corresponds to the C_2H_2 class consensus and is unusually rich in amino acids potentially coordinating zinc ions: five cysteines and two histidines (Fig. 1B). As demonstrated in this work, the kin17 zinc finger is functional and promotes efficient kin17 binding to DNA.

The search for a protein homologous to kin17 in the NBRF data bank pointed to the mouse poly(ADP-ribose) polymerase, an enzyme involved in the detection of DNA strand-breaks in eukaryotes (3,19). Two kin17 regions, the zinc finger and the NLS, are homologous to those observed in PARP (Fig. 1A and B). Interestingly, the kin17 zinc finger shares homology with the second zinc finger located in the DNA-binding domain of poly(ADP-ribose) polymerase. The PARP second zinc finger recognizes and binds specifically DNA at single-strand breaks (20, 21).

Binding specificity of kin17 for curved DNA

Even though there was a slight impurity in the kin17, kin17Δ1, kin17Δ2 preparations, we were able to circumvent it. We were able to demonstrate in three ways that the binding properties to curved DNA pertained to kin17 protein. (i) By using Southwestern blotting we obtained a satisfactory resolution of our kin17 preparation. The assay is not precise enough for determining binding constants but we were nevertheless able to prove the specific binding of kin17 to curved DNA. Binding specificity for DNA of several other proteins was also demonstrated with the use of Southwestern blotting (22–26). (ii) By using another protocol (EMSA/Western blotting) in which the protein DNA-binding assay precedes gel electrophoresis of the binding complexes and the identification of the components of the complexes, we demonstrated the existence of specific kin17–curved-DNA complexes with the help of anti-RecA antibodies that strongly react with kin17 (1). (iii) By constructing kin17Δ2, a mutant protein whose molecular weight was lower than kin17, we showed that they have a similar preferential binding to curved DNA.

Kin17 possible domains involved in binding curved DNA

The preferential binding to curved DNA of kin17Δ1, devoid of its zinc finger motif, and of kin17Δ2, deleted of the C-terminal 110 amino acids, indicate that: (i) kin17 possesses a domain recognizing curved DNA, other than the zinc finger; and (ii) the domain resides outside the 110 amino acid C-terminal region. The domain recognizing curved DNA must be located between amino acids 71 and 281, that is, within the protein core.

The efficiency of mouse kin17 protein in binding curved DNA might result overall from the activity of two protein domains, the zinc finger would first ensure binding of the protein to double-stranded DNA and then, the second domain in the protein core would direct the protein to a curved DNA structure.

Nature of kin17 binding to curved DNA

Kin17 preferential binding to curved DNA is associated with the presence of homopolymeric A-tracts. The curved C42 synthetic oligonucleotide 5′-AAAAATCTCTAAAAAATCTCTAAAAA-

TCTCTAAAAAATCTCT-3′ is made of four homopolymeric A-tracts, spaced in phase with the double helix, and flanked with TCTC sequences. The non-curved NC42 synthetic oligonucleotide 5′-TCTAATCTCTCTCTAATCTCTTCTAATCTCTCTC-TAATCTCT-3′ is composed of interspersed TCTC sequences within four TAAT blocks. Both synthetic oligonucleotides are AT-rich but the presence in C42 of homopolymeric A-tracts determines kin17 preferential binding.

Intrinsic DNA curvature is correlated with the presence in the DNA of homopolymeric A- and T-tracts (27–30). We have not addressed the question whether kin17 might recognize (i) poly(A) sequences themselves, or (ii) DNA curvature produced by the poly(A) sequences.

In any case, we determined that kin17 shows preferential binding to curved DNA by using different substrates. Not only was pBR322 DNA used here but we also performed a reconstruction experiment using as substrate a non-curved DNA fragment into which we inserted a short curved synthetic oligonucleotide. It has been observed that curved DNA regions are present at hot spots of illegitimate recombination in rodent and human HeLa cells (31,32). Kin17 binds also preferentially to those curved DNAs of mammalian cells (33).

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